

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

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

A Thesis Presented in Partial Fulfilment  
of the Requirements for the Degree of

**MASTER OF SCIENCE**  
in Microbiology

at Massey University

**GRAHAM ERNEST NAYLOR**

1998

## 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<sub>2</sub>. 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<sub>2</sub>. 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.

## ACKNOWLEDGEMENTS

It is a great pleasure to say thanks to so many friends for their help and kindness during the preparation of this thesis.

To my supervisors, Dr George Ionas, Institute of Molecular BioSciences, Massey University, Palmerston North and Dr Keith Joblin, Rumen Microbiology Unit, AgResearch, Grasslands Research Centre, Palmerston North, for their advice, encouragement and support throughout this research project. Special thanks to Keith for allowing me to include his SEM images of isolate RM13.

To Dr Graeme Attwood, Bev Breslin, Diana Burgess, Dr Graeme Jarvis, Dr Brieuc Morvan and Kerri Reilly of the Rumen Microbiology Unit, AgResearch, Grasslands Research Centre, Palmerston North, for their friendship, advice and help in all sorts of ways. Special thanks to those whose proof-reading skills and sense of humour with regard to my scribbles, provided light-relief from what, at times, had become a chore.

To Helen Little and Joanne Morris of the Information Technology Group, AgResearch, Grasslands Research Centre, Palmerston North, for teaching me how to use a computer more effectively. Special thanks to Helen for her help with the final assembly of the thesis.

To Doug Hopcroft and Crunch Bennett of the Keith Williamson Electron Microscopy Unit, HortResearch, Palmerston North, for their ever friendly advice and help with TEM images of the ruminal mycoplasmas.

To Ann Ainscough, Barbara McPhee, Sarah Nation and Stephen Northover of the Crown Research Institutes' Library, Palmerston North, for their help, friendship and for finding those difficult to locate references.

To my Mum and Dad for their love and encouragement, and who in 1959 had the foresight to bring our family to live in New Zealand, with all the benefits that has provided.

To my wife Ann, for her love, support and patience; for help with proof reading and typing and for providing ever-welcome breaks.

This has been a real team effort.

Thanks for all your help.

## 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)



# CONTENTS

ABSTRACT .....	i
ACKNOWLEDGEMENTS .....	iii
DEDICATION .....	v
THE MICROBE .....	vi
CONTENTS .....	vii
LIST OF FIGURES.....	x
LIST OF TABLES.....	xi
ABBREVIATIONS .....	xii
1 INTRODUCTION .....	1
2 LITERATURE REVIEW .....	5
2.1 Introduction .....	5
2.2 The Rumen Microbial Ecosystem .....	5
2.3 Fibrolytic Ruminal Microorganisms.....	6
2.3.1 Fibrolytic Ruminal Bacteria .....	6
2.3.2 Fibrolytic Ruminal Fungi.....	7
2.3.3 Ruminal Protozoa .....	9
2.4 Ruminal Bacteriophages.....	10
2.5 Ruminal Yeasts.....	10
2.6 The Class <i>Mollicutes</i> .....	11
2.6.1 Genetics of the Class <i>Mollicutes</i> .....	13
2.6.2 Pathogenicity of <i>Mycoplasma</i> spp.....	14
2.6.3 Ruminal Mycoplasmas .....	15
2.6.4 Pathogenicity of <i>Anaeroplasma</i> spp.....	18
2.6.5 Metabolism of <i>Anaeroplasma intermedium</i> and <i>Asteroleplasma anaerobium</i> .....	18
2.6.6 Non-Ruminal Anaerobic Mycoplasmas.....	19
3 MATERIALS AND METHODS .....	21
3.1 Introduction .....	21
3.2 Isolation of Ruminal Mycoplasmas .....	22
3.2.1 Media for the Isolation and Purification of Ruminal Mycoplasmas .....	22
3.3 Characteristics of Ruminal Mycoplasmas .....	23
3.3.1 Morphology of Ruminal Mycoplasma Cells and Colonies .....	23
3.3.2 Filtration of Ruminal Mycoplasma Cells.....	25
3.3.3 Relationship between Temperature and Growth of Ruminal Mycoplasmas .....	25
3.3.4 Effect of pH on Growth of Ruminal Mycoplasmas .....	25

3.3.5 Growth Substrates for the Ruminal Mycoplasma Isolates .....	25
3.3.6 Analyses of Fermentation End-Products.....	26
3.3.7 Analyses of Fermentation Gases.....	26
3.3.8 Antibiotic Sensitivity of Ruminal Mycoplasmas .....	27
3.3.9 Lysis of Bacteria by Ruminal Mycoplasmas.....	28
3.3.10 Proteolysis by Ruminal Mycoplasmas .....	29
3.3.11 Lysis of Fungal Cell-Walls by Ruminal Mycoplasmas.....	29
3.3.12 Chitinase Activity of Ruminal Mycoplasmas .....	29
3.3.13 Sterol Requirements of Ruminal Mycoplasmas .....	30
3.3.14 RAPD Analysis of Ruminal Mycoplasma DNA.....	31
3.3.15 PAGE Analysis of Ruminal Mycoplasma Proteins .....	34
3.3.15.1 Extraction and Measurement of Mycoplasma Cellular Proteins .....	35
3.3.15.2 Electrophoresis of Mycoplasma Cellular Proteins .....	36
3.4 Coculture Studies .....	37
3.4.1 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Bacteria .....	37
3.4.2 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Fungi .....	38
3.4.3 Effect of Ruminal Mycoplasmas on <i>N. frontalis</i> Growing on Cellobiose.....	39
3.4.4 SEM Studies of Ruminal Mycoplasma RM13 in Coculture with <i>N. frontalis</i> .....	39
3.5 Enumeration of Bovine Ruminal Mycoplasmas .....	40
4 RESULTS AND DISCUSSION .....	41
4.1 Isolation of Ruminal Mycoplasmas .....	41
4.2 Characteristics of Ruminal mycoplasmas .....	41
4.2.1 Morphology of Ruminal Mycoplasmas .....	42
4.2.2 Filtration of Ruminal Mycoplasma Cells.....	46
4.2.3 Relationship between Temperature and Growth of Ruminal Mycoplasmas .....	47
4.2.4 Effect of pH on the Growth of Ruminal Mycoplasmas.....	52
4.2.5 Growth Substrates for the Ruminal Mycoplasma Isolates .....	56
4.2.6 Analyses of Fermentation End-Products.....	58
4.2.7 Antibiotic Sensitivity of Ruminal Mycoplasmas .....	59
4.2.8 Lysis of Bacteria by Ruminal Mycoplasmas.....	61
4.2.9 Proteolysis by Ruminal Mycoplasmas .....	63
4.2.10 Lysis of Fungal Cell-Walls by Ruminal Mycoplasmas.....	64
4.2.11 Chitinase Activity of Ruminal Mycoplasmas .....	64
4.2.12 Sterol Requirements of Ruminal Mycoplasmas.....	65
4.2.13 Identification of the Ruminal Mycoplasma Isolates .....	66
4.2.14 RAPD Analysis of Ruminal Mycoplasma DNA.....	67
4.2.15 PAGE Analysis of Ruminal Mycoplasma Proteins.....	69
4.3 Coculture Studies .....	71
4.3.1 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Bacteria .....	71

4.3.2 Effect of Ruminal Mycoplasmas on Cellulolysis by Ruminal Fungi .....	74
4.3.3 Effect of Ruminal Mycoplasmas on <i>N. frontalis</i> Growing on Cellobiose.....	77
4.3.4 SEM Studies of Ruminal Mycoplasma RM13 in Coculture with <i>N. frontalis</i> .....	78
4.4 Enumeration of Bovine Ruminal Mycoplasmas .....	79
5 CONCLUSIONS .....	83
6 APPENDIX: MICROORGANISMS USED IN THIS STUDY.....	87
6.1 Bacteria.....	87
6.2 Ruminal Fungi .....	87
7 BIBLIOGRAPHY .....	88

## LIST OF FIGURES

<b>Fig 4.1</b>	Subsurface (left) and surface (right) colonies of isolate RM10.....	43
<b>Fig 4.2</b>	Scanning electron micrograph to demonstrate the pleomorphic nature of isolate RM13. Note the nodulated, doughnut and dumbbell forms of the cells (arrowed) .....	44
<b>Fig 4.3</b>	Transmission electron micrograph of isolate RM10. Note the trilaminar cell membrane (arrowed), lack of a cell wall and unevenly stained cytoplasm. ....	45
<b>Fig 4.4</b>	Transmission electron micrograph of isolate RM14 showing the unevenly stained cytoplasm and the pleomorphic nature of the cells.....	46
<b>Fig 4.5</b>	Effect of temperature on growth of isolate RM10.....	47
<b>Fig 4.6</b>	Effect of temperature on growth of isolate RM11.....	48
<b>Fig 4.7</b>	Effect of temperature on growth of isolate RM12.....	49
<b>Fig 4.8</b>	Effect of temperature on growth of isolate RM13.....	49
<b>Fig 4.9</b>	Effect of temperature on growth of isolate RM14.....	50
<b>Fig 4.10</b>	Effect of temperature on growth of isolate RM15.....	51
<b>Fig 4.11</b>	Effect of pH on growth of isolate RM10.....	53
<b>Fig 4.12</b>	Effect of pH on growth of isolate RM11.....	53
<b>Fig 4.13</b>	Effect of pH on growth of isolate RM12.....	54
<b>Fig 4.14</b>	Effect of pH on growth of isolate RM13.....	54
<b>Fig 4.15</b>	Effect of pH on growth of isolate RM14.....	55
<b>Fig 4.16</b>	Effect of pH on growth of isolate RM15.....	55
<b>Fig 4.17</b>	RAPD profiles of DNA from isolates RM10 to RM15. For lane contents see Table 4.5.....	68
<b>Fig 4.18</b>	PAGE profiles of the proteins from isolates RM10 to RM15 .....	70
<b>Fig 4.19</b>	Photograph to illustrate the effect on cellulolysis by ruminal fungi in the presence of a ruminal mycoplasma. Tube 1. <i>N. frontalis</i> + RM14. Tube 2. <i>N. frontalis</i> alone. Tube 3. Control.....	75
<b>Fig 4.20</b>	Scanning electron micrograph of isolate RM13 in coculture with the fungus <i>N. frontalis</i> . RM13 is attached evenly to the thallus tissue of the fungus (arrowed) and to the paper support. ....	79
<b>Fig 4.21</b>	Total population density of ruminal mycoplasmas in 3 cows, per gram of ruminal digesta. ....	80
<b>Fig 4.22</b>	Population density of <i>Asteroleplasma</i> species in 3 cows, per gram of ruminal digesta.....	80
<b>Fig 4.23</b>	Total population density of ruminal mycoplasmas in 3 cows, per gram of ruminal digesta, determined 14 days after those shown in Fig 4.21.....	81
<b>Fig 4.24</b>	Population density of <i>Asteroleplasma</i> species in 3 cows, per gram of ruminal digesta, determined 14 days after those shown in Fig 4.22.....	81

## LIST OF TABLES

<b>Table 2.1</b> Classification of ruminal fungi .....	9
<b>Table 2.2</b> Taxonomy and characteristics of the class <i>Mollicutes</i> <sup>a</sup> .....	13
<b>Table 2.3</b> Taxonomy of the ruminal mycoplasmas <sup>a</sup> .....	17
<b>Table 2.4</b> Type Strains and G+C mol% of Ruminal Mycoplasma DNA <sup>a</sup> .....	18
<b>Table 3.1</b> Antibiotic stock solutions and final concentration of each antibiotic in media. ....	27
<b>Table 4.1</b> Substrate utilisation by type strains of ruminal mycoplasmas and the isolates from this study .	57
<b>Table 4.2</b> Antibiotic sensitivities <sup>a</sup> of ruminal mycoplasmas .....	59
<b>Table 4.3</b> Lysis of autoclaved bacterial cells by ruminal mycoplasmas in agar culture .....	62
<b>Table 4.4</b> Lysis of autoclaved bacterial cells by ruminal mycoplasmas in broth culture .....	63
<b>Table 4.5</b> DNA and primer combinations for the RAPD analysis .....	68
<b>Table 4.6</b> Protein concentration of cell lysates and lane-loading of the PAGE gel .....	70
<b>Table 4.7</b> Cellulolysis and H <sub>2</sub> production by ruminal bacteria growing on cellulose in the presence of ruminal mycoplasmas .....	73
<b>Table 4.8</b> Effect of ruminal mycoplasmas on cellulolysis by the fungus <i>N. frontalis</i> .....	75
<b>Table 4.9</b> Effect of ruminal mycoplasmas on cellulolysis by the fungus <i>P. communis</i> .....	76
<b>Table 4.10</b> Effect of ruminal mycoplasmas on cellulolysis by the fungus <i>C. communis</i> .....	77
<b>Table 4.11</b> Effect of ruminal mycoplasmas on the fungus <i>N. frontalis</i> growing on cellobiose .....	78

## 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 <sub>4</sub>	methane
cm	centimetre
cm <sup>-2</sup>	per square centimetre
cm <sup>-3</sup>	per cubic centimetre
CO <sub>2</sub>	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 <sup>-1</sup>	per gram
GC	gas chromatograph
H <sub>2</sub>	hydrogen
HPLC	high pressure liquid chromatography
hr	hour
ICSBSTM:	International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of <i>Mollicutes</i> , 1995.
IU	international unit
kbp	kilobase pairs
kDa	kiloDaltons
l	litre
LDH	lactic dehydrogenase
M	molar
mA	milliampere
mg	milligram
min	minute
min <sup>-1</sup>	per minute
ml	millilitre
ml <sup>-1</sup>	per millilitre
mm	millimetre
mM	millimolar
Mmix	mastermix

$\mu\text{g}$	microgram
$\mu\text{l}$	microlitre
$\mu\text{m}$	micrometre
$\mu\text{M}$	micromolar
na	not applicable
nd	not determined
ng	nanogram
$\text{NH}_3$	ammonia
nm	nanometre
dNTP	2'-deoxynucleoside 5'-triphosphate
$\text{O}_2$	oxygen
$\text{OD}_x$	absorbance of light; x is the incident-light wavelength in nm
$\text{OsO}_4$	osmium tetroxide
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
$\text{pH}_{\text{opt}}$	optimum pH for culture growth
PIM	primary isolation medium
$\text{P}_2\text{O}_5$	phosphorus pentaoxide
$\text{PP}_i$	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	<i>Thermus aquaticus</i> DNA polymerase
TCA	tricarboxylic acid cycle
TE	tris-EDTA buffer
TEM	transmission electron microscope
TEMED	N,N,N',N' tetramethylethylenediamine
TLC	thin-layer chromatography
$T_{\text{opt}}$	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