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