

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.

**16S Ribosomal DNA Probes for the Detection and Enumeration of
Proteolytic Rumen Bacteria**

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

Master of Science
in Molecular Biology
at Massey University

Kerri Reilly

1999

ABSTRACT

Bacterial degradation of protein causes inefficient nitrogen retention in New Zealand ruminants. The 16S rRNA genes of a *Butyrivibrio fibrisolvens*-like strain and three *Streptococcus bovis* strains, isolated from New Zealand cattle were sequenced to further characterise these isolates. Based on 16S rDNA analysis the *B. fibrisolvens*-like isolate was classified as *Clostridium proteoclasticum*, while the three *S. bovis* isolates were confirmed as *S. bovis* strains.

In the absence of selective media for enumeration of these bacteria, a competitive PCR (cPCR) approach was developed for enumeration of these bacteria from rumen samples. PCR primers were designed to variable regions within the 16S ribosomal RNA genes of both *S. bovis* and *C. proteoclasticum*. These primers were used in conjunction with the universal forward primer fD1*, to allow amplification of 16S rDNA fragments from these organisms. DNA database searches revealed that the B316 830 primer sequence was present in four *B. fibrisolvens* strains. Analysis of 16S rDNA sequences indicated that these *B. fibrisolvens* strains are closely related to *C. proteoclasticum* and that the B316 830 primer circumscribes these five strains.. The B315 454 primer sequence was found in the 16S rDNA of 10 *Streptococcus* species. Primer specificity was tested in amplification reactions with DNA extracted from 85 bacterial isolates, mainly of rumen origin. The *C. proteoclasticum* primer B316 830 and fD1* produced a specific PCR product from *C. proteoclasticum* DNA only, while the *S. bovis* primer B315 454 and fD1* gave specific PCR product from DNA of all strains of *S. bovis* tested but from no other rumen bacterium. An internal control was developed for both *S. bovis* and *C. proteoclasticum* to use in cPCR reactions for quantitation. Standard curves were constructed relating the PCR product intensity of target DNA extracted from a known number of cells and the intensity of internal control DNA PCR product. The standard curves were used to quantitate populations of *S. bovis* and *C. proteoclasticum* in rumen samples collected from eight dairy cows fed a rotation of four diets. Populations detected ranged from 2×10^6 to 2.8×10^7 for *C. proteoclasticum* and 1.7×10^7 to 1.3×10^8 for *S. bovis*. Diet had no significant effect on the populations of either of these proteolytic bacteria.

ACKNOWLEDGEMENTS

To my supervisors Dr Graeme Attwood, Rumen Microbiology Unit, AgResearch, Grasslands Research Centre, Palmerston North and Professor Barry Scott, Institute of Molecular Biosciences, Massey University, Palmerston North for their advice, support and encouragement throughout this research project.

I wish also to thank Keith Joblin, Brieuc Morvan, Graham Naylor, Diana Burgess, Bev Breslin and Graeme Jarvis of the Rumen Microbiology Unit for their encouragement, advice and friendship throughout the course of this study.

To Dr Peter Lockhart of the Institute of Molecular Biosciences for his advice and patience in the construction of the phylogenetic trees.

Thanks to my family and friends, your love and support is always appreciated. A special thanks goes to Paul Cole, my partner, who gave me lots of encouragement, motivation and love during the course of this study.

I also wish to thank AgResearch for allowing me to undertake this study.

CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	ii
CONTENTS	iii
LIST OF FIGURES	vi
LIST OF TABLES	vii
Chapter 1 - INTRODUCTION AND LITERATURE REVIEW	1
1.1 Introduction	1
1.2 Nitrogen Metabolism in the Rumen	3
1.3 Identification and Classification of Bacteria in the Rumen	7
1.3.1 Molecular Methods for Phylogenetic Analysis	7
1.3.2 Phylogenetic Analysis of Rumen Bacteria	11
1.3.3 Competitive PCR	14
1.4 Aims and Objectives	17
Chapter 2 - METHODS AND MATERIALS	19
2.1 Bacterial Strains	19
2.2 Chemicals	20
2.3 Media	20
2.4 Buffers and Solutions	20
2.5 Bacteria Growth	20
2.6 Phenol/Chloroform/Isoamyl Alcohol Extractions	21
2.7 Ethanol Precipitations	21
2.8 DNA Extraction	21
2.8.1 Enzymatic Lysis	21
2.8.2 Physical Disruption	21
2.8.3 Chemical Extraction	22
2.9 DNA Quantitation	22
2.10 Agarose Gel Electrophoresis	22
2.11 Extraction of DNA from Agarose	23

2.13 Ligations	23
2.14 Transformations	24
2.15 Plasmid DNA Minipreparations	24
2.16 PCR Primers and Amplification	25
2.17 Cloning of the 16S rRNA Genes	26
2.18 16S rDNA Sequencing	26
2.19 Sequence Analysis	29
2.20 Construction of the Internal Controls	30
2.21 Preparation of Bacteria Cells for Sensitivity Testing	31
2.22 Quantitation of PCR Products	31
2.23 Rumen Samples	31
Chapter 3 – 16S rDNA DETERMINATION, ANALYSIS, PRIMER DESIGN AND INTERNAL CONTROL CONSTRUCTION	33
3.1 Introduction	33
3.2 Results	34
3.2.1 Cloning and Sequence Analysis of the 16S rRNA genes	34
3.2.2 Primer Design	34
3.2.3 Prime Specificity	41
3.2.4 Internal Control Construction	41
3.2.5 Internal Control Amplification Efficiency	48
3.3 Discussion	57
Chapter 4 – ENUMERATION OF <i>C. proteoclasticum</i> AND STREPTOCOCCI FROM RUMEN SAMPLES BY COMPETITIVE PCR	61
4.1 Introduction	61
4.2 Results	61
4.2.1 Co-amplification Detection Limit	61
4.2.2 DNA Extraction Efficiency	64
4.2.3 Standard Curve Construction	64
4.2.4 Detection of <i>C. proteoclasticum</i> added to Rumen Fluid	71
4.2.5 Detection of <i>C. proteoclasticum</i> and <i>S. bovis</i> <i>in vivo</i>	71
4.3 Discussion	78

Chapter 5 – GENERAL DISCUSSION AND CONCLUSIONS	83
APPENDIX A	89
APPENDIX B	92
APPENDIX C	96
APPENDIX D	116
REFERENCES	117

LIST OF FIGURES

Figure 2.1 Cloning of 16S rRNA genes.	27
Figure 3.1 Phylogenetic analysis of <i>C. proteoclasticum</i>	36
Figure 3.2 Phylogenetic analysis of streptococcal strains	38
Figure 3.3 Primer Design for <i>C. proteoclasticum</i> and <i>S. bovis</i>	40
Figure 3.4 B316 830 primer specificity	43
Figure 3.5 B315 454 primer specificity	45
Figure 3.6 Verification of amplification in template DNA	47
Figure 3.7 Construction of the <i>C. proteoclasticum</i> internal control.	50
Figure 3.8 Construction of the <i>Streptococcus</i> internal control.	52
Figure 3.9 <i>C. proteoclasticum</i> internal control amplification efficiency	54
Figure 3.10 <i>S. bovis</i> internal control amplification efficiency.	56
Figure 4.1 Detection limit of cPCR	63
Figure 4.2 <i>C. proteoclasticum</i> standard curve construction	66
Figure 4.3 <i>Streptococcus</i> standard curve construction	68
Figure 4.4 <i>Streptococcus</i> standard curve construction	70
Figure 4.5 <i>In vivo</i> detection of <i>C. proteoclasticum</i>	72
Figure 4.6 Populations of <i>C. proteoclasticum</i> in dairy cows under four different feeding regimes.	75
Figure 4.7 Populations of streptococci in dairy cows under four different feeding regimes.	77

LIST OF TABLES

Table 2.1 Bacterial Strains	19
Table 2.2 PCR Primers	25
Table 2.3 Sequencing Primers	29
Table 2.4 Rotation of Diet	32