Homoacetogenesis as an alternative hydrogen sink in the rumen

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Microbiology and Genetics

at Massey University, Palmerston North, New Zealand.

Preeti Raju

2016

Abstract

Ruminant livestock contribute significantly to global greenhouse gas emissions. This is due to microorganisms, known as methanogens that generate methane from hydrogen and carbon dioxide during feed fermentation in the rumen. Mitigation strategies are being developed to reduce methane emissions from ruminants. However, inhibiting methane production may cause accumulation of unused hydrogen in the rumen, which may slow down rumen fermentation and affect animal productivity. Homoacetogens, microbes known to reside in the rumen, can use hydrogen and carbon dioxide to form acetate. Homoacetogens could take over the role of ruminal hydrogen disposal following inhibition of methanogens. The aims of this study were to quantify the involvement of alternative hydrogen utilisers, such as homoacetogens, in hydrogen or electron utilisation. Chemical compounds were screened to identify specific inhibitors of methanogens (BES, acetylene), and both methanogens and homoacetogens (chloroform). Homoacetogenesis was measured via incorporation of $^{13}CO_2$ into ^{13}C-acetate using a short-term *in vitro* assay. This short-term *in vitro* assay measured and confirmed the occurrence of homoacetogenesis in sheep rumen fluid, and it accounted for 1.67% of electron utilisation in fresh rumen fluid. Homoacetogenesis increased in the assay when BES was added, suggesting homoacetogens could increase their activity in the absence of methanogens. Homoacetogenesis decreased with the addition of chloroform, which is known to partially inhibit homoacetogens. Methane formation was inhibited by acetylene in an *in vitro* serial batch fermentation inoculated with sheep rumen fluid. Homoacetogenesis did not increase, but the homoacetogens were able to grow and maintain themselves as the rumen material was repeatedly diluted and supplemented with fresh feed. Their activity accounted for 2.32% of electron utilisation. To study their significance in the rumen, methane formation was inhibited in sheep using acetylene. Homoacetogenesis increased and accounted for 6.53% of electron utilisation. However, propionate appeared to be the major electron sink (58-88%) in the absence of methanogenesis both *in vitro* and *in vivo*. In the future, knowledge of these hydrogen-utilising microorganisms could be used to divert hydrogen or electrons into more beneficial end-products, leading to the transition from a normal methane-producing rumen to an equally or even more productive low methane one.

Acknowledgements

It is rightly said that life is a constant learning process, and the quest for knowledge is a never-ending process, as it is fuelled by its own curiosity. The learning process that I have begun long back has continued throughout my PhD tenure and towards the end of this course. I utilize this opportunity to thank everyone who has assisted me. Foremost, I would like to extend my deepest gratitude to Dr. Peter H. Janssen, who has contributed enormously to the genesis of this thesis, both in terms of intellectual input and encouragement. His extraordinary scientific acumen has chiselled me as a budding researcher. My PhD studies would not have been possible without his constant support and expert guidance. I am indebted to his role as a mentor.

I duly acknowledge Dr. Gemma Henderson for her continuous support throughout my PhD. Her guidance, insightful suggestions, motivation and immense knowledge made a profound difference to my studies. My sincere thanks goes to Dr. Michael Tavendale for help in planning, supervision, being approachable and timely advice throughout my studies. I would also like to thank Associate Professor Jasna Rakonjac, Massey University, for attending my monthly supervisor meetings and her valuable suggestions, and timely support. I highly appreciate the consistent feedback, support and expert advices from all my four supervisors. I also convey my thanks to Dr. Graeme Attwood and Dr. Sinead Leahy for attending my monthly supervisor meetings and their extended interest in my project.

My thesis would not have been possible without the technical help and encouragement by many people in the Rumen Microbiology Team, AgResearch, especially Debjit Dey, Faith Cox, Michelle Kirk and Priya Soni, and all the other lab members for their friendly and fantastic nature. I am highly grateful to Dr. Stefan Muetzel, from the Animal Nutrition and Physiology team, for his valuable suggestions and for helping me with the *in vitro* and sheep trials. I am also thankful to Sarah Lewis, Sarah Maclean, Greg Skelton, Dan Robinson, German Molano and Edgar Sandoval Rodriguez, members of the Animal Nutrition and Physiology team, for helping me with rumen sample collection and GC analysis, and in various ways during the course of my study.

Brian Treloar provided expert help with VFA analysis using HPLC. I am greatly thankful to Dr. Siva Ganesh and Catherine Lloyd-West for helping me with statistical analysis of my study. I owe a great deal of gratitude to Sharron Smith and Caroline Costall for their effort to administer necessary formalities during my PhD tenure. My journey was continued to be pleasant with the friends Lovepreet Kaur, Sandeep Kumar, Navjot Kaur, Harinder Kaur, Dr. Helal Ansari, Dr. Wajid Hussain, Dr. Ajmal Khan, Phil Theobald, Won Hong, Lydia Koolaard and my office buddies Nikola Palevich and Li Yang. I will ever cherish the convivial moments that I spent with them during my stay.

The financial assistance provided by New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) and Pastoral Greenhouse Gas Research Consortium (PGgRc) is duly acknowledged. I thank Kate Parlane and Dr. Heather Went (NZAGRC) for assistance with NZAGRC formalities during my studies.

Last but not the least, my family is my perpetual source of inspiration and driving force. My mother, brother and my best friend Sandeep Singh were invaluable for their spiritual support and encouragement during my tough times. I must not forget to acknowledge my Dad, whose memories are always with me, will remain forever and keep encouraging me to work hard.

Contents

List of Tables

List of Figures

Abbreviations

With a few exceptions (to avoid confusion), standard SI units are not defined here.

ACS	acetyl-CoA synthetase
ATP	adenosine triphosphate
atm	atmosphere
BES	2-bromoethanesulfonate
bp	base pair
BSA	bovine serum albumin
CH_4	methane
$CHCl_3$	chloroform
CoM	coenzyme M
CO_2	carbon dioxide
CODH	carbon monoxide dehydrogenase
Conc.	concentration, concentrated
dNTP	deoxynucleotide triphosphate
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FTHFS	formyltetrahydrofolate synthetase
g	gravity
$\Delta G°'$	Gibb's (free) energy change
GC	gas chromatography
GC-FID	gas chromatography with flame ionisation detector
GC-MS	gas chromatography mass spectrometry
GC-IRMS	gas chromatography with isotope ratio mass spectrometry
2GenRFV	GCXAL-CPY-rumen fluid-vitamin mix with double substrate concentrations
GHG	greenhouse gases
GP	general purpose diet
H_2	hydrogen
HPLC	high performance liquid chromatography
HMM	hidden Markov model

HMMER	profile hidden Markov model software
IPTG	isopropyl β-D-1-thiogalactopyranoside
kJ/mol	kilojoules per mole
K_s	half saturation constant (also referred to as Monod's constant)
LB	Luria-Bertani
Ltd.	limited
M	molar
min	minutes
m/z	mass-to-charge ratio
N_2	nitrogen
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAD(P)H	NADH or NADPH
$NaHCO_3$	sodium hydrogen carbonate
NoSubRFV	rumen fluid vitamin mix with no added growth substrates
PCR	polymerase chain reaction
psi	pounds per square inch
qPCR	quantitative real-time polymerase chain reaction
QIIME	quantitative insights into microbial ecology
RCC	Rumen Cluster C
RF	rumen fluid
RM02	rumen medium number 2
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
S	dissolved substrate concentration
S_{min}	minimum threshold substrate concentration
SPME	solid-phase micro extraction
SD	standard deviation
SEM	standard error of the mean
TAE	tris-acetate-EDTA
U/µl	units/microlitre
v/v	volume per volume
V	relative rate of metabolism
V_{max}	maximum rate of metabolism

VFA	volatile fatty acid(s)
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Mathematical abbreviations

A	amount of acetate produced
A_f	amount of acetate formed from fermentation
A_{ha}'	amount of acetate produced from homoacetogenesis (VFA inter-conversion uncorrected)
A_{ha}	amount of acetate (unlabelled and labelled) produced via homoacetogenesis (VFA inter-conversion corrected)
A^*	$^{13}C_1$-acetate
A^{**}	$^{13}C_2$-acetate
^{13}A	amount of excess labelled acetate
$^{13}A_{ha}$	amount of ^{13}C-labelled acetate produced via homoacetogenesis
B	amount of butyrate produced
B_f	amount of butyrate formed from fermentation
B_{hb}'	amount of butyrate produced from homobutyrogenesis (VFA inter-conversion uncorrected)
B_{hb}	amount of butyrate (unlabelled and labelled) produced via homobutyrogenesis (VFA inter-conversion corrected)
B^*	$^{13}C_1$-butyrate
B^{**}	$^{13}C_2$-butyrate
B^{***}	$^{13}C_3$-butyrate
B^{****}	$^{13}C_4$-butyrate
^{13}B	amount of excess labelled butyrate
$^{13}B_{hb}$	amount of ^{13}C-labelled butyrate produced via homobutyrogenesis
f_{ab}	fractional amount of ^{13}C-acetate converted to ^{13}C-butyrate
f_{ap}	fractional amount of ^{13}C-acetate converted to ^{13}C-propionate
f_{ba}	fractional amount of ^{13}C-butyrate converted to ^{13}C-acetate
f_{bp}	fractional amount of ^{13}C-butyrate converted to ^{13}C-propionate
f_{pa}	fractional amount of ^{13}C-propionate converted to ^{13}C-acetate
f_{pb}	fractional amount of ^{13}C-propionate converted to ^{13}C-butyrate
H_2	amount of hydrogen formed from fermentation
$2H$	two reduced protons, representing two electrons
m/z	mass-to-charge ratio
M	amount of methane formed from fermentation

MPE_i	mole percent excess for any species containing *i* labelled carbons
MPE_0	mole percent excess for any species containing no labelled carbon
MPE_1	mole percent excess for any species containing one labelled carbon
MPE_2	mole percent excess for any species containing two labelled carbons
MPE_3	mole percent excess for any species containing three labelled carbons
MPE_4	mole percent excess for any species containing four labelled carbons
P	amount of propionate produced
P_f	amount of propionate formed from fermentation
P^*	$^{13}C_1$-propionate
P^{**}	$^{13}C_2$-propionate
P^{***}	$^{13}C_3$-propionate
^{13}P	amount of excess labelled propionate
rA_{Lferm}	ratio of $^{13}C/^{12}C$ in acetate in fermentations with $NaH^{13}CO_3$
rA_{Uferm}	ratio of $^{13}C/^{12}C$ in acetate in fermentations with unlabelled $NaHCO_3$
rB_{Lferm}	ratio of $^{13}C/^{12}C$ in butyrate in fermentations with $NaH^{13}CO_3$
rB_{Uferm}	ratio of $^{13}C/^{12}C$ in butyrate in fermentations with unlabelled $NaHCO_3$
$rCO_{2\ Lferm}$	ratio of $^{13}C/^{12}C$ in CO_2 in fermentations with $NaH^{13}CO_3$
$rCO_{2\ Uferm}$	ratio of $^{13}C/^{12}C$ in CO_2 in fermentations with unlabelled $NaHCO_3$
rP_{Lferm}	ratio of $^{13}C/^{12}C$ in propionate in fermentations with $NaH^{13}CO_3$
rP_{Uferm}	ratio of $^{13}C/^{12}C$ in propionate in fermentations with unlabelled $NaHCO_3$
$r^{13}CO_2$	ratio of excess of dissolved $^{13}CO_2$ relative to control without $NaH^{13}CO_3$
R_1	selected ion peak area ratios for ions m/z 108:107 for sample
R_{10}	selected ion peak area ratios for ions m/z 108:107 for control
R_2	selected ion peak area ratios for ions m/z 109:107 for sample
R_{20}	selected ion peak area ratios for ions m/z 109:107 for control
R_3	selected ion peak area ratios for ions m/z 110:107 for sample
R_{30}	selected ion peak area ratios for ions m/z 110:107 for control
R_4	selected ion peak area ratios for ions m/z 111:107 for sample
R_{40}	selected ion peak area ratios for ions m/z 111:107 for control
V_f	amount of valerate formed from fermentation
X_a	fraction of acetate coming from CO_2
X_b	fraction of butyrate formed from homobutyrogenesis