THE ISOLATION AND CHARACTERISATION OF RUMINAL MYCOPLASMAS AND THEIR INTERACTIONS WITH RUMINAL CELLULOLYTIC MICROORGANISMS

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ABSTRACT

Six ruminal mycoplasmas (RM10, RM11, RM12, RM13, RM14 and RM15) were isolated from the ruminal digesta of Friesian cows using anaerobic techniques. The isolates were characterised using a range of culture media and molecular techniques to provide phenotypic data. Cellular and colonial morphology were elucidated using light microscopy, scanning electron microscopy and transmission electron microscopy. It was very apparent the isolates fell into two distinct groups. Isolates RM10, RM11 and RM12 had a growth requirement for sterol, were strictly anaerobic, were able to lyse the cell wall of the Gram-negative bacterium *E. coli*, were proteolytic, were non-motile, had trilaminar cell membranes, had no distinguishable cell wall, were pleomorphic, were able to pass through 0.45 μm filters, were Gram-negative and produced H_2. Therefore, they were classified as members of the genus *Anaeroplasma,* within the class *Mollicutes.* In the absence of 16S rRNA sequence data, it was not possible to determine the phylogeny of the isolates. Analysis of cellular proteins by SDS-PAGE demonstrated differences among the three isolates. Random Amplified Polymorphic DNA (RAPD) profiles showed RM11 and RM12 were more closely related to each other than to RM10. Isolates RM13, RM14 and RM15 had the same characteristics as isolates RM10, RM11 and RM12 except they were not able to lyse *E. coli* cell walls, were not proteolytic and did not produce H_2. These phenotypic characteristics identified them as *Anaeroplasma abactoclasticum.* Analysis of cellular proteins by SDS-PAGE showed variation in high molecular weight bands which suggested RM14 and RM15 were more closely related to each other than to RM13. Evidence based on RAPD profiles of DNA confirmed these relationships.

The range of carbohydrates used for growth was small and varied among the isolates. Antibiotics to which both groups were sensitive were those which inhibit protein synthesis and included, chloramphenicol, lincomycin-HCl and tetracycline. All isolates had an optimum growth pH in the range pH 6.0 to 6.8 and an optimum growth temperature in the range 42°C to 45°C.

The population density of ruminal mycoplasmas in hay-fed Friesian cows was between 10^7-10^8 g^{-1} of ruminal digesta. A similar population density was observed in grass-fed Friesian cows. The population density of *Asteroleplasma* species in both sets of animals was between 10^5-10^6 g^{-1}. Therefore, ruminal mycoplasmas represent between 0.1-1.0% of the total bacterial population in the bovine rumen.

Experimental evidence showed, that when grown in coculture with ruminal cellulolytic fungi, some isolates reduced the extent of cellulose digestion by the fungus as follows; *Caecomyces communis* (80%), *Neocallimastix frontalis* (60%) and *Piromyces communis* (70%). Inhibition of fungal cellulolysis was most marked when the fungi were grown in coculture with *An. abactoclasticum* (isolates RM13, RM14 and RM15). The isolates were also examined in coculture with ruminal cellulolytic bacteria. Cellulolysis by *Ruminococcus* species, in coculture with ruminal mycoplasmas, was inhibited by 30-70% when growing on paper. Cellulolysis by *Fibrobacter succinogenes* and *Clostridium chartatabidum* was not inhibited, and may have been slightly stimulated. The mechanisms for the observed effects are not known.

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DEDICATION

The late **CECIL WILLIAM LEA**, BSc (NZ), was a teacher who truly loved and lived his vocation. He was a remarkable man and even today, 33 years since I last talked with him, I remember him and his teaching with affection.

Cecil Lea taught at Feilding Agricultural High School from 1936 until 1965 having graduated Bachelor of Science from the University of New Zealand in the 1920s. A colleague once said of him, "He was a stern taskmaster with little patience for "loungers and loafers, and those allergic to work!" Yet underneath that rather stern countenance was fostered a kindness and sympathy for the keen student, for the industrious pupil, and a real sense of duty towards those given into his charge."

It is a pleasure to dedicate this thesis to his memory.

THE MICROBE

The microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.
His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All of these have never yet been seen-
But scientists, who ought to know,
Assure us that it must be so....
Oh! let us never, never doubt,
What nobody is sure about!

from

The Bad Child's Book of Beasts
by
Hilaire Belloc (1870-1953)

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ABBREVIATIONS

ABS	anaerobic buffer solution
ATP	adenosine triphosphate
BCRFB	basal clarified rumen fluid broth
bp	base pair
BSA	bovine serum albumin
°C	degrees Celcius
CBR	Coomassie Blue Reagent
CbRFB	cellobiose rumen fluid broth
CBS	Coomassie Blue Stain
CH_4	methane
cm	centimetre
cm^{-2}	per square centimetre
cm^{-3}	per cubic centimetre
CO_2	carbon dioxide
CRFA	clarified rumen fluid agar
CRFB	clarified rumen fluid broth
CsB	cellulose broth
CtSA	chitin starch agar
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid, disodium salt
FCWA	fungal cell wall agar
g	gram
g^{-1}	per gram
GC	gas chromatograph
H_2	hydrogen
HPLC	high pressure liquid chromatography
hr	hour
ICSBSTM:	International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995.
IU	international unit
kbp	kilobase pairs
kDa	kiloDaltons
l	litre
LDH	lactic dehydrogenase
M	molar
mA	milliampere
mg	milligram
min	minute
min^{-1}	per minute
ml	millilitre
ml^{-1}	per millilitre
mm	millimetre
mM	millimolar
Mmix	mastermix

µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
na	not applicable
nd	not determined
ng	nanogram
NH_3	ammonia
nm	nanometre
dNTP	2′-deoxynucleoside 5′-triphosphate
O_2	oxygen
OD_x	absorbence of light; x is the incident-light wavelength in nm
OsO_4	osmium tetraoxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
PEP	phosphoenol pyruvate
PFK	phosphofructokinase
pH	indicator of acidity or alkalinity
pH_{opt}	optimum pH for culture growth
PIM	primary isolation medium
P_2O_5	phosphorus pentaoxide
PP_i	pyrophosphate
PSM	paper strip medium
RAPD	random amplified polymorphic DNA
RFCbB	rumen fluid cellobiose broth
RFLP	restriction fragment length polymorphism
RM	ruminal mycoplasma
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second
SEM	scanning electron microscope
TAE	tris-acetic acid EDTA buffer
Taq	*Thermus aquaticus* DNA polymerase
TCA	tricarboxylic acid cycle
TE	tris-EDTA buffer
TEM	transmission electron microscope
TEMED	N,N,N^1,N^1 tetramethylethylenediamine
TLC	thin-layer chromatography
T_{opt}	optimum temperature for culture growth
TRIS	(tris[hydroxymethyl]aminomethane)
UV	ultra-violet light
VFA	volatile fatty acid
v	volt
v/v	volume : volume ratio
w/v	weight : volume ratio

1 INTRODUCTION

Ruminants are cloven-hoofed mammals which obtain food by browsing or grazing on plants (Hungate, 1966). They are the earth's dominant herbivores, due in part to the evolution within this group of a mechanism utilising microorganisms to digest plant components (Hungate, 1975). Sheep, cattle, goats, deer and buffalo are all ruminants (Hungate, 1966). There are 184 species of ruminating mammals, representing 74 genera and 6 families within the kingdom *Animalia.* A feature common to most ruminants is a four-chambered stomach comprising the reticulum, rumen, omasum and abomasum. Exceptions to this general rule are found in the family *Tragulidae* (chevrotains and mouse deer) where the stomach has just three chambers (Clarke, 1968). All ruminant nutrition relies on the presence of the symbiotic intestinal microflora and microfauna. Without this, ruminants would be incapable of digesting the plant materials which constitute their diet (Hungate, 1966).

Fermentation of the digesta is carried out in the first two chambers (reticulum and rumen) of the four-chambered stomach (Clarke, 1968). The rumen provides conditions where moisture, pH, temperature, anaerobiosis and food supply are nearly constant. These near constant conditions, lack of oxygen, and types of food, prevent many microorganisms from growing within the rumen microbial ecosystem (Hungate, 1960). Conversely, these same conditions allow microorganisms adapted to the rumen environment, to proliferate to high numbers.

The temperature within the rumen is usually 39°C, although this may rise slightly in the period after feeding when fermentation is maximal. However, the temperature will fall with water intake (Dehority, 1991; Theodorou *et al,* 1992). The rumen pH usually lies in the range 6.0 to 6.7 and is maintained by the secretion of an alkaline-buffered saliva which neutralises the short-chain fatty acids produced during fermentation (Hungate, 1988). The atmosphere within the rumen comprises 65% CO_2, 27% CH_4, 7% N_2; with H_2, H_2S, and O_2 being present in trace amounts (Hungate, 1966). Methane is formed in

the rumen by the reduction of CO_2 with H_2, and not from volatile fatty acids (Hungate, 1988).

Significant variation occurs in the numbers and types of microorganisms found within the rumens of individual animals in a herd, and there are also marked differences in the rumen populations of different ruminant species. This occurs because of host specificity, dietary differences and protozoal predation on bacteria (Clarke, 1968). Some ciliate protozoa in the rumen prevent other protozoa becoming established by predation and competition for food (Eadie, 1967). Deer maintain different ruminal ciliate populations to those in sheep and cattle, in spite of ample opportunity for cross infection (Clarke, 1968). Individuals of the same ruminant species also show gross differences in ruminal ciliate protozoa populations even within a single flock or herd. It is not uncommon to find several sheep in a flock without any holotrich protozoa, or without some entodiniomorph protozoa which are present in all of the other members of the flock (Clarke, 1968).

Swain *et al* (1996), noted that animals on the same diet and penned together, allowing microflora and fauna to be exchanged, had differing bacteriophage (phage) populations. This showed that the phage population of the rumen varies throughout the day, and also that individual animals have unique phage populations. Not only did phage activity vary between animals and groups of animals, but there were distinct diurnal variations in animals fed once daily. Phage numbers fell shortly after feeding and rose to a peak some 8-10 hours later (Swain *et al,* 1996).

In cattle fed once daily, ruminal bacteria population studies showed that regardless of diet, the total bacterial numbers remained fairly constant throughout the day (Leedle *et al,* 1982). The number of viable bacteria fell by 40% to 60% after feeding, but increased to a maximum 16 hr after feeding. Soluble carbohydrate-utilising bacteria predominated at all times (Leedle *et al,* 1982).

Microbial digestion of plant tissues in the rumen produces volatile fatty acids (VFA), principally acetate, propionate and butyrate, and these are used as energy sources by the host. Propionate is a key metabolic intermediate, because it is the only VFA converted

into carbohydrate by the ruminant. Ruminant needs for carbohydrate are less than those of non-ruminants, but carbohydrate is essential especially during periods of lactation. Microbes from the rumen, when digested in the lower intestine, are a major source of protein for the animal. In grazing ruminants, forage is ingested during approximately one third of the 24 hr day. The rumen never empties, even during periods of starvation (Hungate, 1975).

At periodic intervals, the orifice between the reticulum and the omasum opens and liquid and small particles of digesta flow into the omasum (Dehority, 1991). In sheep, the threshold size for digesta to pass from the reticulum to the omasum is between 1-2 mm, while in cattle the size is 2-4 mm (Ulyatt *et al,* 1985). Larger plant fragments are retained in the reticulo-rumen for further digestion. This process involves regurgitation and re-chewing of digesta by the animal and further microbial degradation of lignocellulose in the rumen (Dehority, 1991). In the omasum, fermentation acids and bicarbonate are absorbed by the leaf-like layers of tissues between which the digesta pass. Posterior to the omasum the ruminant alimentary tract is comparable to that of most other animals except that the pancreatic juice contains an exceptionally high concentration of ribonuclease; an adaptation to the abundant ribosomes in the bacteria to be digested (Hungate, 1975).

The particular aspect of rumen microbiology which forms the basis of this thesis, is the study of a group of microorganisms which have received little attention in the past. The class *Mollicutes* comprises eight genera of bacteria, none of which possess a cell wall (Robinson and Freundt, 1987; Dybvig and Voelker, 1996). Only two mollicute genera are found in the rumen and these are loosely termed, ruminal mycoplasmas. The term is misleading since no ruminal mycoplasma belongs to the genus *Mycoplasma*, but belong to the genera, *Anaeroplasma* and *Asteroleplasma* (Robinson and Freundt, 1987). Both genera are obligately anaerobic, in contrast to the other 6 genera of mollicutes, which are facultative anaerobes (Weisburg *et al*, 1989).

There are no studies which have investigated the ecological role of ruminal mycoplasmas in ruminants. Likewise, no information is available as to how these bacteria interact with other microbes, particularly the cellulolytic bacteria and

cellulolytic fungi within the rumen microbial ecosystem. Therefore, the contribution of *Anaeroplasma* and *Asteroleplasma* species to the overall process of fibre digestion and to ruminant nutrition is unknown. The data reported in this thesis will endeavour to answer some of these questions.

2 LITERATURE REVIEW

2.1 INTRODUCTION

Much of the literature relating to the research topic of this thesis uses the term "anaerobic mycoplasma" to describe those members of the class *Mollicutes* (bacteria without cell walls) which can be isolated from the intestinal tract of ruminants. Similarly it uses the terms "mollicutes" or "mycoplasma" to loosely describe any member of the eight genera within the class *Mollicutes*. It is now felt a more precise term for anaerobic mycoplasmas of rumen origin is "ruminal mycoplasma", and this name will be used throughout this thesis. The descriptives, "mollicutes" and "mycoplasma" will be used in their generic sense, unless specifically discussing the class *Mollicutes* or the genus *Mycoplasma*, when the name will be italicised. To give an overall picture, the review includes a brief description of the rumen microbial ecosystem and describes a number of ruminal microorganisms. However only fibrolytic bacteria and fungi which have been investigated in cocultural studies with the ruminal mycoplasmas are discussed in any depth. The ruminal mycoplasmas are discussed in detail later, in relation to their physiology, phylogeny and interactions with other ruminal microorganisms.

2.2 THE RUMEN MICROBIAL ECOSYSTEM

The rumen microbial ecosystem is a diverse assemblage of interdependent and interactive microorganisms (see reviews in Hobson and Stewart, 1997). Ruminal microorganisms are found in the liquid phase of the digesta, associated with plant tissues, and attached to the ruminal epithelium (Czerkawski and Cheng, 1988). Microorganisms found within the rumen include bacteria, fungi, protozoa and bacteriophage. They function to digest the host animal's fibrous diet and provide nutrients to the host by way of fermentation end products. Ultimately their cells are digested in the lower intestinal tract, and contribute an important source of protein for the animal. The total number of anaerobic microorganisms in the rumen is between 10^{10} and 10^{11} g^{-1} (Hungate, 1966). Aerobic and facultative microorganisms have also been

isolated but are thought to be mainly transients (Stewart and Bryant, 1988). The temperature of the ruminal digesta is fairly constant at 39°C, although this rises slightly after feeding when the fermentation is at a maximum but falls with water intake (Dehority, 1991; Theodorou *et al,* 1992). The rumen pH usually lies in the range 6.0 to 6.7, and is maintained by the introduction of alkaline-buffered saliva which neutralises VFAs produced by the rumen fermentation (Hungate, 1988). The atmosphere in the rumen comprises 65% CO_2, 27% CH_4, 7% N_2; with H_2, H_2S, and O_2 being present in trace amounts (Hungate, 1966).

2.3 Fibrolytic Ruminal Microorganisms

Ruminal microorganisms may be divided into two major groups; those directly involved in plant tissue degradation, and those which use the end-products of these microorganisms for growth. Fibrolytic microorganisms release sugars as a result of polysaccharide digestion; the sugars are then utilised by non-fibrolytic microorganisms to provide energy for cell growth. Fibrolytic and non-fibrolytic microorganisms produce volatile fatty acids (VFAs), including acetate, propionate and butyrate which are used by the host animal for energy. The capability for breaking down plant fibre in the rumen rests with a relatively small number of bacterial, fungal and protozoal species.

2.3.1 Fibrolytic Ruminal Bacteria

Bacteria are the major group of microorganisms that degrade plant tissues in the rumen (Akin and Benner, 1988). The plant cell-wall degrading bacteria include *Butyrivibrio fibrisolvens, Clostridium* spp., *Eubacterium cellulosolvens, Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens.* *F. succinogenes* and *Ruminococcus* spp. are generally regarded as the most active fibrolytic bacteria. *F. succinogenes* degrades mainly cellulose, while some strains have the ability to degrade storage polysaccharides such as starch, or structural polysaccharides such as pectin (Stewart and Bryant, 1988). Access to plant material is generally gained via damaged tissue or through stomata, while intact plant cuticles appear to be impermeable to either mechanical penetration or enzymatic degradation (Chesson and Forsberg, 1988). Close contact appears to be necessary for plant cell-wall degradation as many of the bacterial enzyme systems involved are cell bound (Chesson and Forsberg, 1988; Dehority, 1991).

Cellulolytic bacteria are sensitive to low pH, so it is essential that fluctuations in rumen pH are avoided if optimum fibre digestion is to be achieved (Wallace, 1992).

Non-fibrolytic bacteria in the rumen have indirect effects on plant tissue degradation by utilising the intermediates and end products produced by plant cell-wall degrading microbes. *Selenomonas ruminantium* for example, is incapable of fermenting cellulose but will utilise the cellodextrins produced during the hydrolysis of cellulose by *F. succinogenes* (Russell, 1985). Also succinate, produced by many plant cell-wall degrading bacteria, never accumulates in the rumen. It is utilised by other bacteria such as *Selenomonas ruminantium* to produce propionate (Scheifinger and Wolin, 1973) which is essential to the ruminant because it is the only metabolic intermediate used in gluconeogenesis (Wolin and Miller, 1988).

2.3.2 Fibrolytic Ruminal Fungi

Ruminal fungi have been the subject of extensive reviews recently. For references see Mountfort and Orpin (1994) and Orpin and Joblin (1997). Ruminal fungi were first observed when fungal rhizoids were seen attached to plant tissue in rumen contents (Orpin, 1975). Previously it had been the practice to examine only the filtrate of rumen contents; the solid fraction being discarded as largely unimportant. Flagellated zoospores of chytridiomycete fungi had been observed earlier in the filtrate, but incorrectly identified as protozoa. Braune (1913), reported by Hungate (1966), observed what he described as flagellates in the rumens of calves and named them *Callimastix frontalis.* These were almost certainly the zoospores of the ruminal fungus *Neocallimastix frontalis*, which were reclassified by Vavra and Joyon (1966) along with flagellates from other environments into the genus *Neocallimastix.* Orpin (1975) observed that "...the vegetative stage of *N. frontalis* bears a strong morphological resemblance to that of certain species of aquatic phycomycete fungi...".

Obligatory anaerobic fungi have been isolated from the rumen, omasum, abomasum, small intestine, caecum, large intestine and faeces of cattle (Davies *et al,* 1993), and from the alimentary tract of a wide variety of other animals (Theodorou *et al,* 1992). These fungi do not possess mitochondria. The life-cycles of anaerobic fungi have three parts; a motile zoospore, a vegetative thallus, and an aerotolerant survival stage. The

thallus carries a sporangia (fruiting body) from which zoospores are released at maturity (Orpin and Joblin, 1997). The classification of the ruminal fungi is shown in Table 2.1.

Ruminal fungi are believed to play an important role in fibre digestion in the rumen (Orpin, 1977(a); Bauchop, 1979), with the initial invasion of the plant material occurring at sites of tissue damage (Bauchop, 1980), and at stomata (Orpin, 1977(a); Ho *et al,* 1988). Following zoospore attachment and encystment, fungi develop highly branched mycelia which grow inside plant fragments, where enzymes active against structural polysaccharides are released. This allows the fungus access to fermentable carbohydrates which are not immediately available to surface-acting ruminal bacteria. It has been shown that fungi colonise fibrous plant tissue, and that lignin-containing tissues are colonised preferentially (Bauchop, 1979; Grenet and Barry, 1988). The evidence for this was the discovery that fungal numbers *in vivo*, as estimated by sporangia counts, are higher in animals fed poor quality, fibrous diets. In contrast, fungi were found to be absent from the rumen of sheep grazing soft, leafy diets, low in fibre (Bauchop, 1979). The smaller populations of fungi found in the rumens of animals fed soft leafy diets, may be due to the short residence time in the digestive tract, leading to incomplete fungal life-cycles (Bauchop, 1989). Counts of 10^5 sporangia cm^{-2} were found on the surface of lucerne (*Medicago sativa*) stems which had been suspended in nylon bags in a sheep rumen (Bauchop, 1979). Estimates of fungal populations have also been made on the basis of counts of zoospores in rumen fluid (Joblin, 1981).

Anaerobic fungi have been seen in the rumens of lambs 8-10 days after birth (Fonty *et al*, 1987). The presence of anaerobic fungi in saliva and faeces suggests these as possible routes of transmission (Trinci *et al,* 1988). Spore-like structures have been found in the rumen (Fonty and Joblin, 1991), and the faeces of cattle (Davies *et al,* 1993). These resting spores are perhaps the way fungi survive outside the rumen, and are possibly one route for transmission between animals (Fonty and Joblin, 1991).

Table 2.1 Classification of ruminal fungi

Monocentric fungi		**References**
Neocallimastix	*N. frontalis*	(Orpin, 1975; Heath *et al*, 1983)
	N. patriciarum	(Orpin and Munn, 1986)
	N. hurleyensis	(Webb and Theodorou, 1991)
Piromyces	*P. communis*	(Orpin, 1977b)
	P. mae	(Li *et al*, 1990)
	P. dumbonica	(Li *et al*, 1990)
	P. rhizinflata	(Breton *et al*, 1991)
Caecomyces	*C. communis*	(Orpin, 1976; Munn *et al*, 1988)
	C. equi	(Gold *et al*, 1988)
Polycentric fungi		
Anaeromyces	*A. mucronatus*	(Breton *et al*, 1990)
Orpinomyces	*O. bovis*	(Barr *et al*, 1989)
	O. intercalaris	(Ho *et al*, 1994)
	O. joyonii	(Breton *et al*, 1989)
Ruminomyces	*R. elegans*	(Ho *et al*, 1990)

2.3.3 Ruminal Protozoa

In 1843, when Gruby and Delafond first observed ruminal protozoa, they noted that some ingested small pieces of plant tissue. They also noted, that while the protozoa in the rumen and reticulum were viable and active, those in the omasum and abomasum were immobile and disintegrating. No protozoa were seen in the duodenum (Hungate, 1966). They concluded that protozoa were a food source for the host animal, and that this was unexpected in the supposedly herbivorous ruminant (Hungate, 1966).

Both ciliate protozoa and flagellate protozoa are present in the rumen, however the ciliates are both the largest and most important in terms of rumen function (Williams and Coleman, 1988). Estimates of the number of ciliate protozoa in the rumen range between 10^5-10^6 ml^{-1} (Hungate, 1966), and are thought to be responsible for 25-33% of the plant tissue degradation occurring in the rumen (Demeyer, 1981).

Two major groups of ciliate protozoa have been described; the entodiniomorphs, and the holotrichs. More than 100 species of entodiniomorph protozoa have been found in the rumen. All are strict anaerobes which feed mainly by engulfing particulate matter, including other ruminal microorganisms (Williams and Coleman, 1988). The entodiniomorphs lack the abundant cilia which cover the surface of holotrichs, but have evolved specialised bands of syncilia (fused ciliary bands) which function both for movement and food ingestion (Hungate, 1966).

Ruminal holotrich protozoa possess cilia over the entire body surface, each inserted singly and not fused with others, except in the region of the mouth (Hungate, 1966). There are two genera of holotrich protozoa found in the rumen; *Isotricha* and *Dasytricha*. These are large microorganisms, with *I. intestinalis* recorded in the range (97-131 μm x 68-87 μm) (Hungate, 1966). Holotrich protozoa are mainly involved in the utilisation of non-structural carbohydrates and soluble sugars, and have only limited ability to degrade plant structural polysaccharides (Demeyer, 1981).

2.4 Ruminal Bacteriophages

Bacteriophage (phage) are an integral part of the rumen microbiota of sheep and cattle, occurring in large numbers and in great diversity (Klieve, 1991). Phage are obligate pathogens of bacteria and their presence leads to the eventual lysis of the bacterial host. Little is known about the effects of phage attack upon ruminal bacteria, or their effect on nutrient cycling within the rumen. The size of the population suggests that they play a significant role in bacterial lysis and the subsequent reduction in the efficiency of feed utilisation (Swain *et al,* 1996). The number of phage found in ruminal fluid from sheep and cattle lies in the range 2×10^7 to 1×10^8 ml^{-1} (Klieve and Bauchop, 1988).

2.5 Ruminal Yeasts

Clarke and di Menna (1961) isolated yeasts from the rumens of cattle in New Zealand, which they believed were true ruminal inhabitants. The yeasts were isolated using aerobic techniques, grew at 39°C, and were not isolated from samples of the feed material. Representatives of the *Candida*, *Trichosporon* and *Rhodotorula* genera were found.

The yeast most commonly isolated from the rumens of cows in Denmark was *Candida krusei* which, like the less commonly isolated *Torulopsis pintolopesii* and *Kluyveromyces bulgaricus*, could reproduce under anaerobic conditions *in vitro* (Lund, 1974).

Lund (1980), reported the isolation of yeasts from ruminal digesta of the Musk Oxen (*Ovibos moschatus)* in Greenland. The isolates most commonly found were *Candida* spp. and *Cryptococcus* spp.. The ruminal digesta of these animals contained predominantly woody plant parts of the *Salix* and *Betula* genera, in sharp contrast to the grass and clover mix found in most New Zealand pasture-fed animals. The yeasts which Lund (1980) described were isolated using aerobic techniques and many of those found in the rumen of the musk oxen were also isolated from samples of peat, soil and algae in a similar geographic location.

2.6 The Class *Mollicutes*

Members of the class *Mollicutes* are small, free-living procaryotes that pass through 0.45 μm filters, have genomes with a very low G+C content, lack cell walls and have unusual nutritional needs (Weisburg *et al*, 1989; Dybvig and Voelker, 1996). Many are pleomorphic, ranging from spherical, coccoid, cocco-bacilliary, ring and dumb-bell forms, to short and long branching filaments. The coccoid forms are approximately 0.3 μm in diameter, while the filamentous forms can be more than 100 μm long (Boatman, 1979). The mollicute genome is believed to be the smallest of any free-living cell, and because of this biosynthetic capacity is limited. More than 100 different species have been isolated from humans, animals, plants and insects.

The class *Mollicutes* contains eight genera (*Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Entomoplasma, Mesoplasma, Mycoplasma*, *Spiroplasma* and *Ureaplasma*), (see Table 2.2) with classification based primarily on differences in morphology, genome size, and some nutritional features (Robinson and Freundt, 1987). The class is phylogenetically quite broad (Weisburg *et al*, 1989; Dybvig and Voelker, 1996). The two anaerobic genera within the class *Mollicutes, Asteroleplasma* and *Anaeroplasma*, are only distantly related (Gundersen *et al,* 1994).

Acholeplasma spp. and *Asteroleplasma anaerobium* are able to grow in the absence of pre-formed sterols (Weisburg *et al,* 1989), while more recently, Toth *et al* (1994) reported a new genus, *Mesoplasma,* which also grow in the absence of sterol. *Mesoplasma* species, of which there are 12, are pathogens of plants and insects. All other mollicute genera require an exogenous supply of sterols for growth (Weisburg *et al,* 1989). *Mycoplasma, Ureaplasma* and *Spiroplasma* spp. are unable to synthesize fatty acids and these must be provided by the host. *Ureaplasma* spp. require exogenous urea for growth, and for this reason are often associated with infections of the genitourinary tract. *M. gallisepticum*, requires pre-formed phospholipids for growth (Razin, 1992). By becoming dependent on host-derived nutrients, a considerable amount of genetic information is saved (Razin, 1992).

The nutritional dependence of some members of the ruminal mycoplasmas on exogenous sterol is a trait that has arisen several times during their evolution (Robinson and Freundt, 1987). Nutritional requirements such as the need for sterol probably reflect a loss of genes, but since different gene functions could be lost and result in similar dependencies, care must be taken when using nutritional requirements as indicators of biosystematic groupings (Toth *et al*, 1994). It has been suggested, that during the evolution of mycoplasmas from Gram-positive bacteria, the loss of cell walls, together with many biosynthetic systems, has necessitated the adoption of a parasitic mode of survival (Woese, 1987; Razin, 1992). All known mycoplasmas are parasites of humans, other vertebrates, plants or arthropods (Razin, 1992). Mycoplasmas have complex nutritional requirements and depend on the host for many amino acids, fatty acids, sterols, and vitamins, (Razin, 1978; Rodwell, 1983; Razin, 1992). Because of this many are not readily culturable.

Table 2.2 Taxonomy and characteristics of the class *Mollicutes*[a]

Class: *Mollicutes*
Order I: *Mycoplasmatales* (sterol required)
Family I: *Mycoplasmataceae* (genome size, 600-1350 kbp)
Genus I: *Mycoplasma* (100 species current)
Genus II: *Ureaplasma* (6 species current)
Order II: *Entomoplasmatales* (sterol may or may not be required)
Family I: *Entomoplasmataceae* (genome size 790-1140)
Genus I: *Entomoplasma* (5 species) (sterol required)
Genus II: *Mesoplasma* (12 species) (sterol not required)
Family II: *Spiroplasmataceae* (genome size 940-2240 kbp)
Genus I: *Spiroplasma* (17 species current) (sterol required)
Order III: *Acholeplasmatales* (sterol not required)
Family I: *Acholeplasmataceae* (genome size 1500-1650 kbp)
Genus I: *Acholeplasma* (13 species current)
Order IV: *Anaeroplasmatales* (sterol may or may not be required)
Family I: *Anaeroplasmataceae* (genome size, 1500-1600 kbp)
Genus I: *Anaeroplasma* (sterol required)
Genus II: *Asteroleplasma* (sterol not required)

[a]Adapted from (Robinson and Freundt, 1987), and the (International Committee on Systematic Bacteriology, Subcommittee on the Taxonomy of *Mollicutes*, 1995).

2.6.1 Genetics of the Class *Mollicutes*

Members of the class *Mollicutes* possess a circular double-stranded DNA chromosome of 600-1800 kb. Yet despite fundamental differences between mollicutes and other bacteria, their phylogeny is quite straightforward and they share a common ancestor with such bacteria as the bacilli, clostridia, enterococci, lactobacilli, staphylococci and streptococci (Dybvig and Voelker, 1996).

Characteristically, mycoplasmal tRNAs have fewer modified nucleosides, reducing the need for modification enzymes and consequently, genomic information. *Mycoplasma* and *Ureaplasma* species have only one DNA polymerase, while *Acholeplasma* and *Spiroplasma* spp. (which have larger genomes of 1600-1700 kb), possess three distinct DNA polymerases, in common with *E. coli* and other eubacteria (Razin, 1992). Analyses of mollicute 16S rRNA sequences indicates a relationship to Gram-positive bacteria whose DNA has a low percentage of guanine and cytosine (% G + C) (Weisburg *et al,* 1989).

Members of the orders *Mycoplasmatales* and *Entomoplasmatales* use the UGA codon to encode tryptophan at a frequency 10 times greater than UGG. The unconventional use of UGA as a tryptophan codon is not shared by all mycoplasmas. *Acholeplasma laidlawii* uses the universal genetic code and has a single tRNA species with an anti-codon CCA that can only translate the normal tryptophan codon UGG (Tanaka *et al,* 1989). This supports the idea that the change in the UGA assignment from a stop codon to a tryptophan codon occurred after separation of the spiroplasma and mycoplasma branches from the acholeplasma branches of the phylogenetic tree (Rogers *et al,* 1985). The International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (ICSBSTM) reported in 1995, that only UGG encodes tryptophan in members of the orders *Acholeplasmatales* and *Anaeroplasmatales.* In the case of anaeroplasmas, little is known about their molecular biology, but since they are closely related to acholeplasmas, it is probable they also use the universal genetic code. The genetic code used by *Asteroleplasma anaerobium* is unknown (Dybvig and Voelker, 1996).

2.6.2 Pathogenicity of *Mycoplasma* spp.

Many members of the genus *Mycoplasma* cause disease in animals and humans, and are usually host specific. Two species known to cause disease in ruminants are *M. bovis* (calf pneumonia, mastitis and arthritis) and *M. agalactiae* (contagious agalactia in goats and sheep) (Pettersson *et al,* 1996). Infections by pathogenic mycoplasmas are rarely of the fulminant type, but are often chronic in nature. The mechanisms of mycoplasma pathogenicity are largely unknown (Razin, 1992).

Mycoplasmas infecting humans and animals are mostly surface parasites, colonising the epithelial linings of the respiratory and genitourinary tracts. Potent toxins have not been associated with mycoplasmas. *M. arthritidis,* which has been implicated in arthritis, produces a potent mitogen; a 27-kDa protein molecule (MAM) which has been defined as one of the so-called superantigens. Superantigens, which are produced by a variety of microbial agents, activate T cells by a unique pathway leading to T cell modification and the induction of autoimmunity. It has been proposed that MAM participates in chronic joint inflammation in rats and mice infected by *M. arthritidis*, not only by activating T cells with the resulting liberation of inflammatory lymphokines, but also by suppressing host defences. The contributions of infection and autoimmunity in the chronic phase of the disease are unknown (Cole and Atkins, 1991). Mycoplasmal infections are often chronic in nature, and it is unclear as to how these wall-less, rather fragile microorganisms resist the immune defence mechanisms of their host. Some pathogenic mycoplasmas are thought to undergo high-frequency phenotypic switching involving variable antigens, which enables them to evade host defences (Razin, 1992).

2.6.3 Ruminal Mycoplasmas

Little is known about the group of microorganisms loosely termed "ruminal mycoplasmas." They are classified within two genera, *Anaeroplasma* and *Asteroleplasma* and are found within the rumen microbial ecosystem. Studies of ruminal mycoplasmas in axenic culture, and in coculture with ruminal cellulolytic bacteria and fungi form the central theme of this thesis and the research reported herein.

An obligately anaerobic microorganism from the rumen of cattle which lysed bacterial cells was described by Hungate in 1966. The microorganism, which lacked a cell wall, was subsequently characterised as a ruminal mycoplasma (Robinson and Hungate, 1973) and classified as *Acholeplasma bactoclasticum.* Later this classification was reviewed and the microorganism reclassified into a new genus and species, *Anaeroplasma bactoclasticum,* on the basis that it was an obligate anaerobe and required sterols for growth (Robinson and Allison, 1975). A feature of the mycoplasma was the production of an extracellular enzyme, which under anaerobic but not aerobic conditions, degraded the peptidoglycan layer in the cell walls of Gram-negative bacteria

such as *E. coli,* causing lysis. Ruminal contents of both cattle and sheep were subsequently found to contain similar mycoplasmas, while other mycoplasmas were found which were abacteriolytic. The abacteriolytic mycoplasmas (10^7 - $10^8 g^{-1}$) were present in higher numbers than the bacteriolytic type (10^5 - $10^7 g^{-1}$) (Robinson *et al,* 1975). Neither of these types of mycoplasma were found in the caecal contents of hamsters, horses, pigs, rabbits or turkeys (Robinson *et al,* 1975). The abacteriolytic ruminal mycoplasmas comprise two types; the sterol-requiring *Anaeroplasma abactoclasticum*, and the sterol non-requiring, *Asteroleplasma anaerobium* (Robinson and Freundt, 1987).

Within the genus *Anaeroplasma*, only one species, *An. bactoclasticum* was originally characterised as being able to lyse the cell-walls of Gram-negative bacteria (Robinson and Allison, 1975). However, later work by Robinson and Rhoades (1977) using gel immunodiffusion precipitation tests, showed that within the species *An. bactoclasticum* three serovars could be distinguished. In contrast *An. abactoclasticum* was represented by only one serovar. Gel diffusion precipitation tests also showed that ruminal mycoplasmas, which were later to be placed into the genus *Asteroleplasma*, did not cross-react with antisera from any of the other groups of ruminal mycoplasma under test. Stephens *et al* (1985) described five distinct groups of ruminal mycoplasma based on [^{3}H] DNA-DNA homology data; four *Anaeroplasma* spp. and one *Asteroleplasma* sp., although not all had been named at that time. These groups were sufficiently diverse for Robinson and Freundt (1987) to propose that ruminal mycoplasmas, previously classified as *An. bactoclasticum,* be reclassified into three separate species - *An. bactoclasticum,* and two new species, *An. varium*, and *An. intermedium.* All three species were capable of lysing the cell walls of Gram-negative bacteria. Based on distance matrix data, *An. bactoclasticum* and *An varium* were more closely related to each other, than they were to *An. intermedium* (Weisburg *et al*, 1989). A fourth species, *An abactoclasticum* was more distantly related (Weisburg *et al*, 1989). The four *Anaeroplasma* spp., and *As. anaerobium* constitute the five groups described by Stephens *et al* (1985). *Asteroleplasma* species are phylogenetically distant from all *Anaeroplasma* species (Gundersen *et al*., 1994; Seemuller *et al*, 1994).

The polar lipids of *Anaeroplasma* spp. contain plasmalogens (alk-1-enyl glyceryl ethers) which are found in anaerobic bacteria but not aerobic bacteria. A bacteriolytic strain 7LA (*An. intermedium*) contained about 50% of the amount of glycolipids present in the non-bacteriolytic strain 6-1 (*An. abactoclasticum*) (Langworthy *et al,* 1975).

The ruminal mycoplasmas (Table 2.3) currently are classified according to the recommendations of Robinson and Freundt (1987) who concluded, "Because of the unique metabolic properties of the obligately anaerobic mycoplasmas, dependence on strict anaerobiosis should outweigh the sterol requirement as the major taxonomic factor. Therefore, we propose to classify the genera *Anaeroplasma* and *Asteroleplasma* in a new family, *Anaeroplasmataceae.* Since this new family is not referable to either of the two established orders of the *Mollicutes*, we propose the assignment of *Anaeroplasmataceae* to a new order *Anaeroplasmatales*, as order III of the class *Mollicutes.*" This classification now has been slightly changed following recommendations of the ICSBSTM, (see Table 2.2); the ruminal mycoplasmas move from Order III to Order IV.

Table 2.3 Taxonomy of the ruminal mycoplasmas[a]

Order IV:	*Anaeroplasmatales* (sterol may or may not be required)
Family I:	*Anaeroplasmataceae* (genome size, 1,000 megadaltons)
Genus I:	*Anaeroplasma* (sterol required)
Species 1:	*An. abactoclasticum*
Species 2:	*An. bactoclasticum*
Species 3:	*An. varium*
Species 4:	*An. intermedium*
Genus II:	*Asteroleplasma* (sterol not required)
Species 1:	*As. anaerobium*

[a] Adapted from Robinson and Freundt (1987).

Details of the type strains of the ruminal mycoplasmas and the percentage G+C of their DNA are shown in Table 2.4

Table 2.4 Type Strains and G+C mol% of Ruminal Mycoplasma DNA[a]

Ruminal Mycoplasma	Type Strain	G+C (mol%)
Anaeroplasma abactoclasticum	(6-1=ATCC 27879)	29.3
Anaeroplasma bactoclasticum	(JR=ATCC 27112)	33.7
Anaeroplasma intermedium	(7LA=ATCC 43166)	32.5
Anaeroplasma varium	(A-2=ATCC 43167)	33.4
Asteroleplasma anaerobium	(161=ATCC 27880)	40.2

[a](Stephens *et al,* 1985; Robinson and Freundt, 1987)

It can be seen from Table 2.4 that the %G+C for *An. bactoclasticum*, *An. intermedium* and *An. varium* are very similar, and the %G+C of *An. abactoclasticum* is somewhat lower. The %G+C of *Asteroleplasma anaerobium* is somewhat higher than all *Anaeroplasma* species to which they are phylogenetically only distantly related (Robinson and Freundt, 1987; Weisburg *et al*, 1989; Toth *et al,* 1994). These groupings are supported by phenotypic evidence, principally because *An. abactoclasticum* is the only species within the genus *Anaeroplasma* not able to lyse the cell walls of Gram-negative bacteria and being non-proteolytic (Robinson and Freundt, 1987). *Asteroleplasma anaerobium* is unique among the ruminal mycoplasmas in being able to grow in the absence of sterols (Robinson and Freundt, 1987).

2.6.4 Pathogenicity of *Anaeroplasma* spp.

Robinson and Hungate (1973) and Robinson and Allison (1975) reported that they observed no deaths in chicken embryos inoculated with active cultures of *Acholeplasma* (syn. *Anaeroplasma*) *bactoclasticum.* Likewise, Truscott (1981) reported no diseases of cattle which could be attributed to the presence of *Anaeroplasma* spp. among their ruminal microflora.

2.6.5 Metabolism of *Anaeroplasma intermedium* and *Asteroleplasma anaerobium*

Cell-free extracts of *An. intermedium* 5LA and *As. anaerobium* 161^T were examined for enzymes of the Embden-Meyerhof-Parnas (EMP) pathway leading from glucose to the triose phosphates and from 3-phosphoglyceric acid to pyruvate. It was shown that *An.*

intermedium 5LA possessed all eight enzymes of the EMP pathway whereas *As. anaerobium* lacked hexokinase activity (Petzel *et al*, 1990). The phosphofructokinase (PFK) of *As. anaerobium* was ATP-dependent in common with most bacteria and *Mycoplasma* spp., but the PFK of *An. intermedium* was PP_i -dependent, in common with *Acholeplasma laidlawii* and the propionibacteria (Petzel *et al*, 1990). The lactic dehydrogenase of both microorganisms was activated by fructose 1,6-diphosphate. *An. intermedium* 5LA and *As. anaerobium* 161^T can therefore be distinguished from each other by their enzyme activities. The occurrence of 3 uncommon PP_i-dependent kinases among the ruminal mycoplasmas may also be useful in determining their phylogenetic relationship to other mycoplasmas, and to the Gram-positive bacteria (Petzel *et al*, 1990).

2.6.6 Non-Ruminal Anaerobic Mycoplasmas

Anaerobic mycoplasmas, which did not require sterol and were abacteriolytic, were isolated from the intestinal tract of pigs (Binder and Kirchhoff, 1988). Like other mycoplasmas, the surface colonies were 'fried egg' in appearance, growth was not inhibited by penicillin and they were bounded by a trilaminar membrane without a distinguishable cell wall (Binder and Kirchhoff, 1988). Resistance to digitonin and growth in the absence of cholesterol, placed them into the genus *Asteroleplasma.* These isolates from the pig were not thought to be *Asteroleplasma anaerobium* , but probably belonged to a new species since they were serologically distinct from anaerobic mycoplasmas previously isolated from the pig, or from the gut of ruminants (Binder and Kirchhoff, 1988). However, they commented that ..."the primary cultivation of these mollicutes was possible only under strict anaerobic conditions.." but then .."with increasing passage the organisms became less sensitive to oxygen." (Binder and Kirchhoff, 1988). Since the fundamental requirement for mollicutes to be classified within the genus *Asteroleplasma* is an absolute need for anaerobic growth conditions, their second admission suggests that this isolate was perhaps a member of the facultative, sterol non-requiring genus *Acholeplasma,* rather than the obligately anaerobic, sterol non-requiring genus *Asteroleplasma* within the class *Mollicutes*.

Two species of obligately anaerobic mycoplasma were the predominant microbes in a methanogenic, glucose-limited, enrichment culture obtained from a sewage sludge

digester (Rose and Pirt, 1981). In pure culture, one of the anaerobic mycoplasmas, tentatively named *Anaeroplasma* sp. strain London, fermented glucose primarily to butyric acid, H_2, and CO_2 while the other produced CH_4 from H_2 and CO_2 and was named *Methanoplasma elizabethii*. Both were classified within the family *Mycoplasmataceae* on the basis of colonial and cellular morphology, ability to pass through 0.45 μm filters, and resistance to penicillin (Rose and Pirt, 1981). Neither microorganism appears in the current literature on the classification of the anaerobic mycoplasmas.

3 MATERIALS AND METHODS

3.1 INTRODUCTION

All chemicals were of analytical grade and obtained from British Drug Houses, UK, unless stated otherwise. All antibiotics were supplied by Sigma Chemicals, USA. All dehydrated microbiological media was supplied by Difco Laboratories, USA. Other chemicals or reagents are as acknowledged in the text.

Unless stated otherwise, all media were prepared under stringent anaerobic conditions using the procedure of Hungate (1969), utilising a gas phase of 100% O_2-free CO_2. All components of the medium, except the reducing agent L-cysteine-HCl, were boiled to remove dissolved O_2 and then saturated with O_2-free CO_2 while cooling to 45°C (agar), or in ice-water (broth), when the L-cysteine-HCl was added. Agar media were dispensed 5 ml per tube and broth media 10 ml per tube in Hungate culture tubes (Bellco Glass, Inc., NJ, USA), under 100% O_2-free CO_2 and sterilised by autoclaving at 121°C for 20 min. All media had a final pH of 6.4-6.6 unless stated otherwise.

Clarified rumen fluid was prepared when ruminal digesta was removed from a Friesian cow via the rumen fistula, strained through several layers of cheesecloth to remove large particulate matter, and clarified by centrifugation at 16,000 x g for 15 min at 4°C. Clarified rumen fluid was stored at -20°C until required.

Mineral solution 1 consisted of: KH_2PO_4 (6.0 g), NaCl (12.0 g), $(NH_4)_2SO_4$ (12.0 g), $CaCl_2\ 2H_2O$ (1.58 g), $MgSO_4 \cdot 7H_2O$ (2.5g) and distilled water to 1,000 ml.
Mineral solution 2 consisted of: K_2HPO_4 (6.0 g) and distilled water to 1,000 ml.

Salt solution A consisted of: KH_2PO_4 (3.0 g), NaCl (6.0 g), $(NH_4)_2SO_4$ (1.5 g), $CaCl_2\ 2H_2O$ (0.79 g), $MgSO_4 \cdot 7H_2O$ (1.2 g) and distilled water to 1,000 ml.
Salt solution B consisted of: K_2HPO_4 (6.0 g) and distilled water to 1,000 ml.

3.2 Isolation of Ruminal Mycoplasmas

All of the ruminal mycoplasma cultures isolated in this study were obtained from the ruminal digesta of non-lactating, hay-fed Friesian cows at AgResearch, Grasslands Research Centre, Palmerston North. The ruminal digesta were removed via the rumen fistula and immediately processed in the laboratory.

3.2.1 Media for the Isolation and Purification of Ruminal Mycoplasmas

Primary Isolation Medium (PIM) consisted of: clarified rumen fluid (40.0 ml), mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (52.5 ml), glucose (0.05 g), cellobiose (0.05 g), starch (0.05 g), tryptone (0.2 g), yeast extract (0.1 g), resazurin (0.1 mg), autoclaved *E. coli* cells (0.5% w/v), Na_2CO_3 (0.4 g), benzylpenicillin (Na^+ salt, 1 x 10^5 IU), L-cysteine-HCl (50 mg) and agar (1.5 g).

Clarified Rumen Fluid Agar (CRFA) and Broth (CRFB) consisted of: clarified rumen fluid (40.0 ml), mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (52.5 ml), glucose (0.2 g), cellobiose (0.2 g), starch (0.2 g), tryptone (0.2 g), yeast extract (0.1 g), resazurin (0.1 mg), Na_2CO_3 (0.4 g), L cysteine-HCl (50 mg) and agar (1.4 g in CRFA).

The *E.coli* used in PIM and used also to supplement CRFB for the bacterial cell-wall lysis studies, was grown aerobically in Brain Heart Infusion Broth (3.7% w/v), for 24 hr at 39°C. Cells were pelleted by centrifugation (15,000 x g for 10 min), resuspended and washed once in phosphate-buffered saline, autoclaved at 121°C for 20 min, cooled to room temperature, recentrifuged and stored as a pellet at -20°C until required.

Ruminal mycoplasmas were isolated from agar roll tubes using the method of Hungate (1969) and the *E. coli*-containing PIM agar medium of Robinson *et al* (1975). The medium was kept at 45°C in a water bath prior to inoculation with ruminal digesta. Sample dilution was performed using hypodermic syringes (1 ml) and needles (21 gauge x 2.5 cm) which were flushed with O_2-free CO_2 prior to each transfer. Particulate plant material in the inoculum often blocked the hypodermic needle during the first transfer, so a reversed sterile 1 ml glass pipette was used for this dilution. In this case the septum

was removed from the first tube of each dilution series to allow the inoculum to be added from the pipette, the septum was replaced and this initial transfer serially diluted to 10^{-10}. Following inoculation, the molten agar tubes were rolled under cold water by hand to solidify the agar in a thin film over the entire internal surface of the tube. All tubes were incubated vertically at 39°C.

After 4 days incubation, discrete colonies could be seen in the roll tubes at dilutions of 10^{-5} and greater. Well separated colonies in the 10^{-7} dilution tubes were picked from the agar using a Pasteur pipette with a tip bent to 90°. By gentle suction using a mouth tube, the colony was drawn into the pipette from where it was transferred to CRFB medium supplemented with benzylpenicillin (800 IU ml^{-1}) and streptomycin sulphate (80 μg ml^{-1}). The change from PIM medium was necessary because although PIM was a suitable medium to isolate ruminal mycoplasmas in agar, turbidity due to the *E.coli* component of the medium made it unsuitable for OD_{600} growth measurements. The two media were essentially the same but with differing carbohydrate concentrations. After 24 hr incubation the CRFB cultures were used to inoculate fresh roll tubes of CRFA medium and a further dilution series prepared. These were again incubated for 4 days, when colony picking was repeated into a further series of CRFA medium, devoid of antibiotics. The culture tubes were rolled as before and incubated at 39°C. This procedure was repeated twice more to ensure pure cultures were obtained.

3.3 Characteristics of Ruminal Mycoplasmas

All isolates obtained during the current study were characterised using the anaerobic procedures of Hungate (1969) and the tests outlined below.

3.3.1 Morphology of Ruminal Mycoplasma Cells and Colonies

The colonial morphology of isolates growing on CRFA medium was established by examination of 72 hr cultures using a 20 x dissecting microscope (Olympus Optical Co. Ltd., Japan). Morphology and motility of individual mycoplasma cells growing in CRFB medium was determined by examination of wet mounts using a Vanox AHBT3 microscope (Olympus Optical Co. Ltd., Japan). Wet mounts of each isolate were prepared under cover slips and examined using phase contrast microscopy and by Nomarski Differential Interference Microscopy at final magnifications of 400 x (dry)

and 1,000 x (oil). Dried and heat-fixed films of cells, stained using Hucker's modification of the Gram stain (Hucker, 1922), were examined at a final magnification of 1,000 x (oil). Details of CRFA and CRFB medium are given in section 3.2.1

Isolates RM10 and RM14 were examined by transmission electron microscopy (TEM) to determine cell morphology and ultrastructure. Cells were fixed in a mixture of 3% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M phosphate buffer pH 7.2, then left for 2 hr at room temperature, mixed with a 20% (w/v) solution of BSA in water and centrifuged to produce a pellet. The pellet was sliced, washed twice in 0.1 M phosphate buffer pH 7.2. and given a secondary fix in 1% OsO_4 in the same buffer for 30 min at room temperature. The fixed pellet was washed twice more in buffer, dehydrated by passage through a graded acetone series, infiltrated in a 50/50 mixture of Procure 812 epoxy resin with acetone and left on a stirrer overnight. Fresh resin (100%) was used to infiltrate the samples for a further 8 hr, after which the samples were embedded in silicone rubber moulds and left to cure at 60°C for 48 hours. Then 1 µm sections were cut and stained with Toluidine Blue and observed using light microscopy to locate the specimens. The block was trimmed to produce EM sections of 90-100 nm thickness. The sections were cut using a diamond knife then stained with uranyl acetate and lead citrate. The sections were observed using a Philips 201C Transmission Electron Microscope (The Philips Company, Eindhoven, The Netherlands).

Scanning Electron Microscope (SEM) studies of one isolate (RM13) were carried out to gain an understanding of the gross morphology of the cells and to assess if ruminal mycoplasmas played any part in the hydrolysis of cellulose. The fixation and dehydration of the samples was as for TEM, and critical point drying was done with liquid CO_2. Specimens were mounted on aluminium stubs, sputter-coated with gold and examined using a Cambridge Model 250 MkIII Scanning Electron Microscope (Cambridge Instruments, UK).

3.3.2 Filtration of Ruminal Mycoplasma Cells

Cultures of ruminal mycoplasmas grown for 24 hr in CRFB medium were filtered through "Supor Acrodisc 32", 0.2 μm syringe filters (Gelman Sciences, MI, USA) and "Minisart NML" 0.45μm syringe filters (Sartorius AG, Germany). Following filtration, 0.5 ml of filtrate was directly injected into pre-warmed CRFB medium at 39°C. Cultures were incubated vertically and unshaken at 39°C for 24-48 hr, and growth assessed by measurement at OD_{600} using a Spectronic 20D spectrophotometer. Details of CRFB medium are given in section 3.2.1.

3.3.3 Relationship between Temperature and Growth of Ruminal Mycoplasmas

Determination of the relationship between temperature and the growth of mycoplasma isolates was carried out in CRFB medium using an Arrhenius Block (Elliot, 1963) to provide temperatures in the range 20°C to 65°C. The actual temperatures of incubation were 20, 25, 31, 34, 36.5, 39.5, 42, 45, 47.5, 52, 58, and 65°C. All culture tubes were incubated in a vertical position and not shaken. Growth was assessed at intervals over a 24 hr period by measurement of OD_{600} made against a medium blank.

3.3.4 Effect of pH on Growth of Ruminal Mycoplasmas

The effect of pH on the growth of ruminal mycoplasmas was determined in CRFB medium where the pH had been adjusted by adding 2M HCl or 2M NaOH, prior to dispensing the medium into Hungate culture tubes. The pH was monitored after autoclaving using a PHM62 pH meter (Radiometer A/S, Denmark). Media at nine pH values between pH 5.0 and pH 8.75 were tested. An inoculum of 0.2 ml of each ruminal mycoplasma grown in CRFB medium, was injected into the test-media and the cultures incubated vertically and unshaken at 39°C. Growth was assessed after 10 hr by changes in OD_{600}. All measurements were made against a medium blank. Details of CRFB medium are given in section 3.2.1.

3.3.5 Growth Substrates for the Ruminal Mycoplasma Isolates

The range of substrates capable of supporting growth of ruminal mycoplasma isolates was determined in Basal Centrifuged Rumen Fluid Broth (BCRFB) containing one of the following: L(+) arabinose, D(+) cellobiose, cellulose, D(-) fructose, D(+) galactose,

D(+) glucose, glycerol, lactose, D-maltose, D(+) mannose, D(+) raffinose, D-salicin, starch, sucrose or D(+) xylose to a final concentration of 0.1%. BCRFB consisted of: clarified rumen fluid (40.0 ml), mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (52.5 ml), tryptone (0.2 g), yeast extract (0.1 g), resazurin (0.1 mg), Na_2CO_3 (0.4 g) and L-cysteine-HCl (50 mg). The substrates were made up as stock solutions (2% w/v) in Anaerobic Buffer Solution (ABS) and sterilised by autoclaving at 115°C for 10 min. ABS consisted of: salt solution A (17 ml), salt solution B (17 ml), distilled water (66 ml), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg) was prepared by the same method used for anaerobic media (see section 3.1). Culture media (9.5 ml) was supplemented with each substrate to a final concentration of 0.1% (w/v) by the addition of 0.5 ml of substrate stock solution. All media were inoculated with 0.1 ml of isolate grown to mid-log phase in CRFB medium (OD_{600} of 0.1) and the cultures incubated 16 hr at 39°C. Growth was assessed by OD_{600} readings made against a medium blank. Growth was measured in cellulose cultures, by analysis of the microbial protein concentration, using the method of Bradford (1976). The pH of all media was monitored after growth.

3.3.6 Analyses of Fermentation End-Products

Fermentation products of the ruminal mycoplasmas were identified qualitatively by HPLC (Bio-Rad Laboratories, USA), using a modified method of Joblin *et al* (1990). Cultures were grown for 24 hr in Cellobiose Rumen Fluid Broth (CbRFB) medium which consisted of: clarified rumen fluid (30 ml), salt solution A (17 ml), salt solution B (17 ml), distilled water (36 ml), cellobiose (0.2 g), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg). Analyses were carried out isocratically using two Bio-Rad Aminex HPX-87H (300 mm x 7.8 mm) ion exclusion columns in series. The mobile phase, 5 mM H_2SO_4, was maintained at a constant flow rate of 0.6 ml min^{-1}. A column temperature of 65°C was maintained throughout. Fermentation products were detected by UV absorption at 210 nm, and a qualitative assessment of end products made by comparison with standards of known retention time.

3.3.7 Analyses of Fermentation Gases

Headspace analyses of cultures were performed using the method of Wallace and Joblin (1985). The H_2 concentration in the culture headspace was determined using an

Aerograph 660 Gas Chromatograph (GC) (Varian Instruments, USA) fitted with a thermal conductivity detector. Gases were separated on a Porapak Q column at ambient temperature, using O_2-free N_2 as the carrier gas at a flow rate of 10 ml min^{-1}. Following incubation, all cultures were equilibrated to room temperature and the fermentation gas volume measured by displacement of a well-lubricated 10 ml glass syringe plunger. A 0.3 ml sample of headspace gas was injected onto the GC, previously calibrated using pure H_2. Peak heights were compared with those of known volumes of pure H_2 and these were used to determine H_2 production.

3.3.8 Antibiotic Sensitivity of Ruminal Mycoplasmas

The ruminal mycoplasma isolates were tested for their antibiotic sensitivities using a method similar to that of Robinson *et al* (1975) but with additional antibiotics included. Studies were carried out in CRFB medium. Details of the antibiotics tested are given in Table 3.1. Stock antibiotic solution (0.1 ml) was added to 9.9 ml of the growth medium. All media were inoculated with 0.3 ml of the isolates grown to an OD_{600} of 0.1 in CRFB medium. Cultures were incubated vertically and unshaken at 39°C and growth assessed after 24 hr by measurement of OD_{600} in a Spectronic 20D Spectrophotometer (Milton Roy Co., NY, USA). Details of ABS are given in section 3.3.5.

Table 3.1 Antibiotic stock solutions and final concentration of each antibiotic in media.

Antibiotic	Stock Solution	Solvent	Final Concentration
Bacitracin	100 mg ml^{-1}	ABS[a]	1.0 mg ml^{-1}
Benzylpenicillin	2 mg ml^{-1}	ABS	20 µg ml^{-1}
Chloramphenicol	2 mg ml^{-1}	ABS	20 µg ml^{-1}
D-Cycloserine	500 µg ml^{-1}	ABS	5.0 µg ml^{-1}
Digitonin[b]	12 mg ml^{-1}	ABS	60 µg ml^{-1}
Lincomycin-HCl	2 mg ml^{-1}	ABS	20 µg ml^{-1}
Streptomycin SO_4^{2+}	2 mg ml^{-1}	ABS	20 µg ml^{-1}
Tetracycline[c]	2 mg ml^{-1}	methanol/ABS	20 µg ml^{-1}
Thallium (1) acetate	200 µg ml^{-1}	ABS	2.0 µg ml^{-1}

[a]Anaerobic Buffer Solution (ABS).[b]Only 50% pure by TLC; [c]Dissolved in 1 ml of methanol and made to 10 ml with ABS.

3.3.9 Lysis of Bacteria by Ruminal Mycoplasmas

The capability of each isolate to lyse bacterial cell walls was examined using two methods.

Serial dilutions from 10^{-1} to 10^{-10} of each isolate in CRFA medium (incorporating 0.5% w/v killed *E. coli* XL1 cells) (see section 3.2.1), were used to examine the ability of the isolates to lyse cell walls of the Gram-negative bacterium *E .coli.* After incubation at 39°C for 7 days, the roll tubes were examined for clearing zones in the *E. coli* suspension surrounding the mycoplasma colonies.

Autoclaved ruminal Gram-negative bacteria were also incorporated into CRFA medium (0.5% w/v). The Gram-negative ruminal bacteria tested were *Butyrivibrio fibrisolvens* H17C, *Lachnospira multiparus* D32, *Prevotella ruminicola ruminicola* ATCC 19189 and *Succinovibrio dextrinosolvens* 24. The Gram-positive ruminal bacterium *Streptococcus bovis* JB1 was included as a negative control. Details of CRFA medium are given in section 3.2.1.

Autoclaved *E. coli* XL1 and autoclaved ruminal bacteria were added to 24 hr broth cultures of ruminal mycoplasmas (OD_{600} of 0.1) in CRFB medium to a final OD_{600} of 0.25. The cultures were incubated for a further 96 hr, mixed by inversion and the OD_{600} measured to determine bacterial cell lysis. All readings of OD_{600} were taken against a medium blank. The ruminal bacteria tested were *Butyrivibrio fibrisolvens* H17C, *Lachnospira multiparus* ATCC 19207, *Megasphaera elsdenii* T81, *Prevotella ruminicola ruminicola* ATCC 19189, *Selenomonas ruminantium ruminantium* HD4 and *Succinovibrio dextrinosolvens* 24. Tubes of media, with autoclaved bacteria but without ruminal mycoplasmas, were used as controls and the Gram-positive ruminal bacterium *Streptococcus bovis* JB1 was included as a negative control. Details of CRFB medium are given in section 3.2.1.

3.3.10 Proteolysis by Ruminal Mycoplasmas

A modification of the CRFB medium of Robinson *et al* (1975), containing agar (1.4% w/v), casein hammersten (0.2% w/v) and starch (0.1% w/v) was used to detect proteolysis by ruminal mycoplasmas. Cultures of each isolate (OD_{600} of 0.1) in CRFB medium, were serially diluted through the agar medium to obtain separate colonies. Following inoculation by syringe, the culture tubes were rolled under a stream of cold water until the agar solidified. After incubation at 39°C for 4 days, the casein suspension was examined for clearing zones surrounding the mycoplasma colonies, indicating casein hydrolysis. Details of CRFB media are given in section 3.2.1.

3.3.11 Lysis of Fungal Cell-Walls by Ruminal Mycoplasmas

Large scale cocultures of the fungus *Neocallimastix frontalis* and the methanogen *Methanobrevibacter smithii* were grown in RFCbB medium to provide fungal cell-wall material for inclusion in Fungal Cell Wall Agar (FCWA) medium. Fungal growth was more luxuriant in the presence of *M. smithii*. RFCbB medium consisted of: salt solution A (340 ml), salt solution B (340 ml), distilled water (720 ml), clarified rumen fluid (600 ml), cellobiose (10 g), $NaHCO_3$ (10 g), resazurin (2.0 mg) and L-cysteine-HCl (1.0 g). After 4 days growth, the fungal tissue was harvested by low speed centrifugation, washed in sterile distilled water and ball-milled for 7 days at 4°C as a 5% w/v suspension. The suspension was incorporated into FCWA medium which consisted of: clarified rumen fluid (40.0 ml), mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (42.5 ml), ball-milled fungal cell wall suspension (10 ml), tryptone (0.2 g), yeast extract (0.1 g), resazurin (0.1 mg), Na_2CO_3 (0.4 g), L-cysteine-HCl (50 mg) and agar (1.4g). Each isolate (0.2 ml) was serially diluted (10^{-1} to 10^{-10}) by syringe in FCWA medium and the agar rolled within the tubes. After 21 days incubation at 39°C, the cultures were examined for any appearance of clearing zones in the fungal cell wall suspension surrounding the mycoplasma colonies.

3.3.12 Chitinase Activity of Ruminal Mycoplasmas

Dilution tubes of Chitin Starch Agar (CtSA) and CtSA lacking added starch were prepared in roll tubes. The media were inoculated with 0.2 ml of each mycoplasma isolate grown to an OD_{600} of 0.1 in CRFB medium. The cultures were serially diluted

(10^{-1} to 10^{-10}) by the transfer of 0.5 ml of inoculum. CtSA consisted of: clarified rumen fluid (40.0 ml), mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (50.5 ml), 3.33% chitin suspension (2 ml), soluble starch (50 mg), tryptone (0.2 g), yeast extract (0.1 g), resazurin (0.1 mg), Na_2CO_3 (0.4 g), L-cysteine-HCl (50 mg) and agar (1.5 g). To prepare the chitin suspension, 10 g of chitin (Sigma C-7170) was ball-milled in 300 ml of distilled water for 4 days at 4°C, then dialysed against 3 changes of distilled water over 3 days at 4°C to remove soluble sugars and salts. The cultures were incubated for 21 days at 39°C and examined using a dissecting microscope to detect zones of clearing in the chitin surrounding the ruminal mycoplasma colonies. Details of CRFB medium are given in section 3.2.1.

3.3.13 Sterol Requirements of Ruminal Mycoplasmas

The sterol requirements of ruminal mycoplasmas were determined using the non-rumen fluid, Modified Medium 10 (MM10) of Robinson *et al* (1975), a modification of Medium 10 of Caldwell and Bryant, (1966). MM10 consisted of: mineral solution 1 (3.75 ml), mineral solution 2 (3.75 ml), distilled water (92.2 ml), soluble starch (0.2 g), glucose (0.2 g), cellobiose (0.2 g), tryptone (0.2 g), yeast extract (0.05 g), volatile fatty acid (VFA) solution (0.31 ml), resazurin (0.1 mg), Na_2CO_3 (0.4 g) and L-cysteine-HCl (50 mg). The VFA solution consisted of: acetic acid (17.0 ml), propionic acid (6.0 ml), n-butyric acid (4.0 ml), iso-butyric acid (1.0 ml), n-valeric acid (1.0 ml), iso-valeric acid (1.0 ml) and DL-α-methylbutyric acid (1.0 ml). The VFA solution was stored at -20°C. To establish the sterol requirements of the isolates, MM10 was supplemented with *E. coli* 055:B5 lipopolysaccharide (LPS) B (Boivin) (Difco) and cholesterol to final concentrations of 0.25 mg ml^{-1} and 20 μg ml^{-1} respectively (Robinson and Allison, 1975). The cholesterol stock solution contained: cholesterol (20 mg) dissolved in 1 ml of ethanol and diluted to 10 ml with Anaerobic Buffer Solution (ABS). This was added to the MM10 prior to autoclaving. The serum bottle containing the *E. coli* LPS (100 mg) was gassed with O_2-free CO_2 before ABS (10 ml) was added by injection. The LPS solution was sterilised by passage through a 0.45μm membrane filter before addition to the sterile MM10. Ruminal mycoplasmas were grown in CRFB medium to mid-log phase, centrifuged and washed in ABS, recentrifuged and resuspended in ABS to an OD_{600} of 0.1. The cells were washed to prevent carry-over of sterol-like material from the rumen fluid in CRFB medium. MM10 and MM10 with added cholesterol and/or

lipopolysaccharide were inoculated with 0.2 ml of the washed mycoplasmas. The extent of growth stimulation in each of the supplemented media was determined by measurement of the OD_{600} after 24 hr incubation at 39°C and confirmed by cellular protein measurements using the method of Bradford (1976). Details of CRFB medium are given in section 3.2.1. Details of ABS are given in section 3.3.5.

To reduce the level of sterol contamination in MM10, a sterol-free anaerobic solution of yeast extract (2%) was prepared. This solution consisted of: yeast extract (2 g) in salt solution A (17 ml), salt solution B (17 ml), distilled water (66 ml) and $NaHCO_3$ (0.5 g) and was extracted five times with diethyl ether in a separating funnel to remove lipids. Following extraction, the aqueous solution was evacuated briefly to remove residual ether, boiled to remove dissolved O_2 and cooled under O_2-free CO_2, in an ice-water bath. 50 mg of L-cysteine-HCl was added, the solution dispensed in 10 ml volumes and sterilised by autoclaving at 121°C for 20 min. To further lower sterol contamination of the medium, a sterol-free anaerobic solution of tryptone (2% w/v) was similarly prepared for inclusion into MM10. These solutions were incorporated into MM10 in place of their solid equivalents and the volume of water in the medium adjusted accordingly.

3.3.14 RAPD Analysis of Ruminal Mycoplasma DNA

Ruminal mycoplasmas were grown in CRFB medium overnight, to a final OD_{600} between 0.1 and 0.12, and the cells pelleted in a Sorvall RC2B Refrigerated Centrifuge fitted with a Sorvall SS34 head (15,000 x g for 15 min at 4°C). Uninoculated medium was treated in the same way to provide a control for the RAPD analysis. Pelleted cells were washed twice by resuspending in TE buffer, centrifuged (15,000 x g for 15 min at 4°C) and then resuspended in TE buffer to a cell density of approximately 10^8 ml^{-1}. The TE buffer consisted of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) in distilled water. Details of CRFB medium are given in section 3.2.1.

DNA was extracted from the pelleted cells using DNAzol reagent (Gibco BRL, NY, USA). Approximately 3 x 10^7 cells (determined by Thoma cell count) of each isolate were transferred to a sterile 1.5 ml microcentrifuge tube, 1.0 ml of DNAzol reagent was added and the cells and reagent mixed by gentle pipetting, which allowed the cells to

lyse and the DNA to be released. Shearing effects on the DNA were minimised by the use of wide mouthed pipette tips. Samples were centrifuged at 10,000 x g for 10 min at 4°C to remove insoluble cell fragments, RNA and polysaccharides from the homogenate. The resulting viscous fluid was transferred to a fresh 1.5 ml microcentrifuge tube. To each 1.0 ml of DNAzol/ cell homogenate, 0.5 ml of 99-100% ethanol was added. The solutions were mixed by inversion and stored 3 min at room temperature. DNA appeared as a cloudy precipitate. This was pelleted by centrifugation at 10,000 x g for 2 min at 4°C and the DNA washed with 1.0 ml of 70% ethanol. The wash solution was carefully removed by decanting, and the pellet dried *in vacuo* at room temperature. DNA samples were rehydrated in 200 µl of sterile TE buffer and allowed to redissolve overnight at 4°C. An equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1, v/v/v) was added to each sample and mixed gently. Samples were centrifuged 10,000 x g for 2 min at 4°C. The aqueous (top) phase was removed for further treatment, taking care to leave the proteinacious interface behind. The phenol/chloroform/iso-amyl alcohol treatment was repeated until the interface was clear. The top layer was again retained and an equal volume of chloroform iso-amyl alcohol added, the samples were mixed and recentrifuged. To the aqueous phase (top layer), 1/10 volume 3M NaOAc pH 5.2 and 1.0 ml of 99-100% ethanol were added. All samples were left at -20°C overnight to allow the DNA to precipitate. The samples were centrifuged for 20 min at 10,000 x g at 4°C to pellet the DNA, the supernatant was removed, the DNA rinsed with 70% ethanol, air dried *in vacuo* and resuspended in 200 µl of sterile TE buffer.

The purity and concentration of the DNA was estimated by measurement of the OD_{260} and OD_{280} using a SpectraMAX 250 microtitre plate reader (Molecular Devices, CA, USA).

RAPD methodology was used to demonstrate polymorphisms in DNA of the ruminal mycoplasmas using the method of Gardiner *et al* (1996). Random primers of 10 nucleotides each (10mers) were employed which had the following nucleotide sequences: OPAA04 (5'-AGGACTGCTC-3'), OPAA06 (5'-GTGGGTGCCA-3') and OPAA09 (5'-AGATGGGCAG-3') (Operon Technologies Inc., CA, USA).

A mastermix (MMix) of reactants was made to reduce pipetting errors and to provide consistency. All reagents were stored on ice. The MMix consisted of: *Taq* 10x buffer (48 µl), 50 mM Mg^{2+} (14 µl), 5 mM dNTP (19 µl), *Taq* DNA polymerase (6 µl) (all Gibco BRL, NY, USA), 16% formamide (48 µl) and sterile distilled water (249 µl). The MMix was divided into 3 equal volumes of 128 µl, and 16 µl of primer solution (2 µM), added to give a total volume of 144 µl. Each Polymerase Chain Reaction (PCR) comprised 18 µl of this solution, and 2 µl of ruminal mycoplasma DNA (3-4 ng) added as the final reactant.

PCR was performed using a Corbett FTS-320 Thermal Sequencer (Corbett Research, Australia), with a melting temperature of 94°C, an annealing temperature of 36°C and an elongation temperature of 72°C. The thermal sequencer was programmed to provide the following conditions: 94/4; [94/1, 36/1, 72/2] x 40; 72/10; 4/1 (°C / min). The final step held the PCR products at 4°C.

PCR products were separated on a 2% agarose gel comprising: 1% NuSeive agarose (FMC BioProducts, ME, USA) and 1% Ultrapure agarose, (Gibco BRL, NY, USA) in TAE buffer. TAE Buffer consisted of: Trizma base (48.4 g), glacial acetic acid (11.42 ml), 0.5 M EDTA (pH 8.0) (20 ml) and distilled water to 1,000 ml. The Loading Buffer consisted of: bromophenol blue (0.25 g), xylene cyanol FF (0.25 g), glycerol (30 ml) and distilled water to 100 ml. The solutions were stored at room temperature. Loading buffer (4 µl) was added to each PCR product prior to loading 18 µl of product/buffer onto the gel. A 100 bp ladder (Pharmacia Biotech, Sweden) was included for PCR product size-comparison purposes. PCR products were electrophoresed at 100 volts (about 150 mA) for 3.00 hr using a Model 1000/500 Power Supply (Bio-Rad Laboratories, CA, USA).

Following electrophoresis, gels were stained in an aqueous solution of ethidium bromide (5 µg ml^{-1}) for 10 min, followed by a wash in cold tap water for 1 hr. PCR products were visualised using a UV transilluminator (Spectronics Corporation, NY, USA) and photographed with Polaroid 667 (ISO 3000) black and white film (Polaroid, UK).

3.3.15 PAGE Analysis of Ruminal Mycoplasma Proteins

The method used to electrophoretically separate proteins according to their molecular size was that of Laemmli (1970). A polyacrylamide gel solution was polymerized between glass plates and placed between upper (anode) and lower (cathode) reservoirs of Tris-Glycine Reservoir Buffer (pH 8.3). The polyacrylamide gel comprised two parts; a lower running gel, overlaid by the stacking gel.

The running gel consisted of the following: Lower Tris Buffer (5.0 ml), Running Gel Solution (6.7 ml), distilled water (8.3 ml), NH_4^{2+} persulphate solution (0.1 ml) and TEMED (0.01 ml). The solutions were mixed thoroughly after each addition to ensure even polymerization. The gel solution was poured between the glass plates of the electrophoresis apparatus, and when the desired gel length (approximately 11 cm) was attained, distilled water was carefully pipetted on to the top of the mixture. The water had two functions; it allowed any bubbles on the gel surface to escape leaving a flat surface and provided anaerobic conditions, essential for complete gel polymerization. Polymerization was complete when an interface appeared between the gel and the water.

The stacking gel was composed of the following: Upper Tris buffer (2.5 ml), Stacking Gel Solution (1.5 ml), distilled water (6.0 ml), NH_4^{2+} persulphate solution (0.05 ml) and TEMED (0.01 ml). The distilled water overlaying the polymerized running gel was decanted and the surface of the gel rinsed with 1-2 ml of freshly prepared, but unpolymerised stacking gel solution. More stacking gel solution was added to fill the remaining space between the glass plates i.e. approximately 5 ml. A perspex comb used to form the sample wells was inserted into the stacking gel between the glass plates and the stacking gel solution left to polymerise for a further 5-10 min.

The solutions required to make the running and stacking gels were as follows: NH_4^{2+} persulphate solution which consisted of: NH_4^{2+} persulphate (0.1 g) and distilled water to 1.0 ml. This solution was freshly made for each polyacrylamide gel. Lower Tris Buffer which consisted of: Trizma Base (18.17 g), 10% solution of SDS in distilled water (4.0 ml) and distilled water to 100 ml. The Trizma Base was added to 70 ml of distilled water; the pH adjusted with 12M HCl to pH 8.8; the SDS solution added and the final volume made to 100 ml with distilled water. Running Gel Solution which consisted of:

acrylamide (30 g), methylene-bis-acrylamide (0.5 g) and distilled water to 100 ml. The solution was filtered through a single layer of Whatman No 1 filter paper prior to storage at 4°C. Stacking Gel Solution which consisted of: acrylamide (30 g) dissolved in 70 ml of distilled water which was allowed to return to room temperature before methylene-bis-acrylamide (1.6 g) was added, and the solution made to 100 ml. The solution was filtered through a single layer of Whatman No 1 filter paper prior to storage at 4°C. Upper Tris Buffer which consisted of: Trizma Base (6.06 g), 10% solution of SDS in distilled water (4.0 ml) and distilled water to 100 ml. The Trizma Base was added to 70 ml of the distilled water; adjusted with 12M HCl to pH 6.8; the SDS solution was added and the volume made to 100 ml with distilled water. TEMED is the acronym for N,N,N^{1},N^{1} tetramethylethylenediamine.

Tris-Glycine Reservoir Buffer consisted of: Trizma Base (6.07 g), glycine (28.8 g) and SDS (2.0 g) in distilled water. The pH was adjusted to pH 8.3, prior to the volume being made to 2,000 ml. The solution was stored at room temperature.

3.3.15.1 Extraction and Measurement of Mycoplasma Cellular Proteins

A batch culture of each ruminal mycoplasma was grown in 300 ml of CRFB medium for 24 hr at 39°C, then centrifuged at 15,500 x g for 10 min at 4°C. The cell pellet was washed twice in 0.15M phosphate buffered saline (PBS), recentrifuged and finally resuspended in 300 µl of PBS. An aliquot (0.1 ml) of ruminal mycoplasma cells was diluted 3 fold and 10 fold in 0.2 M NaOH and heated at 100°C for 3 min to release cellular proteins. The protein solutions were cooled to room temperature and further diluted 10 x in 0.2M NaOH to give 30 x and 100 x dilutions. A sub-sample (0.1 ml) from each of the four dilutions was mixed with 5 ml of Coomassie Blue Reagent (CBR) and transferred to spectrophotometer tubes. The OD_{595} was measured and the protein concentration determined from the standard curve.

The protein standard curve was prepared using a modification of the method of Bradford (1976). Bovine serum albumin (BSA) standards consisting of 0 to 100 µg in a total volume of 0.1 ml were prepared in 0.2 M NaOH and heated to 100°C for 3 min. The BSA solutions were cooled to room temperature and 5 ml of Coomassie Blue Reagent (CBR) added to each sample. The tubes were covered with Parafilm to prevent

contamination with other protein then mixed by inversion. A sub-sample of this solution was transferred to spectrophotometer tubes, or alternatively to microtitre plates and the OD_{595} measured. A standard curve for the determination of protein concentration was constructed.

The solutions required for this section comprised: Phosphate Buffered Saline (PBS) which consisted of: NaCl (8.5 g), Na_2HPO_4 (2.7 g), NaH_2PO_4 (0.39 g) and distilled water to 1,000 ml. The final pH was 7.2. Coomassie Blue Reagent (CBR), which consisted of: Brilliant Blue G-250 (0.1 g), 95% ethanol (50 ml), 85% (w/v) phosphoric acid (100 ml) and distilled water to 1,000 ml. The Brilliant Blue G-250 dye was dissolved in the mixture of ethanol and phosphoric acid and diluted to 1,000 ml with distilled water. The reagent was filtered through two layers of Whatman No 1 filter paper and stored in an amber-glass bottle at room temperature.

3.3.15.2 Electrophoresis of Mycoplasma Cellular Proteins

Aliquots of SDS-sample buffer (25 µl), and bromophenol blue tracking dye (12.5 µl), were added to 0.1 ml of disrupted ruminal mycoplasma cells and heated to 100°C for 3 min to solublise cellular protein. The solutions were cooled to room temperature, centrifuged at 4,000 x g for 15 min and then loaded into wells on the gel. 80 µg of protein, solublised in Bromophenol-Blue tracking dye, was added to each well. Glycerol 8% (v/v) present in the tracking dye ensured that samples settled to the bottom of each well. The volumes of lysate required are reported in the results and were different for each isolate. The position and identity of each lane was marked on the glass plate using a felt pen.

The solutions required for this section were: SDS-Sample Buffer which consisted of: β-mercaptoethanol (10 ml), SDS (6.0 g), Upper Tris Buffer (25 ml) and distilled water to 100 ml. Bromophenol Blue Tracking Dye which consisted of: bromophenol blue (0.25 g), glycerol (40 ml) and distilled water to 50 ml.

The protein lysates, in 50 µl increments, were loaded onto the stacking gel and electrophoresed at 15 mA for 60 min. The gel was run with the anode at the origin. During this time the wells were topped up with protein solution until the total protein

added to each lane reached 80 µg. When the tracking dye migrated to the stacking gel/running gel interface, the current was decreased to 10 mA. It was maintained at this value until the tracking dye was about 0.5 cm from the bottom edge of the running gel (approximately 4.5 hr).

Following electrophoresis, the glass plates were carefully separated and a reference mark made on the gel by removing the lower left hand corner. The gel was placed into the staining bath containing the Coomassie Blue Stain (CBS) and left to stain overnight. The gel was removed from the CBS solution and destained in 200 ml of 10% (v/v) acetic acid while being gently agitated on a rocking table. The acetic acid solution was replaced several times over the next 24 hr until the gel background was transparent, leaving only the protein bands stained blue. Finally the gel was washed in water to protect the optical surfaces of the imaging system from acetic acid fumes. Gel details were recorded using an Alpha Innotech Gel Doc Imaging System (Alpha Innotech Corporation).

Coomassie Blue Stain (CBS) consisted of: Brilliant Blue R-250 (0.4 g), isopropanol (250 ml) glacial acetic acid (100 ml) and distilled water to 1,000 ml. The stain was filtered through two layers of Whatman No 1 filter paper before being stored at room temperature.

3.4 COCULTURE STUDIES

3.4.1 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Bacteria

To determine if ruminal mycoplasmas interacted with cellulolytic bacteria, cocultures of each bacterium with each ruminal mycoplasma isolate were established in Paper Strip Medium (PSM). PSM consisted of: salt solution A (17 ml), salt solution B (17 ml), clarified rumen fluid (30 ml), distilled water (36 ml), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg). 80 mg of Whatman No.1 filter paper (cellulose) in 1 cm strips was added as sole carbon source. Bacterial cultures in PSM, without added ruminal mycoplasmas were used as controls. All of the cellulolytic bacteria were

obtained from the Rumen Microbiology Unit Culture Collection, AgResearch, Grasslands Research Centre, Palmerston North.

The bacterial isolates used in this study were; *Clostridium chartatabidum* DSM 5482, *Fibrobacter succinogenes* S85, *Ruminococcus albus* RA7 and *Ruminococcus flavefaciens* FD1

An inoculum of 0.2 ml of each bacterium, grown in Cellulose Broth (CsB) medium, was inoculated into pre-warmed PSM containing 80 mg of Whatman No. 1 filter paper per tube, and left 24 hr for the bacteria to establish. Then 0.2 ml of each ruminal mycoplasma isolate, grown overnight in CRFB medium, was inoculated into the bacterial cultures in PSM. All cocultures were gently mixed and incubated vertically and unshaken at 39°C for 7 days. The degree of interaction between the bacteria and the ruminal mycoplasmas was assessed by measuring H_2 production and by re-weighing the washed and dried paper residue following fermentation. CsB medium consisted of: salt solution A (17 ml), salt solution B (17 ml), distilled water (21 ml), clarified rumen fluid (30 ml), 2% cellulose suspension (15 ml), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg). The 2% cellulose suspension used in CsB medium was prepared by ball-milling 12 g of Whatman No.1 filter paper in 600 ml of distilled water for 96 hr at 4°C. The suspension was dialysed at 4°C against several changes of distilled water for 72 hr to remove soluble sugars.

3.4.2 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Fungi

To determine if ruminal mycoplasmas interact with ruminal fungi, cocultures of each fungus with each ruminal mycoplasma were established in PSM. These cocultures, with pure fungal cultures in PSM as controls, would allow the extent of ruminal mycoplasma inhibition of the fungus to be assessed. All ruminal fungi were obtained from the Rumen Microbiology Unit Culture Collection, AgResearch, Grasslands Research Centre, Palmerston North and were maintained on Sisal Broth (SB) medium, prior to the coculture studies. SB medium consisted of: salt solution A (17 ml), salt solution B (17 ml), clarified rumen fluid (30 ml), distilled water (36 ml), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg). A 2 cm piece of sisal twine (*Agave sisalone* L) was added as sole carbon source. Details of PSM are given in section 3.4.1.

The fungal cultures used in this study were; *Caecomyces communis* strain B7, *Neocallimastix frontalis* strain Rold, and *Piromyces communis* strain B19.

Zoospores of each fungus were inoculated from SB cultures (3 days old) into triplicate tubes of PSM, pre-warmed to 39°C and the cultures incubated for 24 hr to allow fungal zoospores to encyst and commence fungal growth. 0.2 ml of each CRFB-grown ruminal mycoplasma (OD_{600} of 0.1), was inoculated into the triplicate fungal cultures in PSM. All tubes were gently mixed by inversion and incubated vertically and unshaken at 39°C. After 4 days, the cultures were removed from the incubator and cooled to room temperature. Hydrogen in the head-space was determined using a GC. The final pH of the culture was measured as quickly as possible to avoid any change associated with loss of CO_2 to the atmosphere. The residual paper in each tube was washed twice with distilled water, dried *in vacuo* over phosphorus pentaoxide (P_2O_5) and reweighed.

3.4.3 Effect of Ruminal Mycoplasmas on *N. frontalis* Growing on Cellobiose.

The ruminal fungus *N. frontalis* and ruminal mycoplasmas were grown in cocultures on Rumen Fluid Cellobiose Broth (RFCbB) medium for 4 days at 39°C. RFCbB medium consisted of: salt solution A (17 ml), salt solution B (17 ml), distilled water (36 ml), clarified rumen fluid (30 ml), cellobiose (0.5 g), $NaHCO_3$ (0.5 g), resazurin (0.1 mg) and L-cysteine-HCl (50 mg). When fermentation was complete, the culture head-spaces were analysed for H_2 and the final culture pH recorded.

3.4.4 SEM Studies of Ruminal Mycoplasma RM13 in Coculture with *N. frontalis*

SEM studies were carried out to examine the morphology of isolate RM13 growing in coculture with the ruminal fungus *N. frontalis*. The methods employed to prepare the samples are given in section 3.3.1

3.5 ENUMERATION OF BOVINE RUMINAL MYCOPLASMAS

Ruminal mycoplasmas were enumerated on two occasions, 14 days apart, in the rumens of three non-lactating, Friesian cows being fed a diet of meadow hay *ad libitum*. Enumerations of total populations of ruminal mycoplasmas were carried out using serial dilutions of ruminal digesta from 10^{-1} to 10^{-10} in CRFA roll tubes containing benzylpenicillin (1,000 IU ml^{-1}). For these studies a new syringe was used for each transfer throughout the dilution series, to prevent inoculum carry-over. A second series of CRFA roll tubes with benzylpenicillin (1,000 IU ml^{-1}) and digitonin (60 μg ml^{-1}) was inoculated over the same dilution range. Digitonin inhibits the growth of the sterol-requiring *Anaeroplasma* species, but not the sterol non-requiring *Asteroleplasma anaerobium*. Details of CRFA medium are given in section 3.2.1.

4 RESULTS AND DISCUSSION

4.1 ISOLATION OF RUMINAL MYCOPLASMAS

Ruminal mycoplasmas were isolated from the ruminal digesta of non-lactating, rumen fistulated, hay-fed Friesian cows using the method of Robinson *et al* (1975). The PIM agar used for the isolations contained suspended, autoclaved *E. coli* cells (0.5% wet wgt. /v) which were important in distinguishing ruminal mycoplasmas which lysed Gram-negative bacterial cell walls from those which did not (Robinson *et al*, 1975). After incubation at 39°C for 4 days, colonies of both lytic and non-lytic mycoplasmas were observed up to the 10^{-7} dilution. The *E. coli* suspension gave the agar a slight opacity and lytic colonies were distinguished by zones of lysis in the *E. coli* suspension. Well separated colonies of both types were picked from the anaerobic agar. Colony selection followed by broth culture and further colony isolation was repeated twice more to obtain pure cultures for further study. Culture purity was confirmed by Gram staining of heat-fixed cells and by phase contrast observation of wet mounts. These showed no evidence of culture contamination. Using this approach, eight pure isolates were obtained but two subsequently failed to grow during sub-culturing. The six isolates, RM10, RM11, RM12, RM13, RM14 and RM15 were utilised in the present study.

4.2 CHARACTERISTICS OF RUMINAL MYCOPLASMAS

Anaerobic isolation and culture procedures were shown to be essential for the successful growth and study of all isolates. No ruminal mycoplasma grew in media deliberately exposed to O_2. Efforts were made to obtain cultures of known ruminal mycoplasmas for comparison purposes. However, type cultures of ruminal mycoplasmas are no longer available from the American Type Culture Collection (ATCC) or the culture collection at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) Germany. The type cultures originally deposited with the ATCC were *Anaeroplasma abactoclasticum* (ATCC 27879), *An. bactoclasticum* (ATCC 27112), *An. intermedium* (ATCC 43166), *An. varium* (ATCC 43167) and *Asteroleplasma anaerobium* (ATCC 27880).

Lyophilised type cultures of ruminal mycoplasmas were obtained from Dr Mark Rasmussen, United States Department of Agriculture, but these proved to be non-viable.

4.2.1 Morphology of Ruminal Mycoplasmas

After four days incubation at 39°C, sub-surface colonies of all isolates growing in CRFA medium were golden to fawn in colour, often multilobed, and approximately 0.6-0.8 mm in diameter (Fig 4.1). Surface colonies were approximately 1.0-1.5 mm in diameter, more lightly-coloured than sub-surface colonies, had umbonate elevation and were circular with an undulate edge (Fig 4.1). Surface colonies of ruminal mycoplasmas are often described in the literature as having a "fried-egg" appearance, and this description applies to ruminal, as well as non-ruminal mycoplasmas from a number of genera (Robinson and Freundt, 1987). In the present study, the colonies of all six isolates were similar and had a "fried-egg" appearance.

Phase contrast microscopy examination of the six isolates grown in CRFB medium showed that all had similar morphological features and therefore were not distinguishable using this procedure. Cells from 10 hr cultures of all isolates were coccoid, non-motile and between 0.5-1.5 μm in diameter. Older cells (48-72 hr) had a variety of pleomorphic forms including dumb-bell shaped cells (1-2 μm) and cells with small spherical surface-projections (Fig 4.2). All cultures (10 hr, mid to late log phase) gave a Gram-negative reaction when stained. In the absence of a cell wall, the cells retain the last stain used. These observations are in agreement with those of Robinson and Hungate (1973), and of Robinson *et al* (1975) who described the morphology of *Anaeroplasma* isolates. There is no definitive description in the literature of the cellular or colonial morphology of members of the genus *Asteroleplasma*, but it seems likely that these would be similar to *Anaeroplasma* species. Nomarski Differential Interference Microscopy (NDIM) is a technique which gives cells a three-dimensional appearance, because one side of the specimen appears lighter than the other as if light was falling on it and casting shadows (Rawlins, 1992). The method often gives surface and intracellular detail not observable using phase contrast microscopy. In the present study, the six mycoplasmas were observed using NDIM. This showed cellular detail

already seen by phase contrast microscopy, but revealed nothing new about the cellular morphology of ruminal mycoplasmas.

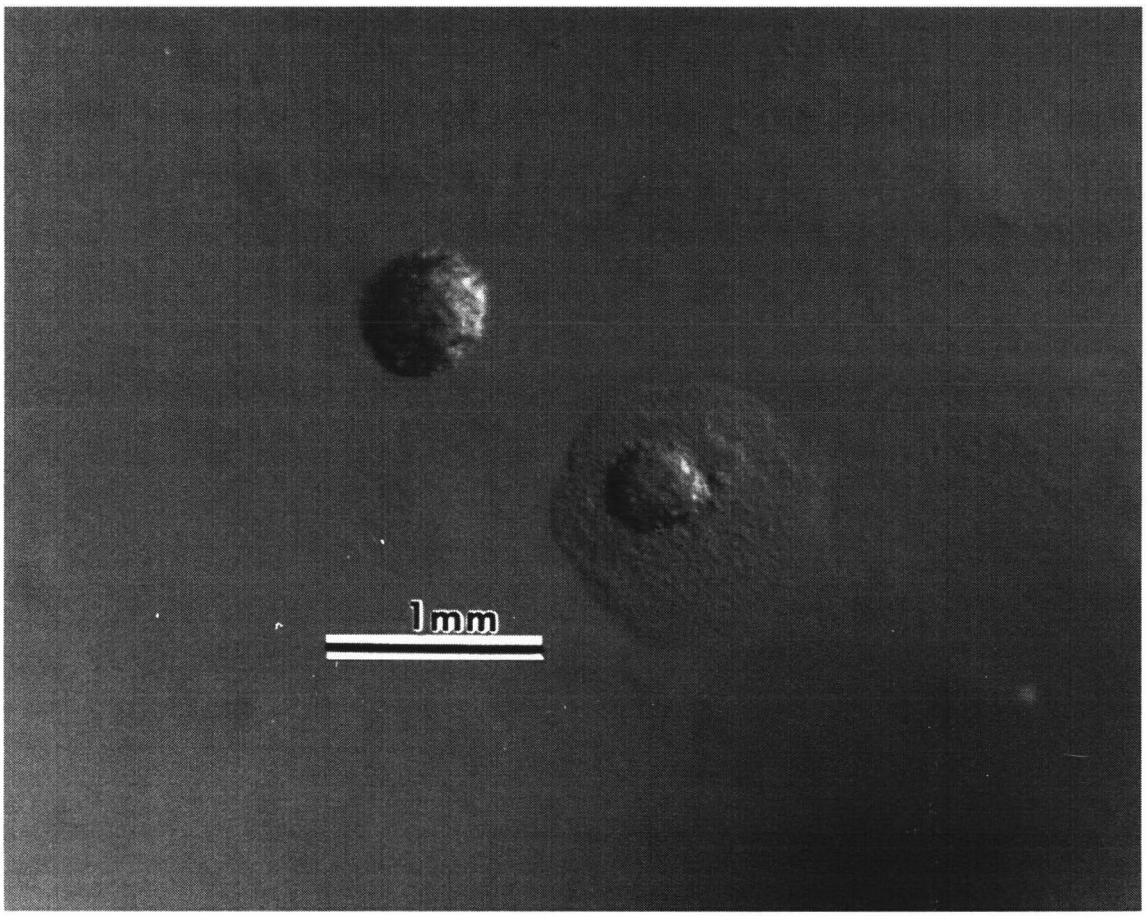

Fig 4.1 Subsurface (left) and surface (right) colonies of isolate RM10

Following multiple sub-culture, in the absence of antibiotics over a 30 month period, there was no evidence of any isolate reverting to a cell-walled bacterial form, which confirmed the isolates as members of the class *Mollicutes* and not bacterial L-forms.
L-forms of bacteria are generally regarded as protoplasmic elements not having a defined shape and lacking the rigid component of a cell wall. The loss of shape can be correlated with the loss of peptidoglycan constituents in the cell wall (Mandelstam and McQuillen, 1973).

Scanning electron microscope (SEM) studies of isolate RM13 were carried out to increase our knowledge of the morphology of ruminal mycoplasmas. A variety of cell morphologies were seen and cells like bi-concave discs, some similar to grains of wheat with longitudinal grooves, and others shaped like dumb-bells were observed. Some

cells appeared to be budding (Fig 4.2). There are no previous SEM studies of ruminal mycoplasmas reported in the literature.

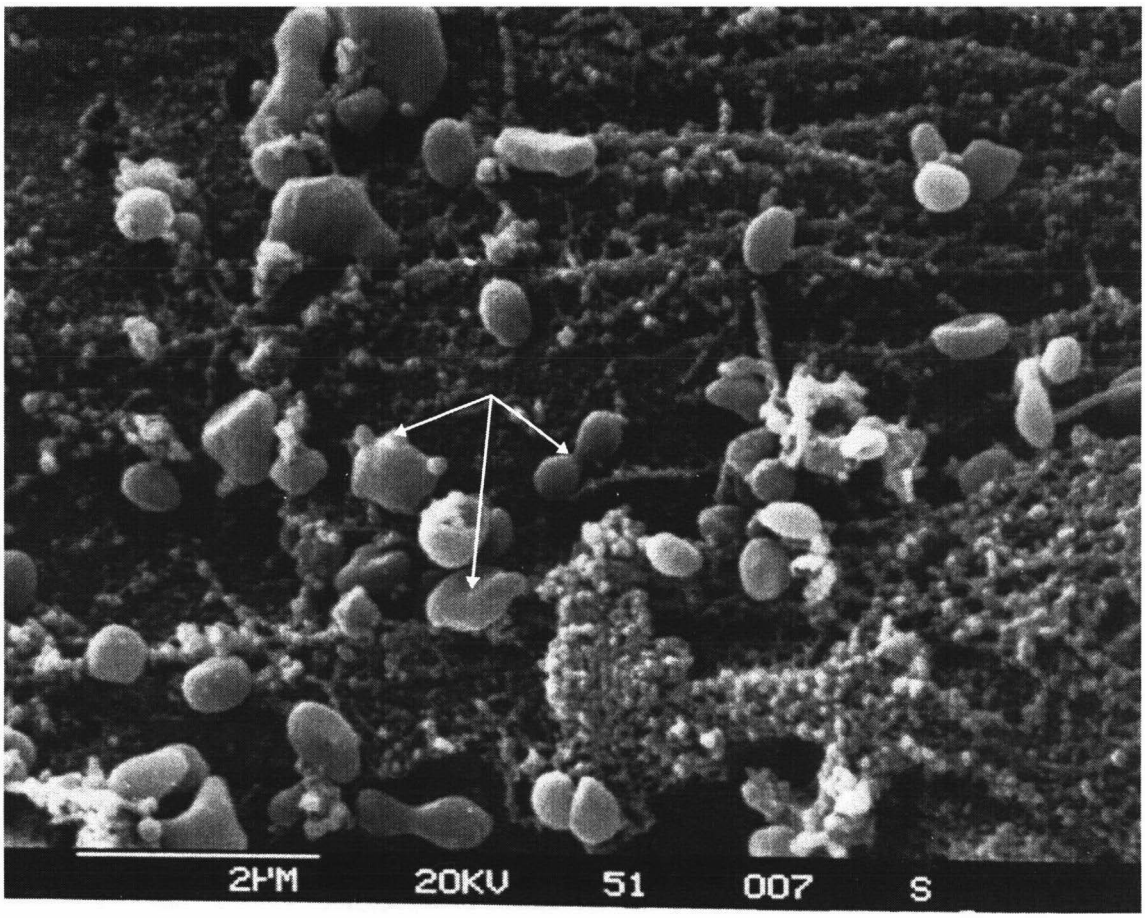

Fig 4.2 Scanning electron micrograph to demonstrate the pleomorphic nature of isolate RM13. Note the nodulated, doughnut and dumbbell forms of the cells (arrowed)

Transmission electron microscope (TEM) studies were conducted on thin sections of two of the isolates RM10 (Fig 4.3) and RM14 (Fig 4.4). The cells were devoid of recognisable internal features, were pleomorphic, bounded by a trilaminar cell membrane, and had no distinguishable cell wall. These observations are in agreement with those of Robinson and Hungate (1973), Robinson *et al* (1975) and Kudo *et al* (1990). Robinson and Allison (1975) were able to demonstrate nuclear material and a cluster of ribosomes in a TEM section (120,000 x) of an *Anaeroplasma* species.

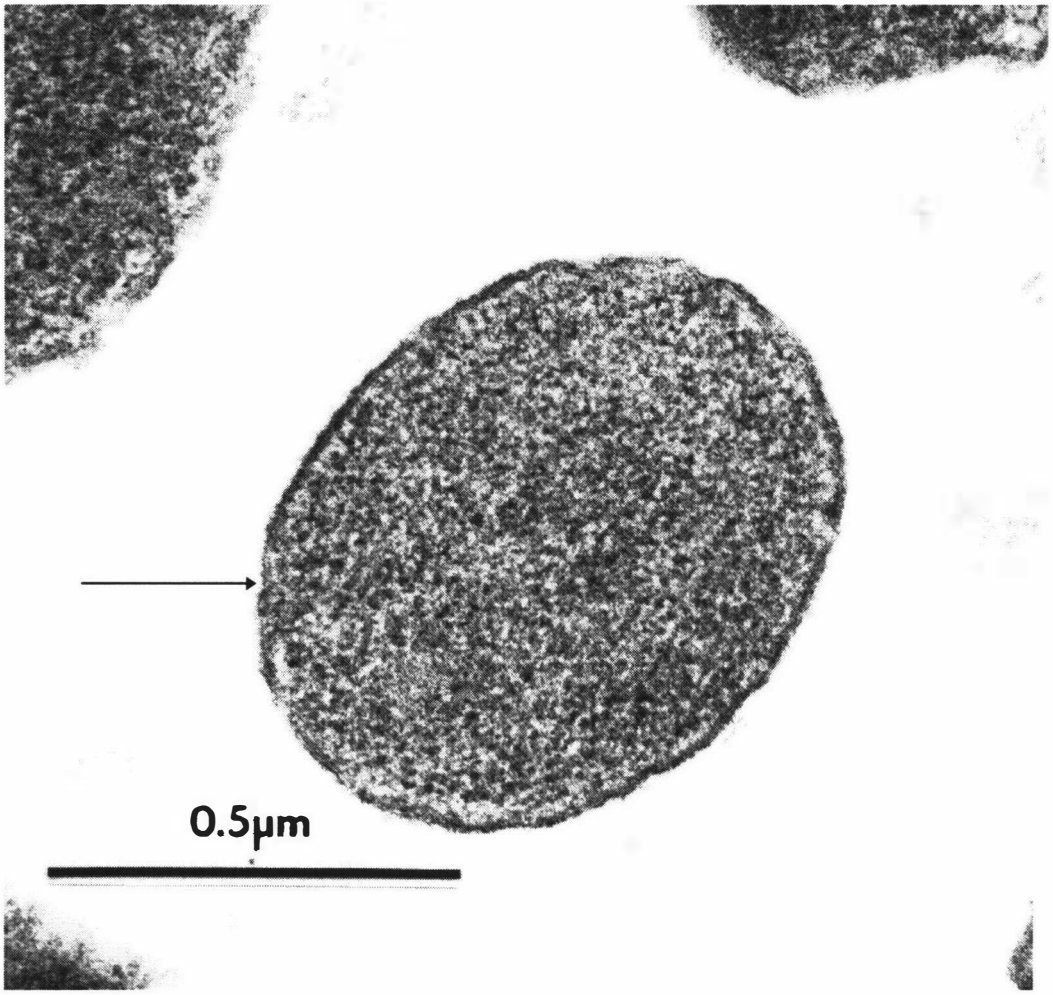

Fig 4.3 Transmission electron micrograph of isolate RM10. Note the trilaminar cell membrane (arrowed), lack of a cell wall and unevenly stained cytoplasm.

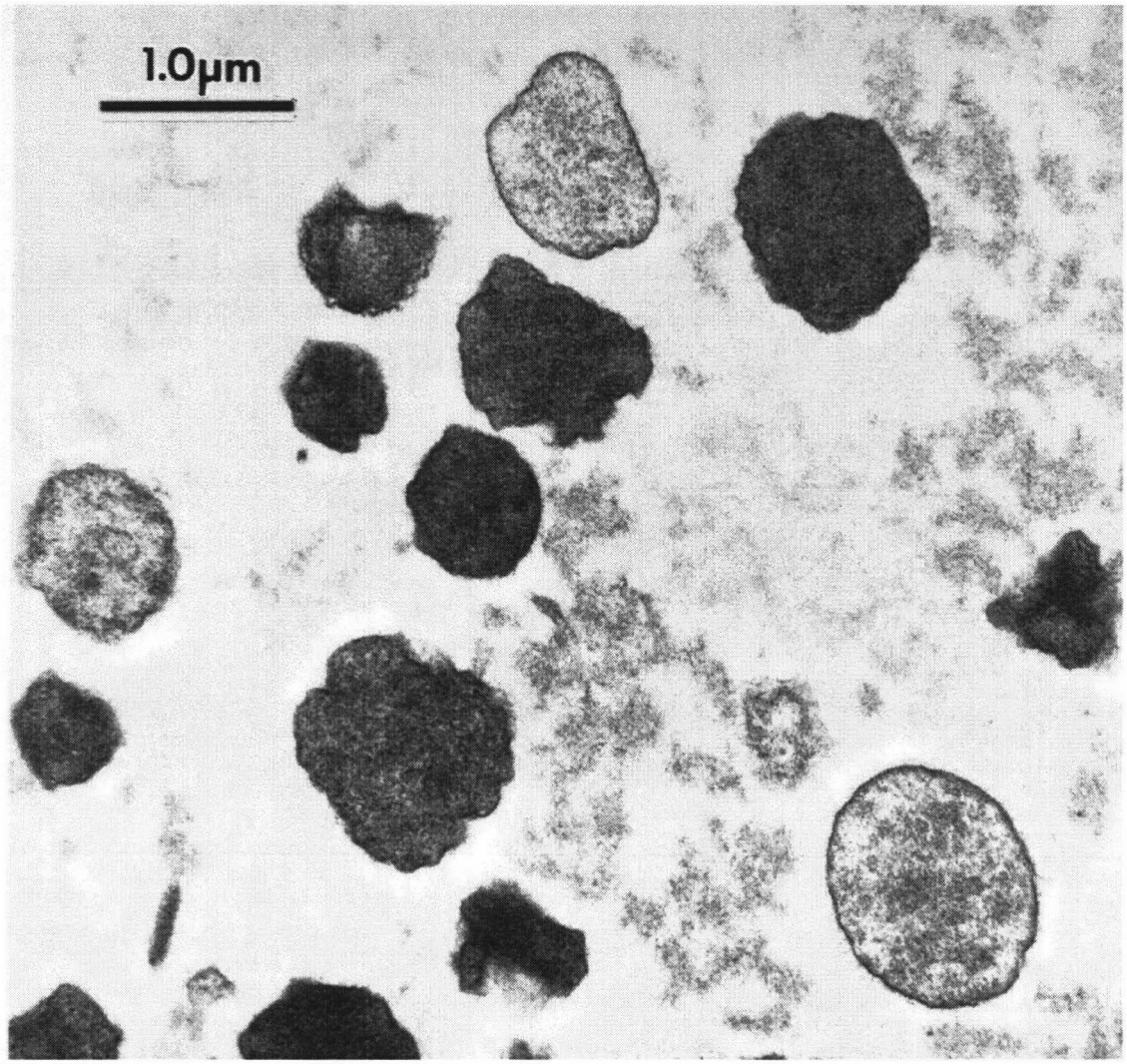

Fig 4.4 Transmission electron micrograph of isolate RM14 showing the unevenly stained cytoplasm and the pleomorphic nature of the cells.

4.2.2 Filtration of Ruminal Mycoplasma Cells

Cells of all isolates were tested for their ability to pass through 0.20 µm and 0.45 µm membrane filters. The ability to pass through 0.20 µm filters appears to be a variable characteristic. All isolates in this study passed through 0.45 µm membrane filters but RM10 was the only isolate which grew following passage through a 0.20 µm filter. Robinson and Hungate (1973) reported their isolate passed through a 0.45 µm filter and through a 0.22 µm filter. Kudo *et al* (1990) reported similar results with their isolates. The small size and lack of a cell wall probably both contributed to the ease with which ruminal mycoplasmas pass through membrane filters. Robinson *et al* (1975) showed that cells of *An. abactoclasticum* did not pass through 0.10 µm membrane filters.

4.2.3 Relationship between Temperature and Growth of Ruminal Mycoplasmas

The optimum temperature for growth (T_{opt}) of isolates was determined from their growth in CRFB medium. Growth was measured at seven different temperatures over a 24 hr period by reading culture OD_{600} against a medium blank. The temperatures at which growth was measured were 31, 34, 36.5, 39.5, 42, 45 and 47.5°C. For the purposes of the following discussion, the optimum growth temperature was considered to be the temperature at which growth increased most rapidly in the shortest time after culture inoculation.

Results for each of the ruminal mycoplasma isolates are given in Figs 4.5 to 4.10.

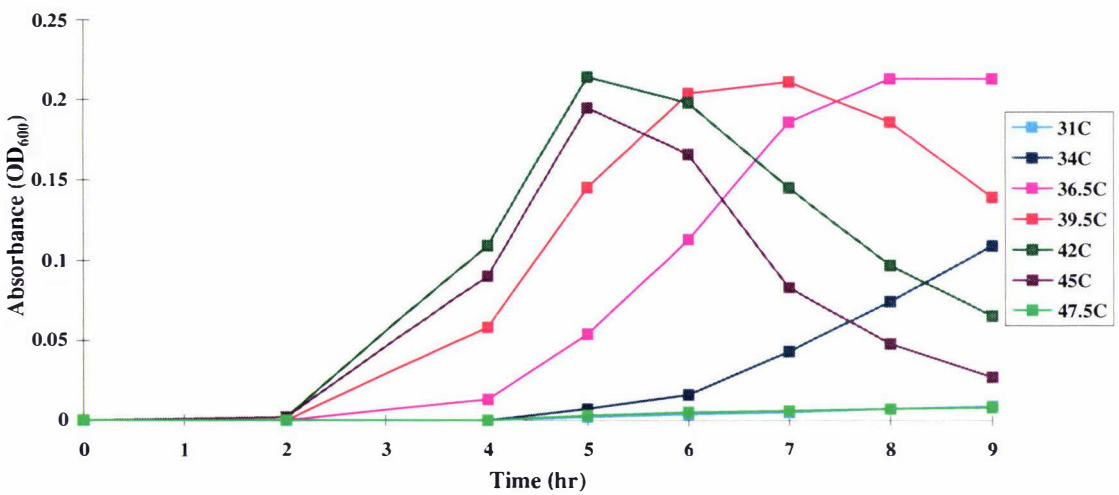

Fig 4.5 Effect of temperature on growth of isolate RM10.

For isolate RM10, the growth curves are given in Fig 4.5. These show that the optimum temperature for the growth of this isolate was 42°C. From Fig 4.5 it can be seen that cells grown at 42°C and 45°C lysed rapidly after reaching maximum growth, as shown by decreasing culture density. Cell lysis increased as the incubation temperature increased. It must be remembered that these growth studies were done in batch culture and do not, in all respects, parallel what would happen in the rumen where the fermentation is closer to being continuous in nature.

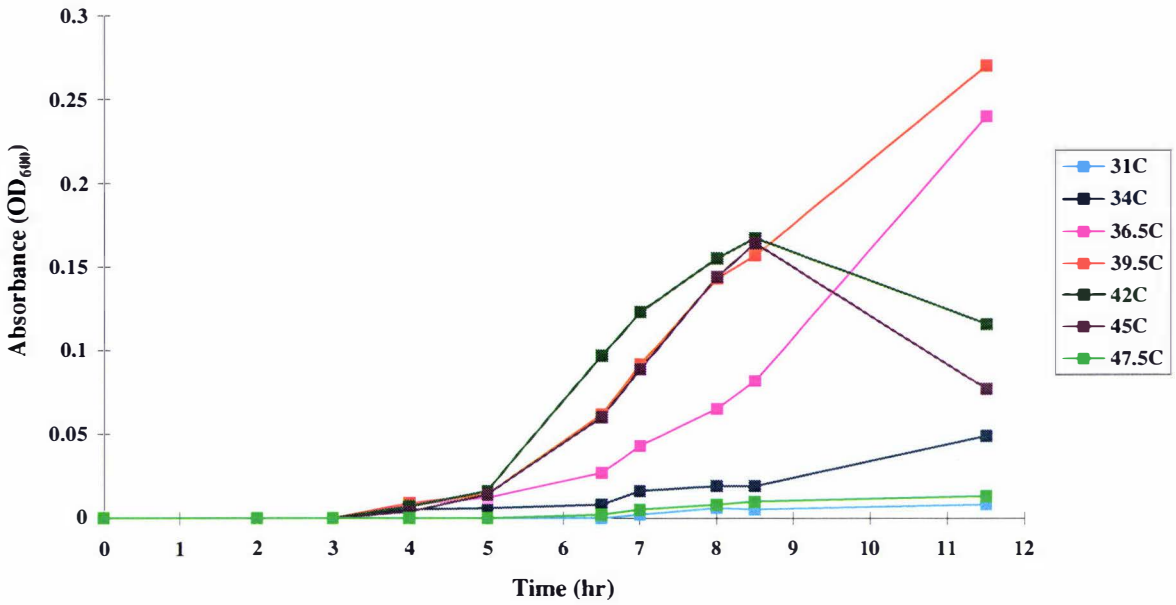

Fig 4.6 Effect of temperature on growth of isolate RM11.

The seven temperature growth curves for isolate RM11 are shown in Fig 4.6. These show that the optimum temperature for the growth of this isolate was 42°C. At 45°C and 42°C, cells began to lyse after 8.5 hr growth. At temperatures below the optimum, growth was slower and cells appeared less prone to lysis. The results in Fig 4.6 clearly show that the lysis of isolate RM11 was temperature dependent rather than growth rate dependent. Although growth rates were similar at 45°C, 42°C and 39.5°C, RM11 lysed readily at 45°C and 42°C but not at 39.5°C, the temperature nearest that found in the rumen. There was no growth at 31°C or 47.5°C.

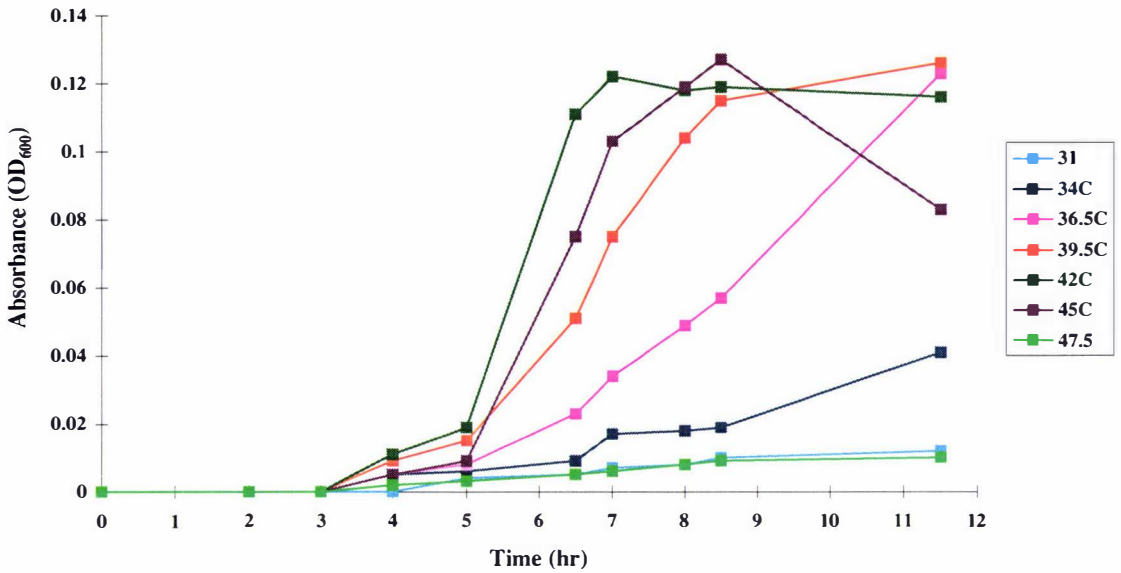

Fig 4.7 Effect of temperature on growth of isolate RM12.

The growth curves for isolate RM12 (Fig 4.7) show that the optimum temperature for this isolate was 42°C. Cell lysis is clearly temperature dependent, only occurring when the cells were growing at 45°C. There was no growth at 31°C or 47.5°C.

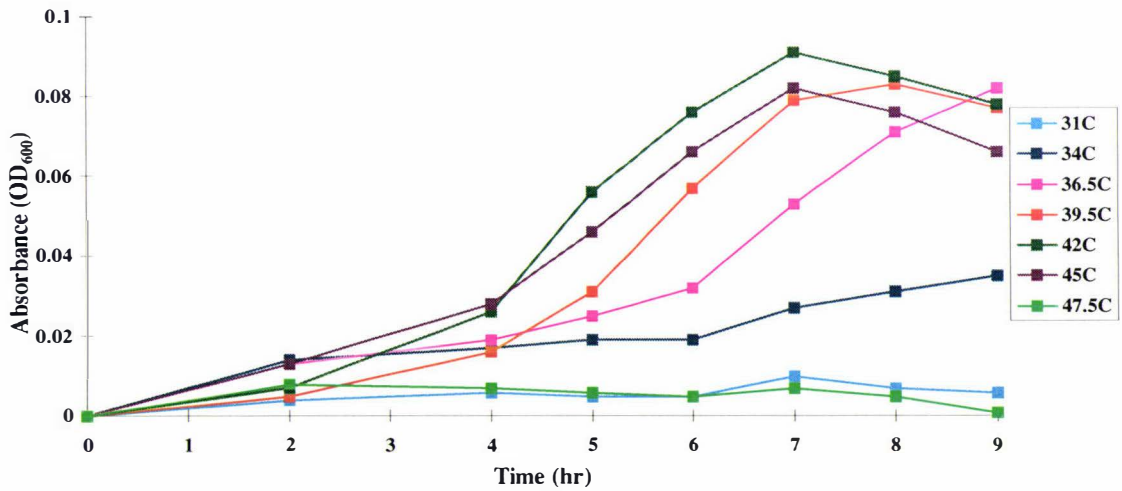

Fig 4.8 Effect of temperature on growth of isolate RM13.

The growth curves for isolate RM13 (Fig 4.8) illustrate that the optimum temperature of this isolate was 42°C. It can also be seen from Fig 4.8 that cell lysis occurs at 39.5, 42

and 45°C, but that the onset of cell lysis was most rapid at 42°C and 45°C. There was no measurable growth at 31°C or 47.5°C.

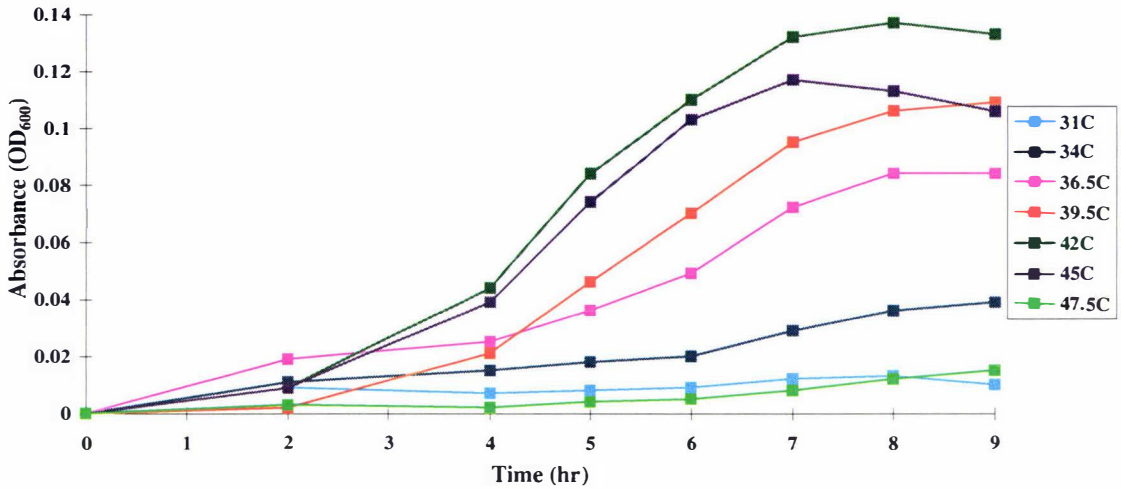

Fig 4.9 Effect of temperature on growth of isolate RM14.

The effect of temperature on the growth of mycoplasma RM14 is shown in Fig 4.9. The data clearly shows that the optimum temperature for this isolate was 42°C. The growth rates were similar at 42°C and 45°C, but cell lysis appeared to be occurring more rapidly at 45°C. The temperature of the rumen is 38°C-40°C (Hobson, 1997). In general, for cells of isolate RM14 growing at temperatures close to that of the rumen, there was no evidence of cell lysis. No measurable growth was seen at 31°C or 47.5°C.

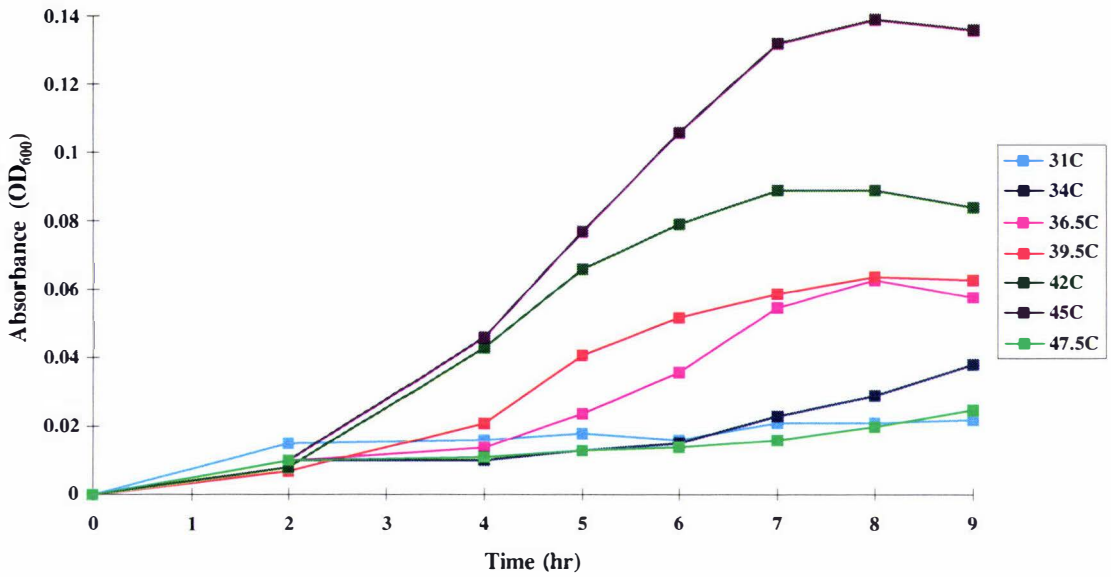

Fig 4.10 Effect of temperature on growth of isolate RM15.

The effect of temperature on the growth of mycoplasma RM15 is illustrated in Fig 4.10. The optimum temperature for the growth of this isolate was 45°C. Growth at all other temperatures was noticeably less than that at the optimum, with little cell lysis occurring at any of the growth temperatures tested. Cells growing at the temperature of the rumen appeared to be very stable. No growth occurred at 47.5°C or 31°C, with only a little at 34°C.

From Figs 4.5 to Figs 4.10 it is apparent that most of the isolates have a T_{opt} around 42°C. RM15 is the exception having a T_{opt} of 45°C. The temperature of the rumen is around 38°C-40°C (Hobson, 1997) and this is somewhat lower than the T_{opt} found in the present study. All of the isolates had unique growth temperature profiles. Robinson and Hungate (1973) noted that *Anaeroplasma bactoclasticum* grew at 36°C, 42°C and 45°C, but found no growth at 30°C or 47°C. The results in this study are in agreement with these observations and others in the literature. The data in Figs 4.5 to 4.10 show that none of the mycoplasmas grew at 31°C or 47.5°C and little growth occurred at 34°C. Robinson and Allison (1975), working with the same isolate as Robinson and Hungate (1973) found the T_{opt} for *An. bactoclasticum* was between 30°C and 47°C. In a separate study, Robinson *et al* (1975) showed that *An. abactoclasticum* Strain 6-1 failed to grow at 26°C and 47°C, but grew at 37°C. In terms of cell growth, isolates RM10 and RM11

grew to a maximum OD_{600} between 0.2 and 0.3, whereas all other isolates failed to grow to an OD_{600} greater than 0.14. This suggests two things; that some isolates were better adapted to CRFB medium or some isolates were lysing faster than their cells were dividing. All isolates became more prone to lysis as the incubation temperature increased. Also, all isolates had a broader temperature growth range below their optimum than above it. A comparison of the effects of temperature on growth for the six isolates shows that the growth curves for isolates RM13, RM14 and RM15 are similar to each other but very different to those of RM10, RM11 and RM12. This may indicate that isolates RM13, RM14 and RM15 are related species.

4.2.4 Effect of pH on the Growth of Ruminal Mycoplasmas

The six isolates were each inoculated into nine CRFB-based media adjusted to a final pH of 5.0 to 8.75 and growth measured after 10 hr incubation. 39°C was chosen as the growth temperature for these experiments because, for most isolates, little cell lysis occurred at this temperature. Uninoculated medium was used as the reference for growth measurements.

The effects of pH of culture media on the growth of isolates RM10, RM11 and RM12 are shown in Figs 4.11, 4.12 and 4.13 respectively. RM10, RM11 and RM12 grew in the pH range 5.5 to 7.2 with a pH_{opt} of 6.5 for RM10 and RM11 but with a pH_{opt} closer to pH 6.0 for RM12. The pH growth profiles of RM10 and RM11 were similar but differed significantly from that of RM12. The optical densities reached by RM10 and RM11 at their pH_{opt} were considerably greater than that reached by RM12.

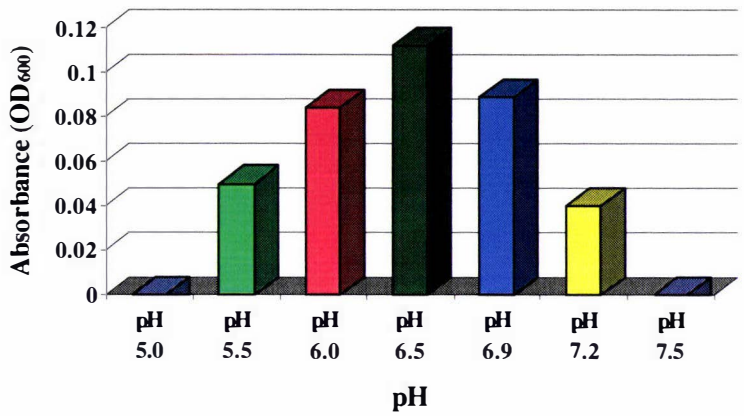

Fig 4.11 Effect of pH on growth of isolate RM10

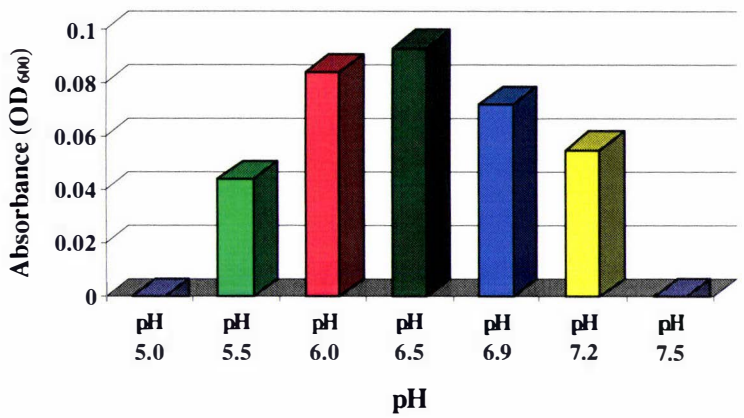

Fig 4.12 Effect of pH on growth of isolate RM11

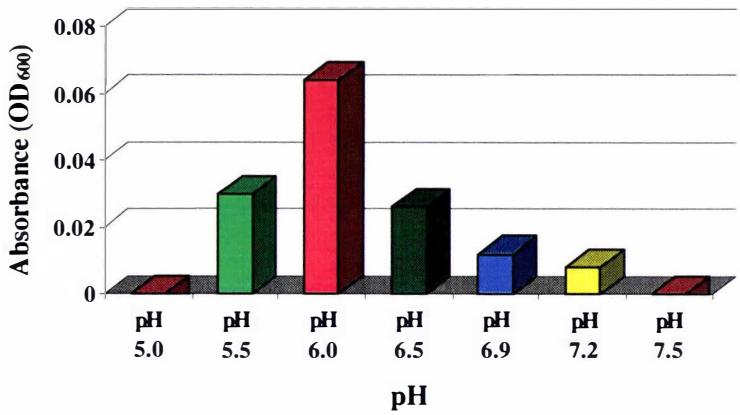

Fig 4.13 Effect of pH on growth of isolate RM12

Figs 4.14 to 4.16 show the effects of culture pH on the growth of isolates RM13, RM14 and RM15 respectively. Isolate RM13 grew within the range pH 5.5 to 7.9 whereas isolates RM14 and RM15 grew over the wider range of pH 5.0 to 7.9. Isolates RM13, RM14 and RM15 all had a pH_{opt} of 6.5 to 6.9, and all grew to a similar culture density at this pH. The similar responses to culture pH for isolates RM13, RM14 and RM15 suggest that they all may be similar. A comparison of these pH profiles with those in Figs 4.11 to 4.13 indicates that they are markedly different from isolates RM10, RM11 and RM12.

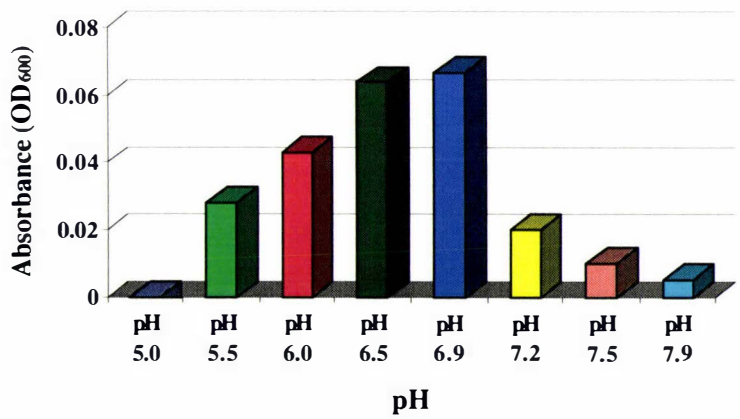

Fig 4.14 Effect of pH on growth of isolate RM13.

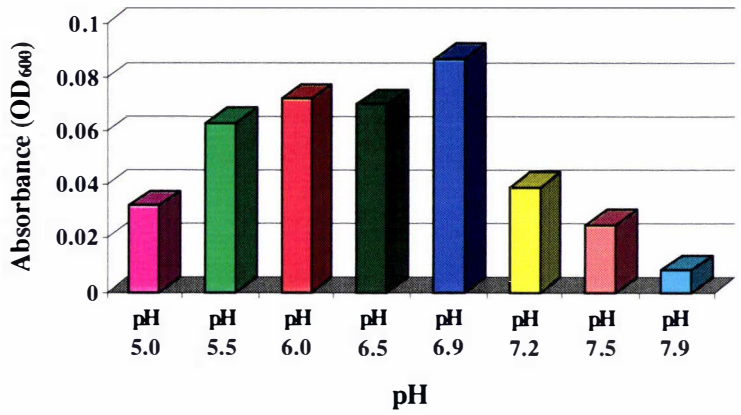

Fig 4.15 Effect of pH on growth of isolate RM14.

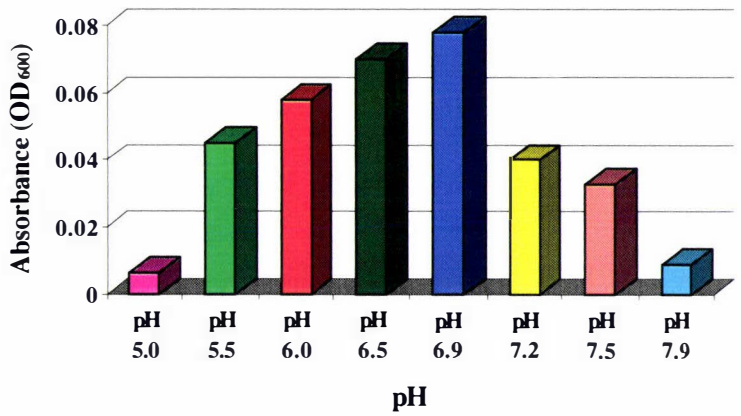

Fig 4.16 Effect of pH on growth of isolate RM15.

Based on the growth response to culture pH, there appeared to be two groups of ruminal mycoplasma in this study; one consisting of isolates RM10, RM11 and RM12 (Figs 4.11 to 4.13) and the other of isolates RM13, RM14 and RM15 (Figs 4.14 to 4.16). The differences between the two groups were particularly evident in media at higher pH with isolates RM13, RM14 and RM15 all showing measurable growth at pH 7.5 and 7.9 in contrast to isolates RM10, RM11 and RM12 which did not grow at these pH values. None of the isolates grew at pH 8.75. The pH of the rumen is usually in the range of pH

6.0-6.8 (Hungate, 1966). It is not surprising therefore that all of the isolates in this study grew well in media with mid-range pH values. This is the first time the effects of culture pH on the growth of ruminal mycoplasmas has been recorded.

4.2.5 Growth Substrates for the Ruminal Mycoplasma Isolates

Ruminal mycoplasmas vary in their ability to ferment carbohydrates (Robinson and Freundt, 1987). The range of substrates utilised by *An. abactoclasticum* is shown in Table 4.1 (Robinson *et al*, 1975). The differences in substrate utilisation between strains of what were considered to be *An. bactoclasticum* (Robinson and Allison, 1975) are also shown in Table 4.1. These differences may in part be explained by the fact, that in 1975 when this paper was published, it was not appreciated that ruminal mycoplasmas, identified then as *An. bactoclasticum*, were later to be reclassified into three new *Anaeroplasma* species. (Robinson and Freundt, 1987). An appreciation of this is required in order to interpret Table 4.1 where strains JR and 7LA were originally both classified as *An. bactoclasticum.* There is no information in the literature on the substrates used by *An. varium,* or *Asteroleplasma anaerobium.*

Table 4.1 Substrate utilisation by type strains of ruminal mycoplasmas and the isolates from this study

Substrate	*Anaeroplasma. abactoclasticum*[a] Strain6-1	*Anaeroplasma bactoclasticum*[b] Strain JR	*Anaeroplasma intermedium*[b] Strain 7LA	Isolate RM10	Isolate RM11	Isolate RM12	Isolate RM13	Isolate RM14	Isolate RM15
L(+) Arabinose	–[c]	±	–	–	–	–	–	–	–
D(+) Cellobiose	+	nd	nd	+	+	+	+	+	+
Cellulose	nd	nd	nd	–	–	–	–	–	–
D(-) Fructose	–	–	–	–	–	–	–	–	–
D(+) Galactose	–	+	–	+	+	–	+	–	–
D(+) Glucose	+	±	+	+	+	+	+	–	–
Glycerol	–	–	–	–	–	–	–	–	–
Lactose	nd	±	–	–	–	–	–	–	–
D-Maltose	+	+	+	+	+	+	+	+	+
D(+) Mannose	–	–	–	–	–	–	–	–	–
D(+) Raffinose	–	–	–	–	–	–	–	–	–
D-Salicin	–	–	–	–	–	–	+	+	+
Starch	+	+	+	+	+	+	+	+	+
Sucrose	–	±	–	–	–	–	–	–	–
D(+) Xylose	–	±	–	–	–	–	–	+	–

[a] Data for *Anaeroplasma abactoclasticum* adapted from Robinson *et al* (1975) and[b] for the two lytic *Anaeroplasma* species from Robinson and Hungate (1973), and Robinson and Allison (1975).

[c] + indicates growth; - indicates no growth; ± indicates where there is disagreement between authors on the use or non-use of a substrate by the same isolate.

nd: not determined.

The range of substrates used by isolates in the present study is shown in Table 4.1. Cellobiose, maltose and starch were utilised by all isolates. Arabinose, cellulose, fructose, glycerol, lactose, mannose, raffinose, and sucrose were not metabolised by any isolate. RM14 was the only isolate to utilise xylose. Isolates RM13, RM14 and RM15 utilised salicin, while isolates RM10, RM11 and RM12 did not. The use of salicin could provide a useful selection medium for this group of isolates. It can also be seen from Table 4.1, that the substrates used by isolates RM10, RM11, RM12, RM13, RM14 and RM15 are the same as those used by the type strains; the use of salicin by isolates RM13, RM14 and RM15 being an exception.

4.2.6 Analyses of Fermentation End-Products

The fermentation products of ruminal mycoplasmas grown in Cellobiose Rumen Fluid Broth (CbRFB) medium were identified by HPLC comparison. End-products were qualitatively assessed by UV detection at a wavelength of 210 nm by comparison with standards of known concentration and retention time. Software problems and the failure of the refractive index detector made quantitative analysis difficult. Because the refractive index detector failed, it was not possible to analyse culture supernatants for ethanol, or trace the disappearance of cellobiose from the cultures. Hydrogen was analysed by gas chromatography. Although small changes in some fermentation end-products were difficult to assess because of the relatively high background levels of VFAs in the culture medium, it was found that the major end-products produced by isolates RM10, RM11 and RM12 were formate, acetate, lactate, and H_2. The major end-products produced by isolates RM13, RM14 and RM15 were formate, acetate and lactate. These isolates did not produce H_2. The results agree with those of Robinson and Hungate (1973) who found that *Acholeplasma* (syn. *Anaeroplasma*) *bactoclasticum* produced formate, acetate, lactate, H_2 and CO_2 following growth on galactose. They noted that because of a high VFA background in their medium, more accurate results were achieved using ^{14}C-galactose as the growth substrate and radiochemical methods to assay end-products. Robinson *et al* (1975) employed ^{14}C-starch to examine the end-products of *An. abactoclasticum* and found formate, acetate and lactate as the major products. Small amounts of ^{14}C were detected also in succinate, CO_2 and several unidentified products.

4.2.7 Antibiotic Sensitivity of Ruminal Mycoplasmas

The susceptibility of isolates to antibiotics was assessed after 24 hr growth in CRFB medium which contained the antibiotics listed in Table 4.2 at concentrations described in section 3.3.8.

Table 4.2 Antibiotic sensitivities[a] of ruminal mycoplasmas

Compound	RM10	RM11	RM12	RM13	RM14	RM15
Bacitracin	+	–	–	–	–	+
Benzylpenicillin	+	+	+	+	+	+
Chloramphenicol	–	–	–	–	–	–
D-Cycloserine	+	+	+	+	+	+
Digitonin	–	–	–	–	–	–
Lincomycin-HCl	–	–	–	–	–	–
Streptomycin SO_4^{2-}	+	+	+	+	+	+
Tetracycline	–	–	–	–	–	–
Thallium (I) acetate	+	+	+	+	+	+

[a]Antibiotics were assessed as inhibitory if the OD_{600} of the culture at 24 hr was < 0.03 and are shown as - in Table 4.2. Cultures in which antibiotics showed no inhibition had OD_{600} values at 24 hr > 0.075 and were recorded as + in Table 4.2.

It was observed that in general, antibiotics which inhibited protein synthesis were the most effective inhibitors of ruminal mycoplasmas. Chloramphenicol, digitonin, lincomycin-HCl and tetracycline inhibited all isolates. Chloramphenicol binds to the ribosomal 50S subunit inhibiting protein synthesis (Mandelstam and McQuillen, 1973). Lincomycin-HCl inhibits protein synthesis in Gram positive bacteria, anaerobes and mycoplasmas, but is generally ineffective against Gram negative aerobic bacteria (Mims *et al,* 1993). Tetracycline binds to a protein of the ribosomal 30S subunit and inhibits ribosomal translocation in both Gram-positive and Gram-negative microorganisms (Mandelstam and McQuillen, 1973). Digitonin is a naturally occurring steroid glycoside active in the disruption of plasma membranes (Elias *et al*, 1977). Robinson and Allison (1975) showed that the ruminal mycoplasma, *An. bactoclasticum* was sensitive to digitonin. Robinson *et al* (1975) found that *Anaeroplasma* species were inhibited by digitonin (20 μg ml^{-1}), whereas the sterol non-requiring strains of ruminal mycoplasma

(now reclassified as *Asteroleplasma anaerobium*) were not inhibited by 200 μg ml^{-1} digitonin. All of the ruminal mycoplasmas in this study were sensitive to digitonin (60 μg ml^{-1}) and thus may be members of the genus *Anaeroplasma.* There are no previous studies in the literature on the sensitivities of ruminal mycoplasmas to lincomycin-HCl or to chloramphenicol. In this study, bacitracin was found to inhibit four of the six ruminal mycoplasmas tested (Table 4.2). Bacitracin inhibits bacterial cell wall synthesis by interfering with murein production (Mims *et al,* 1993) and is also an inhibitor of proteases (van Noort *et al*, 1991). The ruminal mycoplasma, *An. abactoclasticum,* has been reported to be sensitive to bacitracin, streptomycin and D-cycloserine (Robinson *et al*, 1975). In the present study, there was no effect on the growth of any isolate by benzylpenicillin or D-cycloserine. Both antibiotics are involved in the inhibition of bacterial cell wall synthesis (Mims *et al,* 1993) and as such would not be expected to inhibit the growth of ruminal mycoplasmas. Robinson and Hungate (1973) reported that *Acholesplasma* (syn. *Anaeroplasma*) *bactoclasticum* was not inhibited by 5000 IU benzylpenicillin ml^{-1}, but was sensitive to 20 μg ml^{-1} terramycin (tetracycline). Although most species within the class *Mollicutes* are susceptible to the tetracyclines, lateral transfer of tetracycline resistance genes from walled bacteria (streptococci) to mollicutes has been shown to occur in isolates from the human genital tract (Roberts, 1990). There was no inhibition by streptomycin sulphate or thallium (I) acetate of any of the isolates in this study. Streptomycin binds to ribosomal components inhibiting protein synthesis by causing mis-reading of m-RNA (Mandelstam and McQuillen, 1973). The negative results for thallium (I) acetate were unexpected; Robinson *et al* (1975) and Robinson and Allison (1975) found that all of their isolates were sensitive to thallium (I) acetate. In contrast, Robinson and Hungate (1973) noted that their isolate was uninhibited by thallium (I) acetate, but in a personal communication to them, Allison and Robinson suggested that ..."the thallium may have been precipitated by the sulfide used as a reducing agent." In this study the medium did not contain added sulphide, but sulphide in the rumen fluid component in the medium, or the L-cysteine-HCl used as a reducing agent, may have had a similar effect.

4.2.8 Lysis of Bacteria by Ruminal Mycoplasmas

The susceptibility of target Gram-negative bacteria to lysis by the six isolates in this study is shown in Table 4.3. The data clearly shows the isolates fall into two groups. In initial experiments, the lytic capabilities of the six ruminal mycoplasmas were determined from clearing zones in agar roll tubes using the method of Robinson *et al* (1975). In the results reported in Table 4.3, autoclaved *E. coli* and four autoclaved Gram-negative ruminal bacteria together with an autoclaved Gram-positive control (*Streptococcus bovis*), were exposed, as suspensions in agar, to viable cultures of the ruminal mycoplasmas. Robinson and Hungate (1973) found in general that autoclaved cells were more extensively lysed than viable cells. Lysis of autoclaved bacteria was demonstrated by clearing zones developing around colonies of the ruminal mycoplasmas in agar roll tube culture. As shown in Table 4.3, isolates RM10, RM11 and RM12 formed a single group capable of the lysis of the cell-walls of both *E. coli* and of Gram-negative ruminal bacteria. In contrast, the group comprising isolates RM13, RM14 and RM15 were incapable of lysing the cell walls of Gram-negative bacteria. No isolate was capable of lysing the cell wall of the control Gram-positive bacterium, *S. bovis*. These results are in keeping with those of Robinson and Hungate (1973) who showed an extracellular, ammonium sulphate-precipitated lytic factor prepared from *Acholesplasma* (syn. *Anaeroplasma*) *bactoclasticum,* lysed both viable and autoclaved Gram-negative ruminal bacteria, *E. coli* and *Salmonella typhimurium.* The factor reduced the OD_{600} of suspended autoclaved ruminal bacteria by a factor of 0.2 in 45 min but it did not lyse the Gram-positive bacterium *Micrococcus lysodeikticus.* Robinson and Hungate (1973) also demonstrated lysis of *E.coli* by the culture supernatant of *An. bactoclasticum,* and showed also that *M. lysodeikticus* was not lysed.

Table 4.3 Lysis of autoclaved bacterial cells by ruminal mycoplasmas in agar culture

Bacterium	Ruminal Mycoplasma					
	RM10	**RM11**	**RM12**	**RM13**	**RM14**	**RM15**
B. fibrisolvens H17C	+[a]	+	+	–	–	–
L. multiparus ATCC 19207	+	+	+	–	–	–
P. ruminicola ATCC 19189	+	+	+	–	–	–
S. bovis JB1	–	–	–	–	–	–
Suc. dextrinosolvens 24	+	+	+	–	–	–
E. coli XL1	+	+	+	–	–	–

[a] +, lysis by mycoplasma; –, no lysis by mycoplasma. For full genus and species names of the bacteria see Chapter 6: "Microorganisms used in this study."

Lysis of bacteria by ruminal mycoplasmas was also tested in broth culture (Table 4.4). This was found to be an easier method for assessment of the lytic properties of ruminal mycoplasmas because it was faster and less labour intensive. The method had the advantage that only one tube of each test bacterium / mycoplasma combination was required, rather than the six or seven required for dilution series using the roll tube method. Lysis was determined by measurement of culture OD_{600} after 96 hr incubation at 39°C. The results in Table 4.4 show that *E. coli* and six Gram-negative ruminal bacteria, exposed to the six ruminal mycoplasmas in broth culture, were lysed by isolates RM10, RM11 and RM12, but not lysed by isolates RM13, RM14 and RM15. The data in Table 4.4 also show that the Gram-negative ruminal bacteria *Megasphaera elsdenii* and *Selenomonas ruminantium,* not tested using the agar method, were also lysed by isolates RM10, RM11 and RM12. The Gram-positive cell wall of *S. bovis* was resistant to lysis by all isolates. Examination of the data in Tables 4.3 and 4.4 confirmed that both methods used to study cell wall lysis gave identical results.

Table 4.4 Lysis of autoclaved bacterial cells by ruminal mycoplasmas in broth culture

Bacterium	Ruminal Mycoplasma					
	RM10	**RM11**	**RM12**	**RM13**	**RM14**	**RM15**
B. fibrisolvens H17C	+[a]	+	+	–	–	–
L. multiparus ATCC 19207	+	+	+	–	–	–
M. elsdenii T81	+	+	+	–	–	–
P. ruminicola ATCC 19189	+	+	+	–	–	–
Sel. ruminantium HD4	+	+	+	–	–	–
S. bovis JB1	–	–	–	–	–	–
Suc. dextrinosolvens 24	+	+	+	–	–	–
E. coli XL1	+	+	+	–	–	–

[a] +, lysis by mycoplasma; –, no lysis by mycoplasma. For full genus and species names of the bacteria see Chapter 6: "Microorganisms used in this study."

One of the principal characteristics which divides species within the genus *Anaeroplasma* is the ability (or inability) of isolates to lyse Gram-negative bacterial cell walls (Robinson and Freundt, 1987). The results of both agar and broth lysis assays showed that only bacteria with Gram-negative cell walls were lysed by the ruminal mycoplasmas. When ruminal mycoplasmas were first characterised, all of those which lysed Gram-negative bacterial cell walls were classified as *Anaeroplasma bactoclasticum.* However further analysis, using a variety of tests, indicated that the ruminal mycoplasmas capable of the lysis of Gram-negative cell walls were a heterogenous assemblage of species which were later reclassified as either *An. bactoclasticum*, *An. intermedium* or *An. varium* (Stephens *et al,* 1985; Robinson and Freundt, 1987).

4.2.9 Proteolysis by Ruminal Mycoplasmas

The ability of the six ruminal mycoplasmas to hydrolyse casein (a high molecular weight phosphoprotein) were determined, after 4 days incubation at 39°C, from clearing zones in agar roll tubes using a modified method of Robinson and Hungate (1973). The data clearly showed the isolates fell into two groups. Isolates RM10, RM11 and RM12 produced clearing zones in the casein suspension, while isolates RM13, RM14 and

RM15 did not. These results are in agreement with those of Robinson and Hungate (1973) whose isolate was proteolytic and Robinson *et al* (1975) whose isolate was not.

4.2.10 Lysis of Fungal Cell-Walls by Ruminal Mycoplasmas

To test the hypothesis that the inhibition of ruminal fungi by ruminal mycoplasmas was a consequence of fungal cell wall lysis, required a medium which would support the growth of mycoplasmas and also demonstrate the utilisation of the fungal cell wall as a carbon source. The simplest way was to use the development of clearing zones around mycoplasma colonies growing on agar roll tubes which contained finely ground fungal cell wall as the growth substrate. A major problem was grinding the fungal material fine enough to give an even suspension in an agar medium. Fungal cell walls have a leather-like consistency and in spite of prolonged ball-milling, no really suitable method was found which produced a homogenous product. A fungal cell-wall medium (FCWA) was prepared but this did not produce a truly homogenous agar and under the dissecting microscope, FCWA had a granular appearance. The agar was inoculated with the ruminal mycoplasmas (0.2 ml) (OD_{600} of 0.1), and incubated at 39°C for 21 days. Although the non-uniformity of the agar may have masked hydrolysis of the fungal cell wall, no evidence of clearing zones around any mycoplasma colony was seen. It seems that the inhibition of ruminal fungi by ruminal mycoplasmas is therefore unlikely to involve the degradation of the fungal cell wall.

4.2.11 Chitinase Activity of Ruminal Mycoplasmas

Chitin, a polymer of chitobiose and N-acetylglucosamine, is a major component of the cell wall of ruminal fungi (Kopecny *et al*, 1996). The chitinolytic bacterium *Clostridium tertium* ChK5 has been shown to inhibit cellulolysis by three genera of ruminal fungi (Kopecny *et al*, 1996). Also, chitinases from *Streptomyces griseus* inhibit the cellulases of the fungus *N. frontalis* possibly by disrupting the association between the cellulase and components of the fungal cell wall (Wilson and Wood, 1992). It was not known if ruminal mycoplasmas produce a chitinase, but one possible mechanism whereby ruminal mycoplasmas inhibit ruminal fungi may be via the hydrolysis of chitin in the fungal cell wall (Joblin, pers. comm.,1996).

Cultures of ruminal mycoplasma isolates inoculated into Chitin Starch Agar produced no clearing zones in the chitin after 21 days incubation at 39°C, although colonies were clearly visible. A similar experiment with no starch supplementation, produced poor mycoplasma growth and again no clearing zones were seen in the chitin; the mycoplasmas were probably growing on low levels of an unknown substrate carried over in the rumen fluid component of the medium. This result confirms the FCWA findings (see section 4.2.10). Since no lysis of either fungal cell wall or pure chitin was demonstrated, it is probable the inhibition of ruminal fungi by ruminal mycoplasmas was not as a consequence of a mycoplasmal chitinase. With limited degradative capabilities and a dependence on host-derived enzymes, it would perhaps have been surprising had ruminal mycoplasmas been capable of hydrolysing chitin.

4.2.12 Sterol Requirements of Ruminal Mycoplasmas

The sterol requirement of ruminal mycoplasmas are second only to the requirement for strictly anaerobic growth conditions when identification is being considered. Ruminal mycoplasmas which require sterol for growth belong to the genus *Anaeroplasma*, while those which are sterol non-requiring belong in the genus *Asteroleplasma.* To determine their sterol requirements, all of the isolates in this study were inoculated into medium MM10, supplemented with *E. coli* lipopolysaccharide (LPS) (Boivin) and cholesterol using the method described by Robinson *et al* (1975). Isolates RM10, RM11 and RM12 failed to grow in MM10, either alone, when the medium was supplemented as above or when further supplemented with additional vitamins. The lack of growth of RM10, RM11 and RM12 in MM10 suggested an unidentified growth factor was missing from MM10 or the supplements. Isolates RM13, RM14 and RM15 all grew in MM10 and were all stimulated by LPS and very strongly by LPS and cholesterol together. In a separate experiment in CRFB medium, all six isolates were inhibited by low concentrations of digitonin (60 $\mu g\ ml^{-1}$). Robinson *et al* (1975) stated, when commenting on their isolates..."growth of the sterol requiring strains 6-1 and 171 (*Anaeroplasma* species) were inhibited by digitonin (20 $\mu g\ ml^{-1}$) whereas growth of the sterol non-requiring strain 161 was unaffected by 200 $\mu g\ ml^{-1}$ of digitonin and only slightly by 500 $\mu g\ ml^{-1}$". Robinson *et al* (1975) stated " Cholesterol requirement may also be determined by the selective sensitivity of sterol-requiring mycoplasmas to digitonin. Digitonin sensitivity seems to parallel the ability of ruminal mycoplasmas to

incorporate cholesterol." The indirect evidence based on the sensitivity of the six isolates to low concentrations of digitonin supported the identification of isolates RM10, RM11, RM12, RM13, RM14 and RM15 as members of the genus *Anaeroplasma*.

4.2.13 Identification of the Ruminal Mycoplasma Isolates

The characteristics of isolates RM10, RM11 and RM12, placed them into the genus *Anaeroplasma*. They were classified on the basis that they were strictly anaerobic, were sensitive to low concentrations of digitonin (60 μg ml^{-1}) which indicated a requirement for sterols in growth media, had no cell wall, passed membrane filters with pore sizes of 0.45 μm, produced H_2, were proteolytic and were able to lyse the cell walls of *E. coli* (Robinson *et al,* 1975; Robinson and Freundt, 1987). The evidence for the ability of RM10, RM11 and RM12 to lyse the cell walls of Gram-negative bacteria is given in Table 4.3 and 4.4. The precise identity of the three isolates was not possible because three *Anaeroplasma* species are capable of lysing the cell walls of Gram-negative bacteria, namely, *An. bactoclasticum, An. varium* and *An. intermedium* (Robinson and Freundt, 1987). The isolates may belong to any of these species, or to a new species. 16S rRNA sequence data would help confirm species identification (Weisburg *et al*, 1989; Kuske and Kirkpatrick, 1992).

The characteristics of isolates RM13, RM14 and RM15 place them into the genus *Anaeroplasma.* The characteristics of these isolates were very similar to those of isolates RM10, RM11 and RM12 except they were unable to lyse the cell walls of Gram-negative bacteria, were not proteolytic and did not produce H_2. Additional evidence for a requirement for exogenous sterol was shown by the strongly stimulated growth seen in MM10 supplemented with cholesterol and *E. coli* LPS. The isolates were therefore classified as *Anaeroplasma abactoclasticum*; the only *Anaeroplasma* species not able to lyse the cell walls of Gram-negative bacteria (Robinson *et al*, 1975; Robinson and Freundt, 1987). Confirmation of this identity would have been possible had 16S rRNA sequence data been available (Weisburg *et al*, 1989; Kuske and Kirkpatrick, 1992).

4.2.14 RAPD Analysis of Ruminal Mycoplasma DNA

The RAPD method to examine the DNA of ruminal mycoplasmas for genomic fingerprints was chosen because no prior knowledge of the DNA nucleotide sequence was required, the method was faster than RFLP, no restriction enzyme digestion of DNA was necessary and the process was relatively easy to perform. The RAPD method was developed independently by Williams *et al* (1990) and Welsh and McClelland (1991). Williams *et al* (1990) used single arbitrary primers to demonstrate DNA polymorphisms in DNA samples from humans, soybean, corn and *Neurospora crassa.* Welsh and McClelland (1991) demonstrated DNA polymorphisms in DNA from mice using single random primers, but extended the methodology to show that primers used in pairs would also generate unique banding patterns. The RAPD method has also been successfully applied using DNA from procaryotes to demonstrate intraspecies discrimination in *Listeria monocytogenes* (Czajka *et al*, 1993), to distinguish among strains of *Xanthomonas campestris* (Manulis *et al*, 1994) and to identify conserved regions in actinomycete genomes (Mehling *et al*, 1995). In this study, random oligonucleotide primers of 10 bases (10mers) with 60-70% G+C were used to amplify the DNA of ruminal mycoplasma isolates using the Polymerase Chain Reaction (PCR). The DNA and primer combinations as outlined in Table 4.5. DNA was extracted using DNAzol reagent; the cells lysed readily and good yields of DNA were achieved using the procedures recommended. The DNA was quantified spectrophotometrically using a multiplate reader which measured OD_{260} and OD_{280}. An OD_{260} of 1 is equal to approximately 50 $\mu g\ ml^{-1}$ of double-stranded DNA. The ratio between OD_{260} and OD_{280} gives an estimate of the purity of the nucleic acid. Pure preparations of DNA have a OD_{260}/OD_{280} ratio of 1.8. RNA was also present in the extracts, as shown by agarose gel electrophoresis. The RNA did not interfere with subsequent RAPD analysis which was performed using the method of Gardiner *et al* (1996). The details of the PCR cycle times and temperatures are given in section 3.3.13.

Table 4.5 DNA and primer combinations for the RAPD analysis

Lane	DNA	Primer	Sequence
1	100bp ladder	na	
2	RM10	OPAA04	5'-AGGACTGCTC-3'
3	RM11	OPAA04	
4	RM12	OPAA04	
5	RM13	OPAA04	
6	RM14	OPAA04	
7	RM15	OPAA04	
8	100bp ladder	na	
9	RM10	OPAA06	5'-GTGGGTGCCA-3'
10	RM11	OPAA06	
11	RM12	OPAA06	
12	RM13	OPAA06	
13	RM14	OPAA06	
14	RM15	OPAA06	
15	100bp ladder	na	
16	RM10	OPAA09	5'-AGATGGGCAG-3'
17	RM11	OPAA09	
18	RM12	OPAA09	
19	RM13	OPAA09	
20	RM14	OPAA09	
21	RM15	OPAA09	
22	100bp ladder	na	

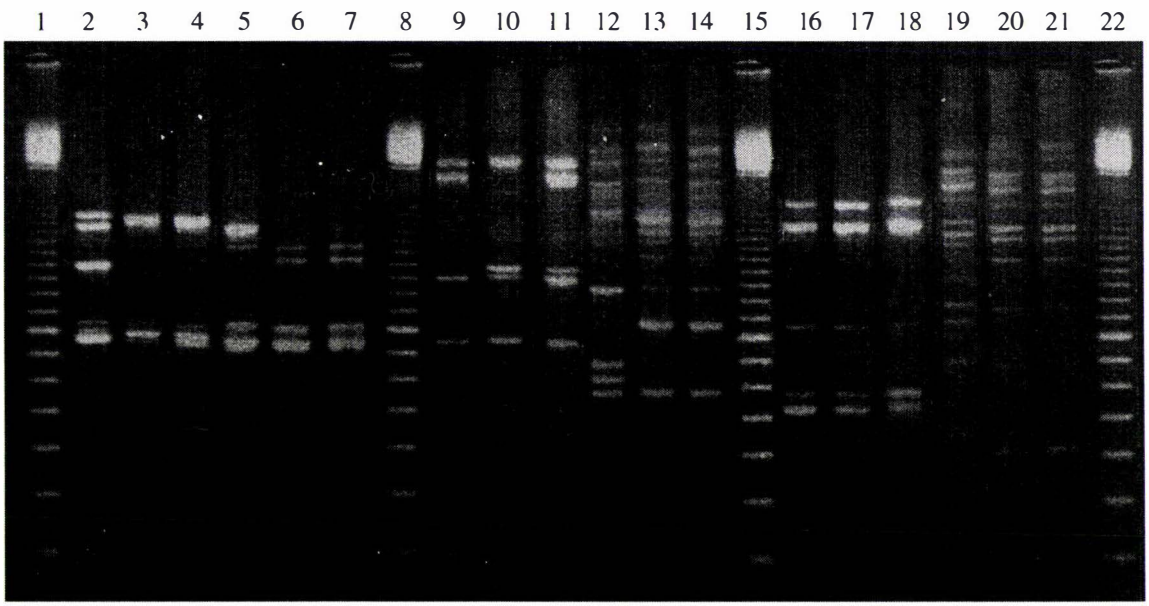

Fig 4.17 RAPD profiles of DNA from isolates RM10 to RM15. For lane contents see Table 4.5

Fig 4.17 illustrates the PCR products of isolate-DNA, amplified using the RAPD technique, after separation on a 2% agarose gel. Isolate RM10 produced unique banding patterns with primers OPAA04 and OPAA06, but with primer OPAA09 gave similar banding patterns to isolates RM11 and RM12. Isolates RM11 and RM12 produced banding patterns which were the same for all three primers tested, which suggested that these two isolates were identical. Isolate RM13 had a unique banding profile with all the primers tested and was not similar to any other isolate. Isolates RM14 and RM15 gave the same banding patterns with all primers tested which suggested that these two isolates were identical. Control reactions were run using distilled water in place of DNA with no bands being produced. The results showed the RAPD procedure to be an effective method for the demonstration of genomic fingerprints in ruminal mycoplasma DNA.

4.2.15 PAGE Analysis of Ruminal Mycoplasma Proteins

The method used to electrophoretically separate ruminal mycoplasma proteins according to their molecular size was that of Laemmli (1970). When the mycoplasma cells were lysed with SDS prior to protein analysis by the method of Bradford (1976), the solutions of RM10, RM11, & RM12 were clear, while those of RM13, RM14 & RM15 were cloudy. This suggested a compositional difference between the two groups of ruminal mycoplasma. This observation may reflect that of Langworthy *et al* (1975) who noted "The bacteriolytic strain 7LA contained only about 50% of the amount of glycolipids present in the non-bacteriolytic strain 6-1". Strain 7LA is now classified as *An. intermedium,* while strain 6-1 is classified as *An. abactoclasticum* (Robinson and Freundt, 1987). The observation does not endow identity to the two groups of ruminal mycoplasma, but may reflect differing glycolipid composition.

Details of the identity of the ruminal mycoplasma lysate in each lane of the gel, the concentration, and volume required to give the necessary 80 µg of protein per lane, are shown in Table 4.6.

Table 4.6 Protein concentration of cell lysates and lane-loading of the PAGE gel

Lane	Isolate	Protein Conc. of Lysate ($\mu g\ ml^{-1}$)	Cell Lysate per Lane[a] (μl)
1	RM10	1500	73.3
2	RM11	1860	59.1
3	RM12	810	135.8
4	RM13	900	122.2
5	RM14	1500	73.3
6	RM15	900	122.2

[a] Volume of lysate to give 80 µg of protein per gel lane.

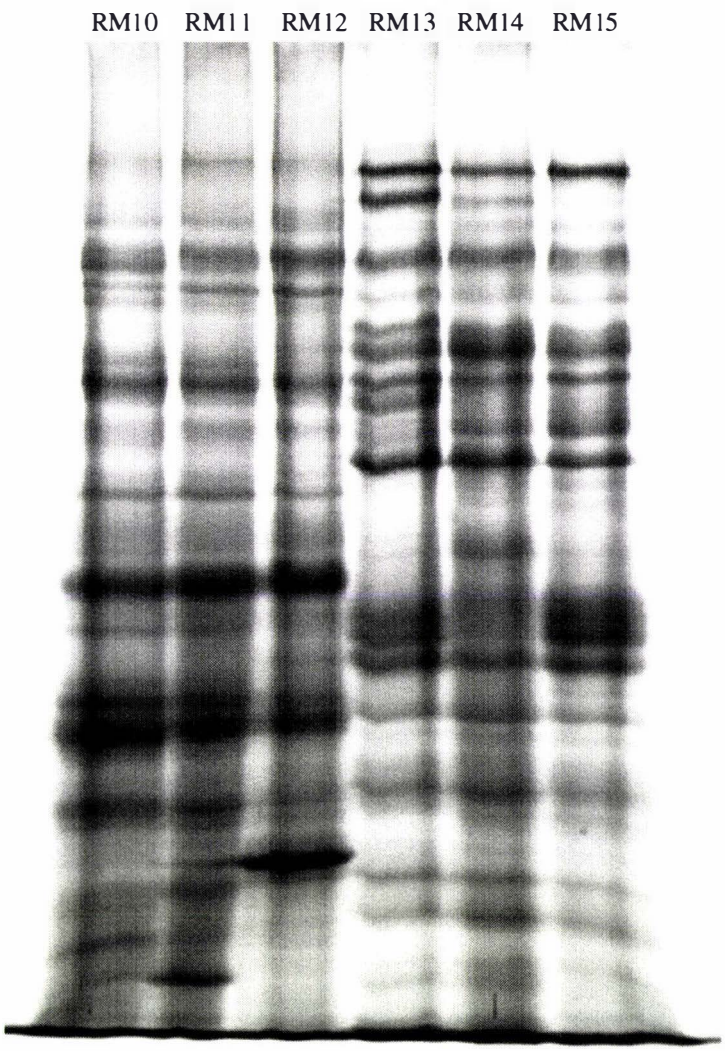

Fig 4.18 PAGE profiles of the proteins from isolates RM10 to RM15

The first impression from the gel was that there were two major groupings within the ruminal mycoplasmas being studied (Fig 4.18). It was clear that isolates RM10, RM11 and RM12 formed one distinct group while isolates RM13, RM14 and RM15 formed the other. Within each group of three isolates, there were minor differences in the protein bands, sufficient to distinguish between the isolates. RM11 and RM12 both possessed one strong, but different low molecular weight band, which RM10 lacked. The presence of a high molecular weight protein band in RM13, which was absent in RM14 and RM15, suggested that RM14 and RM15 were more closely related to each other than they were to RM13.

4.3 COCULTURE STUDIES

Many studies have demonstrated interactions between ruminal microorganisms grown in coculture. Studies have shown that when ruminal bacteria were cocultured with ruminal fungi, the bacteria effected fungal xylanolysis in a variety of ways (Williams *et al*, 1994). When ruminal fungi were cocultured with the methanogen, *Methanobrevibacter smithii,* it was shown that both the rate and extent of xylan utilisation by the fungi were increased in the coculture (Joblin *et al*, 1990). There are no previous coculture studies to show what effect ruminal mycoplasmas have on cellulolysis by ruminal bacteria. Likewise no previous studies have been undertaken to show the effect of ruminal mycoplasmas on cellulolysis by ruminal fungi. However during fungal isolation we have previously noted the co-isolation of ruminal mycoplasmas, suggesting there may be an association between these two groups of microorganisms.

4.3.1 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Bacteria

Cellulolytic ruminal bacteria and the ruminal mycoplasmas were cocultured to establish if there was inhibition of bacterial cellulolysis and was it measurable. The cellulose substrate, paper strip, was digested by the bacteria but not digested by the ruminal mycoplasmas. Growth of the cellulolytic bacteria was monitored by disappearance of the paper and production of H_2. Hydrogen was produced both by the bacteria and isolates RM10, RM11 and RM12. Isolates RM13, RM14 and RM15 did not produce H_2. Hydrogen production by the bacteria was not constitutive, but can be a useful

metabolite to follow as an indicator of growth. The results (Table 4.7) indicate that cellulolysis was inhibited in both *R. albus* (30-60%) and *R. flavefaciens* (50-80%) when grown in coculture with the ruminal mycoplasmas, while *C. chartatabidum* and *F. succinogenes* appeared to be slightly stimulated by the coculture. The H_2 produced in the coculture of *F. succinogenes* with isolates RM10, RM11 and RM12 is a reflection of production by the mycoplasma alone, because *F. succinogenes* does not produce H_2. Similarly, an examination of the results (Table 4.7) show increased H_2 in cocultures of *R. flavefaciens* with RM10, RM11 and RM12 which probably reflect a stimulation of mycoplasmal H_2, rather than a stimulation of H_2 production by the bacteria. This in turn suggested that ruminal mycoplasmas were cross feeding on intermediates produced as a result of the degradation of paper by the ruminococci. The H_2 production results suggest that the cocultures have little effect on the production of H_2 by any of the cellulolytic bacteria. Given that *F. succinogenes* is Gram-negative, it is unclear why on the basis of the demonstrated lysis of other Gram-negative bacteria by isolates RM10, RM11 and RM12, that this bacterium was not also lysed and why cellulolysis was uninhibited. Cellulolytic bacteria often produce extracellular slime when growing on ball-milled cellulose and this may have protected *F. succinogenes* from lysis by these ruminal mycoplasmas. The mechanism whereby *R. albus* and *R. flavefaciens* (both Gram-positive) were inhibited is not known. The inhibition was substantial and may have profound consequences for the rumen fermentation if the *in vitro* observations were also shown to occur *in vivo*.

Table 4.7 Cellulolysis and H_2 production by ruminal bacteria growing on cellulose in the presence of ruminal mycoplasmas

Culture	Cellulose Used		H_2 (ml)[a]
	mg[a]	%[b]	
Clostridium chartatabidum	6.2 ± 0.4	100	2.02 ± 0.18
+RM10	6.3 ± 2.0	100	2.01 ± 0.11
+RM11	7.2 ± 0.5	120	2.02 ± 0.12
+RM12	6.7 ± 1.5	110	1.96 ± 0.12
+RM13	7.0 ± 0.7	115	1.99 ± 0.06
+RM14	7.1 ± 0.8	115	2.10 ± 0.20
+RM15	6.6 ± 0.5	105	2.01 ± 0.15
Fibrobacter succinogenes	32.1 ± 0.8	100	0
+RM10	32.8 ± 1.2	100	0.07 ± 0.02
+RM11	34.2 ± 3.1	105	0.05 ± 0.03
+RM12	34.3 ± 1.3	105	0.03 ± 0.01
+RM13	35.1 ± 2.2	110	0
+RM14	33.5 ± 0.3	105	0
+RM15	33.1 ± 1.8	105	0
Ruminococcus albus	11.2 ± 0.8	100	2.05 ± 0.08
+RM10	6.5 ± 0.6	55	2.03 ± 0.01
+RM11	7.4 ± 1.0	65	1.87 ± 0.07
+RM12	6.9 ± 0.8	60	1.96 ± 0.04
+RM13	7.1 ± 3.3	60	1.89 ± 0.06
+RM14	8.4 ± 2.0	70	1.86 ± 0.04
+RM15	4.4 ± 2.1	40	1.82 ± 0.08
Ruminococcus flavefaciens	4.8 ± 0.7	100	0.09 ± 0.05
+RM10	2.5 ± 1.5	50	0.22 ± 0.14
+RM11	0.9 ± 1.1	20	0.28 ± 0.04
+RM12	2.6 ± 1.4	50	0.29 ± 0.02
+RM13	2.6 ± 0.9	50	0.10 ± 0.02
+RM14	1.8 ± 1.4	35	0.10 ± 0.02
+RM15	1.7 ± 0.7	30	0.10 ± 0.03

[a] Data represents mean and standard deviation of triplicate cultures

[b] Cellulolysis by bacteria in axenic culture taken as 100%.

4.3.2 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Fungi

Ruminal fungi are recognized as producers of some of the most active cellulolytic enzymes (Wood *et al*, 1986). Fungal cellulolysis was inhibited by the action of compounds produced by ruminal bacteria of the *Ruminococcus* and *Butyrivibrio* genera (Stewart *et al*, 1992; Joblin and Naylor, 1994). Previous studies have shown that ruminal mycoplasmas were often co-isolated with ruminal fungi but the degree to which they interacted was unknown (Joblin and Naylor, unpublished data). To establish if there was interaction, cellulolytic fungi and ruminal mycoplasmas were grown in coculture and the effects on fungal cellulolysis monitored. Cocultures of each fungus with each ruminal mycoplasma were established in PSM. These cocultures, with pure fungal cultures on PSM as controls, were examined to give information on substrate utilisation and H_2 production. This allowed an assessment of the extent to which ruminal mycoplasmas and fungi interact in coculture. The substrate, paper strip, was digested by the fungi, but not by the ruminal mycoplasmas. Fungal growth was monitored by disappearance of the paper (cellulose) and by production of H_2. Fungal H_2 production is constitutive. Mycoplasma isolates RM10, RM11 and RM12 also produce H_2, while isolates RM13, RM14 and RM15 do not.

In a preliminary experiment to see if ruminal fungi were inhibited by ruminal mycoplasmas, paper-containing-medium PSM, was inoculated with the fungus *N. frontalis* alone and *N. frontalis* with mycoplasma RM14. The results are shown in Fig 4.19. In the culture inoculated with *N. frontalis* alone (tube 2), the paper was almost totally digested and the paper strip had collapsed completely. In contrast, the paper strip in the culture containing the fungus and the ruminal mycoplasma RM14 (tube 1) remained intact, similar to the paper in the uninoculated control (tube 3). This experiment formed the basis for further experiments involving ruminal fungi of the *Neocallimastix*, *Piromyces* and *Caecomyces* genera cocultured with the six ruminal mycoplasma isolates. The results are shown in Tables 4.8, 4.9 and 4.10 respectively.

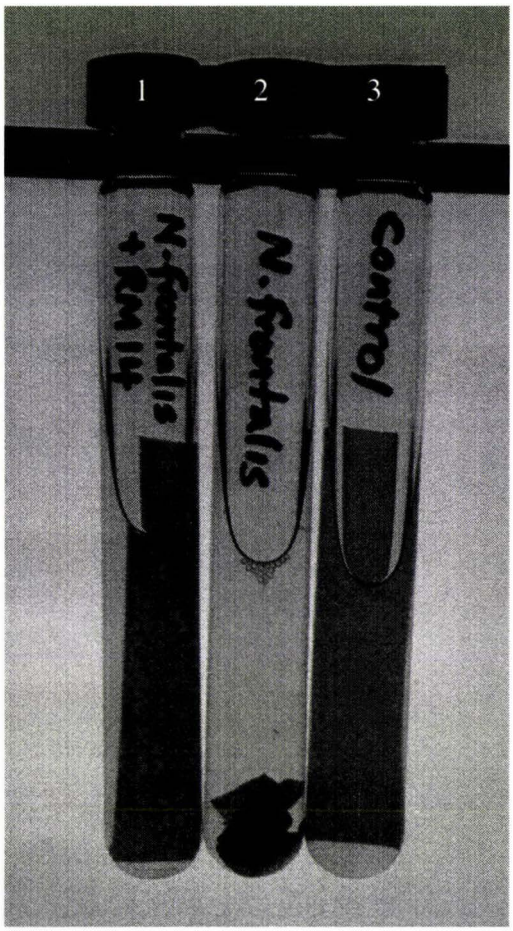

Fig 4.19 Photograph to illustrate the effect on cellulolysis by ruminal fungi in the presence of a ruminal mycoplasma. Tube 1. *N· frontalis* + RM14. Tube 2. *N· frontalis* alone. Tube 3. Control.

Table 4.8 Effect of ruminal mycoplasmas on cellulolysis by the fungus *N. frontalis*

Culture	Cellulose Used		H_2 Produced		pH[c]
	mg[a]	%[b]	ml[a]	%[b]	
N· frontalis	34 ± 6	100	1.85 ± 0.09	100	5.92
+RM10	32 ± 5	95	1.83 ± 0.16	100	6.01
+RM11	36 ± 2	110	1.66 ± 0.04	90	5.94
+RM12	31 ± 4	90	1.54 ± 0.31	85	6.06
+RM13	19 ± 2	55	1.13 ± 0.13	60	6.25
+RM14	15 ± 0	45	1.10 ± 0.11	60	6.34
+RM15	14 ± 3	40	0.94 ± 0.07	50	6.33

[a] Data represents mean and standard deviation of triplicate cultures
[b] Cellulolysis and H_2 production by fungus in axenic culture taken as 100%.
[c] Final culture pH

Not all of the isolates inhibited cellulolysis by *N. frontalis* (Table 4.8). Isolates RM10, RM11 and RM12 did not inhibit *N. frontalis* to any extent, while isolates RM13, RM14 and RM15 inhibited the fungus between 45% and 60% based on cellulose degradation data. The final culture pH also reflected the degree to which each fungus was inhibited by the ruminal mycoplasmas. Fungal H_2 production was also reduced when fungal growth was inhibited.

Table 4.9 Effect of ruminal mycoplasmas on cellulolysis by the fungus *P. communis*

Culture	Cellulose Used		H_2 Produced		pH[c]
	mg[a]	%[b]	ml[a]	%[b]	
P. communis	50 ± 3	100	0.61 ± 0.07	100	5.89
+RM10	45 ± 3	90	0.64 ± 0.04	105	5.82
+RM11	49 ± 2	100	0.61 ± 0.07	100	5.76
+RM12	52 ± 2	105	0.57 ± 0.04	90	5.74
+RM13	19 ± 0	40	0.47 ± 0.01	80	6.25
+RM14	15 ± 4	30	0.56 ± 0.06	90	6.35
+RM15	14 ± 2	30	0.49 ± 0.09	80	6.38

[a] Data represents mean and standard deviation of triplicate cultures
[b] Cellulolysis and H_2 production by fungus in axenic culture taken as 100%.
[c] Final culture pH

The degree to which ruminal mycoplamas inhibited cellulolysis by *P. communis* was variable (Table 4.9). Isolates RM10, RM11 and RM12 did not inhibit *P. communis* to any extent, while isolates RM13, RM14 and RM15 inhibited the fungus between 60% and 70% based on cellulose degradation data. The final culture pH also reflected the degree to which each fungus was inhibited by the ruminal mycoplasmas. The nature of the inhibiting mechanism is not known, but it is interesting to note that the isolates which inhibited cellulolysis by *N. frontalis* and *P. communis* the most, were those which did not lyse the cell walls of Gram-negative bacteria.

Table 4.10 Effect of ruminal mycoplasmas on cellulolysis by the fungus *C. communis*

Culture	Cellulose Used		H_2 Produced		pH[c]
	mg[a]	%[b]	ml[a]	%[b]	
C. communis	22 ± 5	100	0.67 ± 0.17	100	6.35
+RM10	13 ± 1	60	0.53 ± 0.13	80	6.61
+RM11	11 ± 2	50	0.52 ± 0.03	80	6.55
+RM12	8 ± 1	40	0.50 ± 0.06	75	6.62
+RM13	7 ± 3	30	0.48 ± 0.10	70	6.60
+RM14	4 ± 1	20	0.57 ± 0.10	85	6.60
+RM15	4 ± 1	20	0.45 ± 0.08	65	6.62

[a] Data represents mean and standard deviation of triplicate cultures
[b] Cellulolysis and H_2 production by fungus in axenic culture taken as 100%.
[c] Final culture pH

All of the ruminal mycoplasmas inhibited cellulolysis by *C. communis* B7 to varying degrees (Table 4.10). The final culture pH also reflected the degree to which each fungus was inhibited by the ruminal mycoplasmas. All of the coculture pH values lie in the range 6.55 to 6.62, while the final culture pH for *C. communis* in axenic culture had a value of 6.35, indicating greater degradation of cellulose.

4.3.3 Effect of Ruminal Mycoplasmas on *N. frontalis* Growing on Cellobiose.

In cocultures where cellobiose was the sole carbon source, the growth of *N. frontalis* was stimulated by nearly all of the ruminal mycoplasma isolates (Table 4.11), in contrast to the effect seen on cellulose, where fungal growth was often inhibited (Tables 4.8, 4.9 and 4.10). This suggested that the effect of the coculture was substrate dependent. The increased H_2 production and the lower pH seen in the test cultures, both indicate *N. frontalis* was stimulated by the coculture. Isolates RM13, RM14 and RM15 do not produce H_2, and so would not contribute directly to the H_2 produced in cocultures with *N. frontalis*.

Table 4.11 Effect of ruminal mycoplasmas on the fungus *N. frontalis* growing on cellobiose

Culture	H_2 Produced		pH[c]
	ml[a]	%[b]	
N. frontalis	1.08 ± 0.10	100	5.99
+RM10	1.23 ± 0.15	115	5.76
+RM11	1.00 ± 0.07	90	5.81
+RM12	1.31 ± 0.24	120	5.74
+RM13	1.45 ± 0.34	130	5.77
+RM14	1.49 ± 0.19	135	5.80
+RM15	1.51 ± 0.09	140	5.81
Control	0	0	6.79

[a] Data represents mean and standard deviation of triplicate cultures
[b] H_2 production by fungus in axenic culture taken as 100%.
[c] Final culture pH

The final coculture pH for *N. frontalis* and the ruminal mycoplasmas growing on cellobiose, complements the H_2 production data and suggests that ruminal mycoplasmas stimulate the growth of the fungus when growing on cellobiose. Although isolates RM13, RM14 and RM15 did not produce H_2, the stimulatory effect was still apparent, and indicated the increased H_2 production seen in these cocultures was of solely fungal origin.

4.3.4 SEM Studies of Ruminal Mycoplasma RM13 in Coculture with *N. frontalis*

SEM studies were carried out to examine the morphology of isolate RM13 growing in coculture with the ruminal fungus *N. frontalis*. The majority of the RM13 cells were found attached to the paper substrate but in some instances were also seen attached to fungal rhizoids (Fig 4.20). A variety of mycoplasma forms can be seen. There was no evidence the mycoplasma was attacking the cellulose matrix to which it was attached.

Fig 4.20 Scanning electron micrograph of isolate RM13 in coculture with the fungus *N. frontalis*. RM13 is attached evenly to the thallus tissue of the fungus (arrowed) and to the paper support.

4.4 ENUMERATION OF BOVINE RUMINAL MYCOPLASMAS

The population densities of the sterol-requiring *Anaeroplasma* species and the non-sterol requiring *Asteroleplasma* species in New Zealand cattle was unknown. Two separate experiments carried out 14 days apart, determined the total population densities of ruminal mycoplasmas and population densities of *Asteroleplasma* species present in the rumens of 3 Friesian cows on the two days (Figs 4.21 to 4.24). The total population densities of ruminal mycoplasmas found were in the ranges 6.7-8.6 x 10^7 gm^{-1} (day 1) and 6.9-9.4 x 10^7 gm^{-1} (day 14). The population densities of *Asteroleplasma* species found were in the ranges 5.9-8.9 x 10^5 gm^{-1} (day 1) and 6.9-9.1 x 10^5 gm^{-1} (day 14). Population densities were determined by colony counts after dilution of ruminal digesta through CRFB medium containing benzylpenicillin for total population studies, and benzylpenicillin with digitonin for enumeration of *Asteroleplasma* species. The latter medium was developed in the course of this study. The total population densities from

NZ cattle compared well with data published in previous reports (Robinson and Hungate, 1973; Robinson *et al*, 1975). There are no previous reports of population studies on *Asteroleplasma* species in the rumen. Published reports of non-lytic mycoplasma populations do not distinguish between *Asteroleplasma* species and *Anaeroplasma abactoclasticum,* both of which constitute part of the total population of non-lytic ruminal mycoplasmas. Robinson *et al* (1975) reported populations of non-lytic ruminal mycoplasmas of between 10^7 and 10^8 g^{-1} of ruminal digesta.

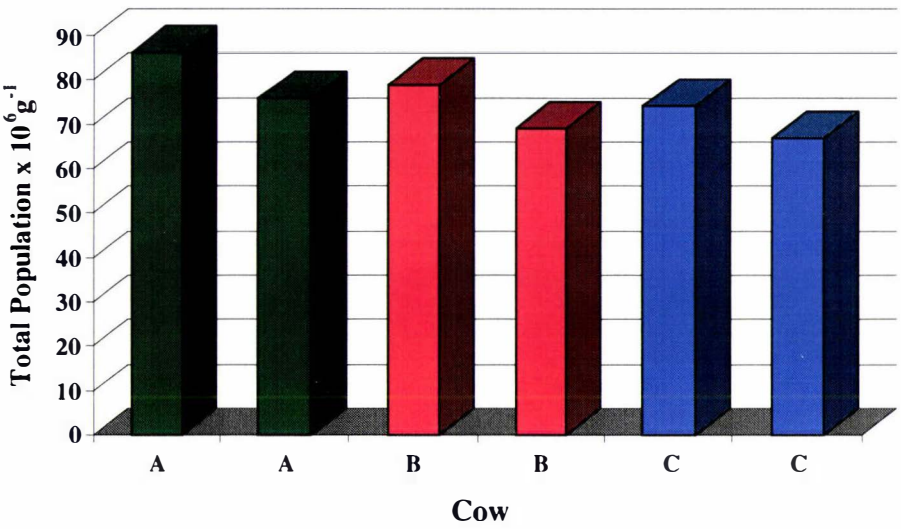

Fig 4.21 Total population density of ruminal mycoplasmas in 3 cows, per gram of ruminal digesta.

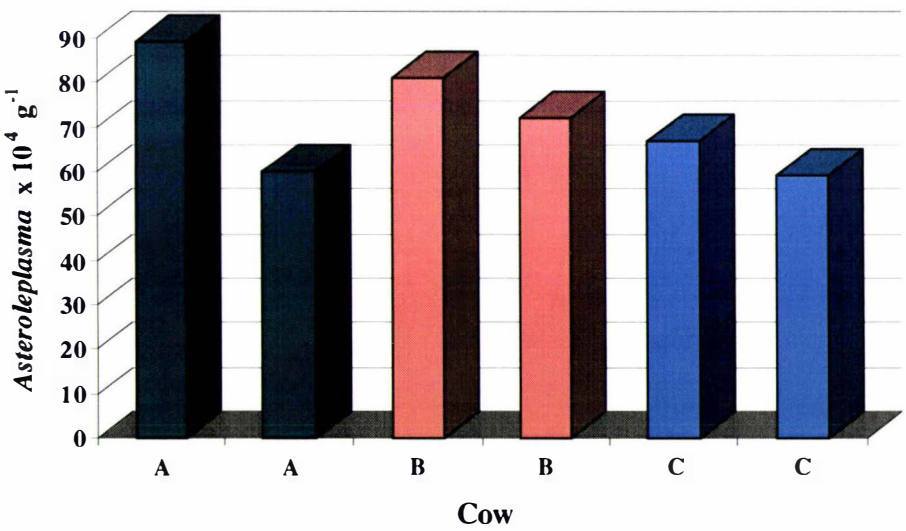

Fig 4.22 Population density of *Asteroleplasma* species in 3 cows, per gram of ruminal digesta.

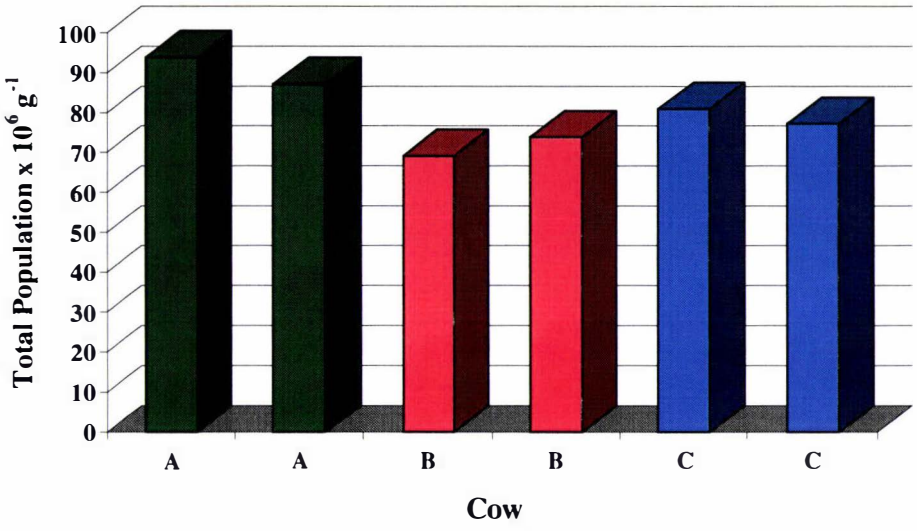

Fig 4.23 Total population density of ruminal mycoplasmas in 3 cows, per gram of ruminal digesta, determined 14 days after those shown in Fig 4.21.

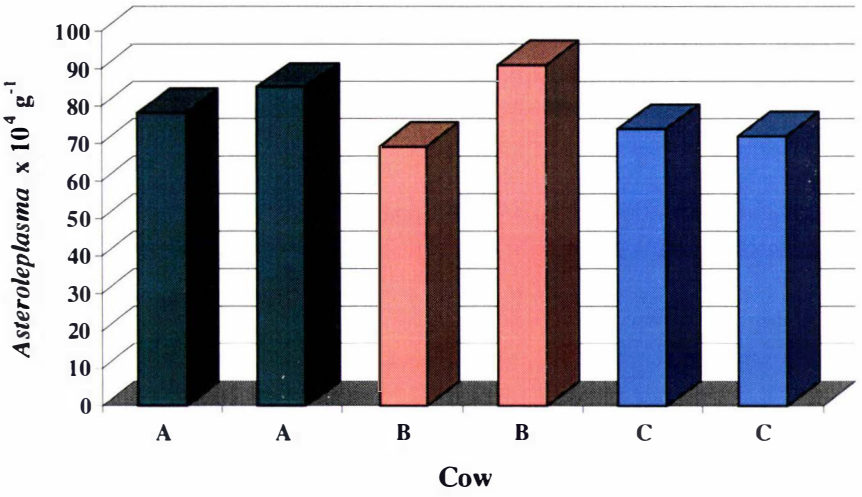

Fig 4.24 Population density of *Asteroleplasma* species in 3 cows, per gram of ruminal digesta, determined 14 days after those shown in Fig 4.22.

Because of the nature of the isolation process, ruminal fungi were often found contaminating tubes of ruminal mycoplasmas at initial isolation. This was generally true in the lower dilution tubes; the ruminal mycoplasmas being present in far greater numbers than the fungi. It was particularly noticeable, that in the tubes used to enumerate *Asteroleplasma* species, that ruminal mycoplasmas appeared to form a close association with the fungal colony, while at more distant points in the culture tube, there

were far fewer colonies of *Asteroleplasma* species. This observation suggests a degree of dependence by the mycoplasma on the fungal colony, and suggests also that the mycoplasma was cross-feeding on fungal metabolites. This observation is in keeping with the generally held belief that because of their small genome, mycoplasmas, both ruminal and non-ruminal, rely on their host for many essential nutrients (Razin, 1992).

5 CONCLUSIONS

A review of the literature revealed less than ten papers exclusively dedicated to the study of ruminal mycoplasmas since the first report published in 1966. A few studies have also been published on their serology, enzymology and molecular biology in order to establish their phylogeny, but only as part of wider-ranging studies on the class *Mollicutes*. The most recent paper on their physiology was published in 1975. From the available literature it must be concluded that ruminal mycoplasmas are a poorly studied group within the rumen microbial ecosystem about which little is known.

From enumeration studies of ruminal mycoplasmas in New Zealand cattle it was found the population density was 10^7-10^8 g^{-1} of ruminal digesta. This is in agreement with data published on overseas cattle studies and leads to the conclusion that New Zealand cattle are not unusual with respect to their populations of this group of ruminal microorganism. Because mycoplasmas constitute about 1% of the total bacterial population of the rumen it is concluded that their impact on the rumen fermentation must be considerable, not only in terms of inhibition of cellulolysis, but also their contribution to the rumen microbial protein pool.

The results presented here indicate that the NZ isolates can pass through 0.45 µm filters, have the same range of growth substrates, the same end products and antibiotic sensitivities, an ability to either lyse or not lyse Gram-negative bacteria, and have a requirement for sterol in growth media. It is concluded that the isolates in this study are similar to ruminal mycoplasmas previously isolated from ruminants overseas and are therefore not unique. Overseas isolates had lower optimum growth temperatures. All NZ isolates belonged to the genus *Anaeroplasma* and each had a unique PAGE protein banding profile, which suggested they belonged to two distinct groups. The optimum pH growth data and RAPD profiles supported this view. There were three isolates in each group. Phenotypic evidence identified three of the six isolates as strains of

Anaeroplasma abactoclasticum having all of the characteristics of ruminal mycoplasmas, not able to lyse Gram-negative bacteria.

The results presented in this thesis indicate that the second group of three isolates were very similar to the first, except they were able to lyse the cell walls of Gram-negative bacteria, were proteolytic and produced hydrogen. From these observations we conclude that they are also members of the genus *Anaeroplasma*, but of unknown species.

In studies investigating cocultures of ruminal mycoplasmas with cellulolytic bacteria it is concluded that mycoplasmas inhibit cellulolysis by some genera of bacteria, but appear to stimulate others. The inhibition of the bacteria does not appear to result from cell lysis since the only bacteria inhibited by ruminal mycoplasmas in this study belonged to two species of the genus *Ruminococcus* which are Gram-positive. Again there is a paradox, in that cultures of the Gram-negative bacterium *Fibrobacter succinogenes*, instead of being lysed and hence inhibited by the coculture with ruminal mycoplasmas, appeared to be slightly stimulated. From these observations we must conclude that the mechanisms of inhibition are unknown.

From studies investigating cocultures of ruminal mycoplasmas with ruminal fungi it is concluded that, while some mycoplasmas inhibited cellulolysis by fungi of the *Neocallimastix* and *Piromyces* genera, all inhibited cellulolysis by fungi of the *Caecomyces* genus. There was evidence to show that ruminal mycoplasmas did not kill the fungi because, although inhibited when growing on cellulose, the mycoplasma enhanced fungal growth on cellobiose. From these observations we conclude that the mechanisms of inhibition are unknown, but that they may be substrate dependent. Of the two groups of ruminal mycoplasma in this study, those most inhibitory to ruminal fungi were *Anaeroplasma abactoclasticum.*

The morphology of the ruminal mycoplasmas, as demonstrated by light microscopy, electron microscopy of thin sections of cells and scanning electron microscopy of whole cells, leads to the conclusion that while these microorganisms are unique in many respects, morphologically they are similar to many mycoplasmas from other mollicute

genera. Because of these morphological similarities, it is not possible to identify ruminal mycoplasmas on the basis of any unique morphological features which they possess.

The use of the RAPD technique to demonstrate polymorphisms in ruminal mycoplasma DNA showed that isolates which gave identical banding patterns with one primer, gave identical but different patterns with another. DNA from unrelated isolates gave different banding patterns when tested against the same primer. The study therefore showed that short primers of arbitrary nucleotide sequence may be used to reproducibly amplify segments of genomic DNA from ruminal mycoplasmas. The RAPD method was therefore able to demonstrate relatedness among the isolates.

The isolation of cellular proteins and their subsequent separation on polyacrylamide gels showed how powerful this technique can be in it's ability to provide relatedness data for the isolates. The clear demonstration of the presence of two groups (RM10, RM11 and RM12) and (RM13, RM14 and RM15) among the isolates also gave relatedness information which was confirmed by growth study data, including the ability of one group to lyse the cell wall of Gram-negative bacteria. The method went further in disclosing subtle differences among the isolates within the two groups. From the RAPD profile it was apparent that isolate RM13 was different from all other isolates, but physiologically similar in many respects to isolates RM14 and RM15. This relatedness was also demonstrated in the protein gel, with RM13 being obviously closely related to RM14 and RM15, but the presence of the very high molecular weight band in RM13 indicated an important difference between these isolates. Likewise, evidence for RM10 being slightly different from RM11 and RM12 is shown by careful examination of the protein profile, but was more obviously seen from the RAPD banding patterns.

The demonstration of the lysis of Gram-negative ruminal bacteria by three of the isolates suggests that *in vivo* these ruminal mycoplasmas may play a significant role in modifying the rumen fermentation. This would occur in a number of ways, but principally by inhibiting cellulolysis by at least three genera of ruminal fungi and one genus of ruminal bacteria. In addition, one of the two groups of ruminal mycoplasma were proteolytic and may play a role in the hydrolysis of protein in the rumen. The

conclusion that this suggests is, that in the absence of ruminal mycoplasmas the rumen would be more efficient both in the rate and extent of cellulose digestion and in the proportion of dietary protein available to the host. Like mycoplasmas from many ecosystems, ruminal mycoplasmas are parasitic, not on the host animal directly but on a number of the bacteria and fungi which populate their rumens and which are involved in forage digestion.

A lack of sensitivity to penicillin was a characteristic which aided the isolation of the first ruminal mycoplasma in 1966. Classified in 1973 as belonging to the genus *Acholeplasma*, it was soon appreciated that as a strict anaerobe the isolate did not conform to the description of this genus. A new genus was proposed in 1975 to encompass the original sterol-requiring isolate, while a second genus was proposed for newly isolated sterol non-requiring ruminal mycoplasmas. A lack of sensitivity to penicillin (and streptomycin sulphate) were characteristics which later facilitated the isolation of ruminal fungi from the ruminal digesta of sheep and cattle. Occasionally, unusual non-fungal colonies were observed in primary cultures, sometimes forming satellite rings of growth around the fungal colony. Careful examination of the colonies and cells, revealed they belonged to an unusual type of bacterium; the presence of characteristic "fried-egg" colonies suggesting they were mycoplasmas. Colonies were isolated and purified and shown to be strictly anaerobic, which further suggested they belonged to the two previously described genera found only in the rumen microbial ecosystem. From this we must conclude that had ruminal mycoplasmas not been resistant to penicillin and streptomycin sulphate and not co-isolated with ruminal fungi, it is unlikely they would have been observed and isolated in New Zealand and therefore would have not available to form the basis of this study.

6 APPENDIX: MICROORGANISMS USED IN THIS STUDY

6.1 Bacteria

- *Anaeroplasma* spp.
- *Butyrivibrio fibrisolvens* H17C
- *Clostridium chartatabidum* DSM 5482
- *Escherichia coli* XL1
- *Fibrobacter succinogenes* S85
- *Lachnospira multiparus* ATCC 19207
- *Megasphaera elsdenii* T81
- *Methanobrevibacter smithii*
- *Prevotella ruminicola ruminicola* ATCC 19189
- *Ruminococcus albus* RA7
- *Ruminococcus flavefaciens* FD1
- *Selenomonas ruminantium ruminantium* HD4
- *Streptococcus bovis* JB1
- *Succinovibrio dextrinosolvens* 24

6.2 Ruminal Fungi

- *Caecomyces communis* strain B7
- *Neocallimastix frontalis* strain Rold
- *Piromyces communis* strain B19

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