

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.

**Investigation of Rumen Methanogens in New Zealand
Livestock**

A thesis presented in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Animal Science

at Massey University, Palmerston North, New Zealand.

Jeyanathan Jeyamalar

2010

Abstract

Methane emitted by farmed ruminants contributes 30.3% to New Zealand's anthropogenic greenhouse gas inventory. Methanogens living in the rumen produce methane from H₂ and CO₂ as a byproduct of feed fermentation. The use of vaccines and small molecule inhibitors against the methanogens are promising methods to reduce methane emissions from extensively-grazed ruminants in New Zealand. Knowledge of the methanogens present in New Zealand ruminants is an important first step for successful vaccine and inhibitor development to target all methanogens.

In this study, the methanogen diversity of farmed ruminants (sheep [*Ovis aries*], cattle [*Bos taurus*] and red deer [*Cervus elaphus*]) was investigated using molecular ecological techniques. Ruminants fed different diets had largely similar rumen methanogen communities. The major methanogen groups identified were from the *Methanobrevibacter ruminantium* clade (*Mbb. ruminantium* and closely-related species), *Methanobrevibacter gottschalkii* clade (*Mbb. gottschalkii* and closely-related species), *Methanosphaera* spp., and the putative methanogens belonging to the group designated Rumen Cluster C. A total of 37.5 - 57% of 16S rRNA genes in the rumen of a group of cows originated from members of Rumen Cluster C. Chloroform treatment of cows increased the abundance of Rumen Cluster C to 82% - 93% of archaeal 16S rRNA genes. In parallel, a total of 22% of *mcrA* genes belonged to an unassigned group of archaea, and chloroform treatment increased the unassigned group of archaea to 92% of all *mcrA* genes. This suggested that Rumen Cluster C archaea contain the gene *mcrA*.

No members of the Rumen Cluster C group have previously been cultured, and currently there is no reported rumen isolate of *Methanosphaera* spp. A strain of *Methanosphaera* sp. was isolated from a sheep rumen and initial characterization suggests that this may be a new species. Three enrichment cultures were obtained containing members of Rumen Cluster C as the only archaea. Initial studies of these enrichment cultures showed that these three isolates were from three different sub-groups of Rumen Cluster C and that they produced methane.

The investigation of methanogen diversity in New Zealand farmed ruminants and isolation of previously uncultured rumen methanogens reported here in this thesis will

significantly aid the development of methane reduction strategies for farmed ruminants in New Zealand.

Acknowledgements

It was my privilege to work under the guidance of Dr. Peter H. Janssen. I am grateful for his excellent guidance, help in planning, supervision, cooperation, morale boost, timely advice and inspiration. He has been very supportive and understanding all this time and it helped me to enjoy my study. I highly appreciate your invaluable help.

This thesis would not have been possible without the unflinching support, cooperation and prompt feedback by Dr Ron Ronimus. His continual enquiry, interest, supports and direction was a big boost and I highly appreciate it.

My sincere thanks are also to Dr. Simone Hoskin for her encouragement and help during my study. I appreciate your help during my study.

The financial assistance provided by NZAID as scholarship is highly appreciated. I am grateful to Pastoral Greenhouse Gas Research Consortium Ltd (PGGRC) for funding for the research and AgResearch for allowing me to conduct the research in their lab.

Many people in Alan Johns building have provided help and friendship during my study. I am very thankful to all of them. You have all been fantastic to work with and have made my time spent in the lab lots of fun. I would like to thank all the members of Rumen Microbiology team in AgResearch, Grasslands, for their continual support. In particular Graham Naylor, you are one of the nicest people I have known, your help over the years is very much appreciated. Sandra Kittelmann and Gemma Henderson, you have been extremely helpful and a great source of inspiration to me. I highly appreciate your help and guidance over the years. Debjit Dey, you have been fantastic and your help over the years is very much appreciated. I also extend my thanks to Marek Kirs and Catherine Tootill for their support at the beginning of this study.

I am thankful to Natasha Swainson for allowing me to collect samples from her experimental animals. I also thankful to Terry Knight, Harry Clark, Cesar Pinares-Patiño and others who collected and processed samples from CHCl_3 trial, for allowing me to use those samples for my study. German Molano and Geoff Purchas, thank you for your help during the sample collection. Special thanks to Kelly Armstrong and

Bryan Treloar for their help in HPLC analysis and Doug Hopcroft and Jianyu Chen for their help in electron microscopy.

My sincere thanks are also to Denise Martin, Catherine Cameron and Debbie Hill for their help during my study. I am also thankful to international student support office, Massey University, especially Sylvia Hooker, Olive Pimental and Sue Flynn for their help and support.

A special thanks to my friend Suganthi Senthilnathan, who has always been like a family to me and thanks to her family who did not mind to look after my little daughter whenever I needed. My special thanks are also conveyed to my family for their help and encouragement and a very special thanks to my little daughter Kaaruniya, who has been just great.

TABLE OF CONTENTS

	Page No
Approval of request to embargo the thesis	iii
Abstract	v
Acknowledgements	vii
Table of contents	ix
List of tables	xvii
List of figures	xxi
List of non-standard abbreviations	xxvii
Chapter 1 Literature review	
1.1 Introduction	1
1.2 Rumen methanogens	3
1.3 Rumen methanogenesis	4
1.4 Techniques for culturing methanogens	6
1.5 Molecular ecology techniques used to identify methanogens	9
1.5.1 16S rRNA gene clone libraries	10
1.5.2 <i>mcrA</i> gene clone libraries	11
1.5.3 DNA probe/fluorescence <i>in situ</i> hybridization (FISH)	12
1.5.4 Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)	13
1.5.5 Quantitative real-time PCR	14
1.6 Cultured methanogens from ruminants	15
1.7 Methanogens identified through molecular ecology techniques	17
1.8 Unidentified archaea in the rumen	21
1.9 Interactions between methanogens and other rumen microbes	27
1.9.1 Bacterial-methanogen interactions	28
1.9.2 Protozoal-methanogen interactions	30
1.9.3 Fungal-methanogen interactions	33
1.10 Methods for controlling rumen methanogenesis	33
1.10.1 Increasing productivity	34
1.10.2 Vaccination against methanogens	35
1.10.3 Defaunation techniques	36
1.10.4 Other approaches	37

1.11	Conclusions	38
1.12	Objectives of the thesis	40

Chapter 2 Materials and methods

2.1	Use of animals	43
2.2	Rumen sample collection and processing	43
2.3	DNA extraction	46
2.3.1	DNA extraction from the rumen samples	46
2.3.2	DNA extraction from cultures	46
2.4	Preparation of DNA-free water	47
2.5	Preparation of Luria-Bertani (LB) medium	47
2.6	Polymerase chain reactions (PCR)	47
2.7	Agarose gel electrophoresis	48
2.8	Purification of PCR products	48
2.9	Purification of plasmids	48
2.10	Extraction and purification of DNA from agarose gels	57
2.11	DNA quantification	57
2.12	Cloning	57
2.13	Sequencing and chimera detection	57
2.14	Phylogenetic analysis	58
2.15	Analysis of clone libraries and the phylogenetic trees	58
2.16	Denaturing gradient gel electrophoresis (DGGE)	59
2.17	DNA extraction from DGGE gels	60
2.18	Analysis of DGGE gels	60
2.19	Quantitative real-time PCR (qPCR)	61
2.19.1	qPCR of total archaea	61
2.19.2	qPCR of Rumen Cluster C	62
2.19.3	qPCR of total bacteria	62
2.20	Fluorescence <i>in situ</i> hybridization (FISH)	63
2.21	Cultivation experiments of methanogens	64
2.22	Media	65
2.22.1	BY medium	65
2.22.2	Modified BY medium (BY ⁺)	66
2.22.3	RM02 medium	66

2.23	Media additives	66
2.23.1	Rumen fluid-yeast extract-vitamin mixture	66
2.23.2	RFgenV	67
2.23.3	Vitamin 10 concentrate	67
2.23.4	Trace element solution (SL10)	67
2.23.5	Volatile fatty acid solution	68
2.23.6	Substrate solutions	68
2.24	Gram staining	69
2.25	Phase contrast microscopy	69
2.26	Electron microscopy	69
2.26.1	Negative staining	70
2.26.2	Transmission electron microscopy (TEM)	70
2.27	G+C mol % analysis of DNA using an HPLC method	71
2.28	Measuring methanogen growth	72
2.28.1	Gas chromatography	72
2.28.2	Spectrophotometer	72
2.29	Long-term preservation of cultures	72
2.29.1	Preservation with dimethyl sulfoxide (DMSO)	72
2.29.2	Preservation with glycerol	73
2.30	Re-generating frozen cultures	73

Chapter 3 Selection of primers for the molecular-based studies of rumen methanogens

3.1	Introduction	75
3.2	Materials and Methods	77
3.2.1	Sample collection	77
3.2.2	Total DNA extraction and PCR	77
3.2.3	Primers used for the construction of clone libraries	78
3.2.4	Clone library construction, chimera detection and phylogeny	78
3.2.5	Analysis of clone libraries and the phylogenetic trees	79
3.2.6	Nucleotide sequences of clones and cultures	79
3.3	Results	80
3.3.1	Variations in the archaeal community structure in the clone libraries from different 16S rRNA gene primer pairs	80

3.3.2	Variation in the methanogen community structure in the clone libraries based on different <i>mcrA</i> gene primer pairs	84
3.3.3	Phylogenetic analysis of 16S rRNA partial genes	87
3.3.4	Phylogenetic analysis of <i>mcrA</i> genes	88
3.4	Discussion	110

Chapter 4 Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets

4.1	Introduction	117
4.2	Materials and methods	119
4.2.1	Use of animals	119
4.2.2	Sample collection	119
4.2.3	Total DNA extraction and PCR	120
4.2.4	Denaturing gradient gel electrophoresis (DGGE)	122
4.2.5	Cloning and sequencing	124
4.2.6	Analysis of electrophoresis patterns	124
4.2.7	Quantitative real-time PCR	125
4.2.8	Phylogenetic analyses	126
4.2.9	Nucleotide sequence accession numbers	126
4.3	Results	128
4.3.1	General archaeal community structure	128
4.3.2	Variations due to host, species or diet	136
4.3.3	Comparison with bacterial communities	140
4.3.4	Identity of dominant archaea	140
4.3.5	Diversity of RCC	144
4.3.6	Validation of DGGE data	145
4.4	Discussion	149
4.4.1	General archaeal community structure	149
4.4.2	Dominant archaea	150
4.4.3	Rumen Cluster C archaea	151
4.4.4	Host and diet specific differences	152

Chapter 5 Effects of rumen-administered chloroform on the abundance and diversity of *mcrA* and 16S rRNA genes of rumen archaea indicate the presence of as-yet uncultured methanogens

5.1	Introduction	155
5.2	Materials and Methods	156
5.2.1	CHCl ₃ treatment and sample collection	156
5.2.2	Total DNA extraction and PCR	157
5.2.3	Clone library construction and phylogeny	157
5.2.4	Phylogenetic analysis	158
5.2.5	Denaturing gradient gel electrophoresis (DGGE)	159
5.2.6	Quantitative real-time PCR analysis	159
5.2.7	Sequences of 16S rRNA and <i>mcrA</i> gene clones	159
5.3	Results	161
5.3.1	Methane production	161
5.3.2	Archaeal and bacterial community sizes	161
5.3.3	Total archaeal community structure	169
5.3.4	Total RCC community structure	173
5.3.5	Gene-based analysis of archaeal community structure	176
5.3.6	Analysis of RCC community structure	184
5.4	Discussion	187

Chapter 6 Isolating uncultured rumen methanogens

6.1	Introduction	191
6.2	Materials and Methods	193
6.2.1	Sample collection	193
6.2.2	Media and blending time	193
6.2.3	Substrates used	194
6.2.4	Isolation experiments	194
6.2.4.1	Isolation Experiment 1 (ISO1)	194
6.2.4.2	Isolation experiment 2 (ISO2)	195
6.2.4.3	Isolation experiment 3 (ISO3)	195
6.2.4.3	Isolation experiment 4 (ISO4)	196
6.2.5	DNA extraction and PCR	201
6.2.6	Cloning and DNA sequencing	201

6.2.7	Phylogenetic analysis of methanogens isolated in this study	201
6.2.8	Quantitative real-time PCR analysis	202
6.2.9	Microscopy	202
6.2.10	Fluorescence <i>in situ</i> hybridization (FISH)	202
6.2.11	Eliminating bacteria	202
6.2.11.1	Dilution	202
6.2.11.2	Using antibiotics	203
6.2.11.3	Heat treatment	203
6.2.11.4	Using lysozyme	203
6.3	Results	204
6.3.1	Isolation experiment 1	204
6.3.2	Isolation experiment 2	204
6.3.3	Isolation experiment 3	206
6.3.4	Isolation experiment 4	207
6.3.5	Archaeal clone library analysis	208
6.3.6	Bacterial clone library analysis	208
6.3.7	Microscopy	210
6.3.8	Fluorescence <i>in situ</i> hybridization (FISH)	212
6.3.9	Phylogenetic position	212
6.4	Discussion	216

Chapter 7 Characterization of *Methanosphaera* isolate ISO3-F5

7.1	Introduction	221
7.2	Materials and Methods	222
7.2.1	Source of inoculums	222
7.2.2	Enrichment and isolation	222
7.2.3	Culture purity	223
7.2.3.1	Microscopy	223
7.2.3.2	Inoculation with mixed sugar, peptone and yeast extract solution (RFgenV)	223
7.2.3.3	PCR with bacterial primers	223
7.2.3.4	Archaeal 16S rRNA gene-based clone analysis	224
7.2.4	Gram stain	224
7.2.5	Electron microscopy	224

7.2.6	Growth factor requirements	224
7.2.7	Substrate requirements	225
7.2.8	Optimum pH for growth	225
7.2.9	Optimum temperature	226
7.2.10	Cell lysis	226
7.2.11	G+C content of genomic DNA	226
7.2.12	Phylogenetic analysis of ISO3-F5	227
7.2.13	Long term preservation of ISO3-F5	228
7.2.14	Gene (16S rRNA and <i>mcrA</i>) sequences	228
7.3	Results	229
7.3.1	Culture purity	229
7.3.2	Morphology	229
7.3.3	RF, YE, CoM, Vit and VFA requirements	230
7.3.4	Substrate requirements	240
7.3.5	Optimum pH	240
7.3.6	Optimum temperature	240
7.3.7	Cell lysis	240
7.3.8	G+C content	240
7.3.9	Phylogenetic position	245
7.3.10	Long term preservation of ISO3-F5	245
7.4	Discussion	250
7.5	Summary	253
7.6	Future work	254
Chapter 8 Summary and general discussion		257
References		265
Appendix		

List of Tables

	Page
Table 1.1 Reaction and standard changes in free energies for methanogenesis	5
Table 1.2 Details of 16S rRNA gene clone library-based surveys of archaea in rumen samples	23
Table 1.3 Deatils of the archaeal 16S rRNA gene primers used to construct clone libraries from rumen samples	27
Table 2.1 The details of the animals and rumen sample collection methods used in this study	45
Table 2.2 Primers used for the PCR amplification to construct 16S rRNA gene clone libraries (Bacteria, Archaea and RCC) and to identify the presence of these organisms in samples or cultures	49
Table 2.3 Primers used for the PCR amplification to construct <i>mcrA</i> gene clone libraries and to identify the presence of methanogens in samples or cultures	53
Table 2.4 Primers used for the amplification of inserts of pCR 2.1 TOPO vector	53
Table 2.5 Primers used to obtain PCR products for analysis by denaturing gradient gel electrophoresis (bacteria, archaea and RCC)	55
Table 2.6 16S rRNA gene-targeted fluorescently- labelled oligonucleotide probes used in this study.	63
Table 3.1 Primer pairs used to construct clone libraries for the comparison study	78
Table 3.2 Pure cultures that were used to validate the primer pair ARC8f and ARC1510r	80

Table 3.3	The number of chimeric sequences detected in each of the primer pair used to construct clone libraries in this chapter	81
Table 3.4	The rumen samples used to validate the primer pair MCRmf and MCRr and the groups of sequences amplified	84
Table 4.1	Primers used in this study to target 16S rRNA genes of total archaea, total bacteria, and archaea of the RCC group	122
Table 4.2	Primer binding sites considered for targeting the archaeal 16S rRNA gene for DGGE analysis	130
Table 4.3	Abundance of different clades of archaea in libraries of PCR-amplified 16S rRNA genes from rumen contents of sheep no. 4 fed winter pasture	147
Table 5.1	Primer pairs selected for qualitative and quantitative study of rumen microbes in CHCl ₃ -treated and control cows	158
Table 5.2	The details of 16S rRNA and <i>mcrA</i> gene clones created from CHCl ₃ -treated and control cows	160
Table 5.3	Abundance of different groups of archaea in libraries of PCR-amplified partial 16S rRNA and <i>mcrA</i> genes generated from DNA extracted from the rumen of a CHCl ₃ -treated cow and a cow from a control group	177
Table 5.4	Abundance of different groups of archaea in libraries of PCR-amplified partial 16S rRNA genes generated from DNA extracted from the rumen of CHCl ₃ -treated cows and control cows	178
Table 6.1	Estimation of methanogen MPN and plan for isolation experiment 1	197
Table 6.2	Estimation of methanogen MPN and plan for isolation experiment 2	198
Table 6.3	Estimation of methanogen MPN and plan for isolation experiment 3	199
Table 6.4	Estimation of methanogen MPN and plan for isolation experiment 4	200

Table 6.5	The number of methane-positive tubes obtained in ISO2 in each dilution with different media after 21 days of incubation	206
Table 6.6	The number of 16S rRNA genes of total archaea and RCC estimated by qPCR from the DNA from methane-positive tubes obtained in isolation experiment 2 (ISO2)	207
Table 6.7	The different groups of methanogens identified in the methane-positive tubes from isolation experiment 3 (ISO3)	207
Table 6.8	The different groups of methanogens identified in the methane-positive tubes from isolation experiment 4	208
Table 6.9	Methanogens identified in the cultures	209
Table 6.10	Bacteria identified in the cultures	209
Table 6.11	Purity checking of cultures	217
Table 7.1	The combinations of growth factors tested for their effects on growth of ISO3-F5	235
Table 7.2	The combinations of substrates tested for growth of ISO3-F5 and the results obtained	241
Table 7.3	Comparison of characteristics of ISO3-F5 with <i>Methanosphaera stadtmanae</i> and <i>Methanosphaera cuniculi</i>	255

List of Figures

		Page
Figure 3.1	The percentages of different archaeal groups identified by six different 16S rRNA gene primer pairs (P1-P6) from the rumen sample obtained from a cow (C2) fed with lucerne silage and barley meal	81
Figure 3.2	Diversity indices of rumen archaeal clone libraries constructed with six 16S rRNA gene primer pairs	83
Figure 3.3	The percentages of different methanogen groups identified by three different <i>mcrA</i> gene primer pairs (M-P1 to M-P3) from the rumen samples obtained from a cow (C2) fed with lucerne silage and barley meal	85
Figure 3.4	Diversity of the methanogen community in the rumen sample detected by three <i>mcrA</i> gene primer pairs	87
Figure 3.5	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 109f and 915r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	90
Figure 3.6	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 630f and 803r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	92
Figure 3.7	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 1af and 1100r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	94

Figure 3.8	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 109f and 1386r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	96
Figure 3.9	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 915af and 1386r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	98
Figure 3.10	Inferred phylogenetic relationships between partial 16S rRNA genes obtained by the primer pair 86f and 1340r from the rumen sample of cow fed with lucerne silage and barley meal and reference 16S rRNA gene sequences	100
Figure 3.11	Inferred phylogenetic relationships between deduced amino acid sequences of <i>mcrA</i> genes obtained by the primer pair ML1 and ML2 from the rumen sample of cow fed with lucerne silage and barley meal and reference <i>mcrA</i> gene amino acid sequences	102
Figure 3.12	Inferred phylogenetic relationships between deduced amino acid sequences of <i>mcrA</i> genes obtained by the primer pair ME1 and ME2 from the rumen sample of cow fed with lucerne silage and barley meal and reference <i>mcrA</i> gene amino acid sequences	104
Figure 3.13	Inferred phylogenetic relationships between deduced amino acid sequences of <i>mcrA</i> genes obtained by the primer pair MCRf and MCRr from the rumen sample of cow fed with lucerne silage and barley meal and reference <i>mcrA</i> gene amino acid sequences	106
Figure 3.14	Inferred phylogenetic relationships between deduced amino acid sequences of <i>mcrA</i> genes obtained by the primer pair MCRmf and MCRr from the rumen sample of cow fed with lucerne silage and barley meal and reference <i>mcrA</i> gene amino acid sequences	108

Figure 4.1	DGGE fingerprints of ruminal archaea in red deer, cattle and sheep fed summer pasture, winter pasture, and silage	132
Figure 4.2	DGGE fingerprints of ruminal archaea and ruminal bacteria in two different flocks of sheep fed winter pasture or a concentrate-based diet	134
Figure 4.3	DGGE fingerprints of ruminal archaea in two different flocks of sheep fed autumn pasture or willow fodder	135
Figure 4.4	Similarities of DGGE profiles of ruminal archaea in the same group of cattle, red deer, and sheep fed summer pasture, winter pasture and silage	138
Figure 4.5	DGGE profiles showing stability of archaeal communities in a cow, a red deer, and a sheep, 2, 4, 6, and 8 hours after feeding silage	139
Figure 4.6	Inferred phylogenetic relationships between partial 16S rRNA genes of derived from excised DGGE bands and reference 16S rRNA gene sequences	142
Figure 4.7	DGGE fingerprints of RCC in sheep, red deer, and cattle fed summer pasture	146
Figure 5.1	Methane emissions from control and CHCl ₃ -treated cows over the experimental period	164
Figure 5.2	Changes in the number of bacterial 16S rRNA genes in control and CHCl ₃ -treated cows over the experimental period	165
Figure 5.3	Changes in the number of archaeal 16S rRNA genes in control and CHCl ₃ -treated cows over the experimental period	166
Figure 5.4	Changes in the number of RCC 16S rRNA genes in control and CHCl ₃ -treated cows over the experimental period	167

Figure 5.5	Changes in the percentages of RCC 16S rRNA genes in the total archaeal 16S rRNA genes in control and CHCl ₃ -treated cows over the experimental period	168
Figure 5.6	DGGE fingerprints of ruminal archaea in control cows and CHCl ₃ -treated cows before and after the CHCl ₃ treatment	171
Figure 5.7	Similarities of DGGE profiles of ruminal archaea in the control and CHCl ₃ -treated cows before and after the CHCl ₃ treatment	172
Figure 5.8	DGGE fingerprints of ruminal RCC in control cows and CHCl ₃ -treated cows before and after the CHCl ₃ treatment	174
Figure 5.9	Similarities of DGGE profiles of ruminal RCC in the control and CHCl ₃ -treated cows before and after the CHCl ₃ treatment	175
Figure 5.10	Comparison of 16S rRNA and <i>mcrA</i> gene phylogenetic trees of the control cow	180
Figure 5.11	Comparison of 16S rRNA and <i>mcrA</i> gene phylogenetic trees of the CHCl ₃ -treated cow	182
Figure 5.12	Phylogenetic tree of 16S rRNA gene sequences constructed using the sequences from RCC group obtained from CHCl ₃ -treated and control cows	185
Figure 5.13	Phylogenetic tree of <i>mcrA</i> gene sequences, constructed using deduced amino acid sequences from the <i>mcrA</i> group 1 obtained from CHCl ₃ -treated and control cows	186
Figure 6.1	Change in the Most Probable Number estimation of methanogens with incubation time for combinations of two media and blending time in isolation experiment 1 (ISO1)	205
Figure 6.2	Photomicrographs of culture ISO4-G16	211
Figure 6.3	Photomicrographs of culture ISO3-F5	211

Figure 6.4	Photomicrographs of culture ISO4-G11	213
Figure 6.5	Phylogenetic relationships of isolated cultures and reference 16S rRNA gene sequences	214
Figure 6.6	Phylogenetic relationships of isolated cultures and reference <i>mcrA</i> amino acid sequences	215
Figure 7.1	Electron photomicrograph of negatively-stained dividing cells of ISO3-F5	231
Figure 7.2	Transmission electron microscopy of ISO3-F5	232
Figure 7.3	Enlargement of cytoplasmic structures <i>csi</i> and <i>csii</i> in the dividing cell of ISO3-F5	234
Figure 7.4	Growth curve of ISO3-F5 with and without 5% rumen fluid in the 3 rd serial transfer	236
Figure 7.5	Growth curve of ISO3-F5 with and without volatile fatty acid mixture in the 3 rd serial transfer	237
Figure 7.6	Growth curve of ISO3-F5 with and without coenzyme M in the 3 rd serial transfer	238
Figure 7.7	Growth curve of ISO3-F5 with and without vitamins in the 3 rd serial transfer	239
Figure 7.8	Growth curve of ISO3-F5 with different substrates	242
Figure 7.9	Effect of pH on maximum culture density	243
Figure 7.10	Effect of incubation temperature on maximum culture density	244
Figure 7.12	Phylogenetic relationships of isolate ISO3-F5 and reference 16S rRNA gene sequences	246
Figure 7.13	Phylogenetic relationships of isolate ISO3-F5 and reference <i>mcrA</i> gene sequences	248

Non-standard abbreviations

BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
C	cow
CoM	coenzyme M
D	red deer
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EM	electron microscopy
FISH	fluorescence <i>in situ</i> hybridization
GHG	greenhouse gas
h	hour
HPLC	high-performance liquid chromatography
IR	infra-red
LB	Luria-Bertani
Ltd	Limited
M	molar
Mbb	<i>Methanobrevibacter</i>
MCR	methyl coenzyme reductase
min	minutes
MOPS	3-(N-morpholino) propanesulfonic acid
MPN	most probable number
PCR	polymerase chain reaction
qPCR	quantitative real-time polymerase chain reaction
RCC	Rumen Cluster C
RF	rumen fluid
RNA	ribonucleic acid
RODS	relative one-dimensional surface

rpm	revolutions per minute
S	sheep
SRB	sulphate reducing bacteria
subs	substrate
TGGE	temperature gradient gel electrophoresis
U	unit
UV	ultraviolet
v/v	volume per volume
VFA	volatile fatty acid
vit	vitamins
w/v	weight per volume
w/w	weight per weight
YE	yeastextract