

PASTORAL GREENHOUSE GAS RESEARCH CONSORTIUM

# 5 YEAR SCIENCE PROGRESS REPORT **2007-2012**

Developing solutions to reduce  
New Zealand agricultural emissions



# New Zealand Pastoral Greenhouse Gas Research Consortium Partners

The PGgRc is an unincorporated Joint Venture operating through its agent company Pastoral Greenhouse Gas Research Ltd.

## PARTICIPANTS



## ASSOCIATE MEMBERS

Ministry for Primary Industries  
Manatū Ahu Matua



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## NZ GOVERNMENT FUNDING PARTNER



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# PGgRc BOARD

As at 30 June 2012

Organisation	Representative
<b>Fonterra</b>	Mark Leslie (D) (Chair)
<b>DairyNZ</b>	Rick Pridmore (D)
<b>Beef+Lamb NZ</b>	Leon Black (D)
<b>AgResearch</b>	Warren McNabb (D)
<b>PGG Wrightson</b>	Damian Lynch (D)
<b>FertResearch</b>	Philip Mladenov (D)
<b>Landcorp Farming</b>	Andrew MacPherson (O)
<b>DEEResearch</b>	Mark O'Connor (O)
<b>MBIE</b>	Marc Lubbers (O)
<b>MPI</b>	Mike Jebson (O)
<b>NIWA</b>	Murray Poulter (O)
<b>NZAGRC</b>	Harry Clark (O)

D=Director O= Observer

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# FOREWORD

Welcome to the second 5 year review of the PGgRc investment covering the period from 2007 to 2012. As with the first review, published in 2007, we have produced this booklet as a reference publication, to summarise a number of key research activities undertaken by the PGgRc. It sets out who the Consortium represents, what research it has invested in to develop methods for New Zealand farmers to reduce livestock GHG emissions and identifies some of our major achievements along with plans for the future.

We have compiled this document to serve a broad audience; ranging from farmers and their investing organisations, through to scientists working in the field and for Government officials seeking further information about our research. Therefore you will note a bit of everything is included. Where we feel the content is specialized or technical in nature we have provided some explanations to provide relevance and context for the reader to decide if they wish continue with that section.

In creating this body of work there has by necessity been many individuals and organisations involved; as Consortium manager I have been privileged to receive their assistance and support throughout the five years and I thank them all. In particular I would like to acknowledge the support and guidance of the PGgRc Board. With its mixture of directors from funding organisations and observers from participating industries the PGgRc has had the benefit of a broad and sound perspective on the investment portfolio from the many parties involved.

Equally I would like to acknowledge and thank the individual scientists and research teams that have been involved in our programme; it is their devotion and continued efforts that have given us the progress we report to you in this review. The Consortium is grateful for those efforts.

This review represents a comprehensive team effort that we believe will enable New Zealand to meet the challenge that it faces in reducing agricultural Methane and Nitrous Oxide emissions—enabling New Zealand Agriculture to remain competitive in the carbon conscious future we face.

We hope that you find this booklet a useful and handy reference. If you require more information on our programme please don't hesitate to contact us.



**Mark Aspin**  
MANAGER PGgRc

# CHAIRMAN'S INTRODUCTION

The Pastoral Greenhouse Gas Research Consortium (PGgRc) is a partnership between the pastoral industries and the New Zealand Government. This is the second 5 year review booklet created by the Consortium and it reports on the investment by the pastoral sector into the mitigation of agricultural greenhouse gases over the 2007-2012 period and introduces the activity and focus for further investment beyond that.

Formed in 2002 to reduce methane emissions from ruminant animals, the PGgRc (then the Methane Consortium) began rebuilding New Zealand's research capabilities and human capital in the field of rumen science. Since 2003 the Consortium's membership has expanded to incorporate nitrous oxide research and with the addition of Landcorp farming as a partner in 2007 has directed the investment of \$45M over the 10 year period into this challenging and vital area of research.

The period has seen considerable change to the landscape in which the PGgRc investment has been made; with the arrival of the NZAGRC, greater investment by the Crown through SLMACC and more latterly the development of the Global Research Alliance. This period also has seen an ETS approach to carbon cost being confirmed and adopted by New Zealand Government as we look to the future of an economy with a carbon cost included. Over this time the PGgRc has maintained its focus, has progressed the science behind methane reduction and continued the development of the understanding of the use of Nitrification Inhibitors to reduce N<sub>2</sub>O.

In April 2006, the research commissioned by the PGgRc and the governance of the Consortium was reviewed by The Crown funding body, The Foundation for Research Science and Technology (FRST). The panel of internationally recognized experts described the PGgRc activities as "World leading research, with excellent productivity for money expended. The Consortium represents the biggest single integrated program globally responding to issues around livestock greenhouse emissions".

The Consortium has continued that momentum and in 2010 was the first group to publish the genome of a ruminant Methanogen detailing the blueprint of the organism responsible for producing methane. From this fundamental knowledge we have developed mitigation programmes that are highly specific to reducing methane. We have also confirmed that low methane emissions is a heritable trait through the respiratory chamber screening of 1300 sheep, resulting in a research flock that will be further investigated to identify accurate and low cost ways to select for this trait in sheep and cattle. These two outstanding results are amongst many and are just a part of progress made across a broad research front.

Looking to the future, the PGgRc's investment will build on the knowledge gained to rapidly develop the most promising technologies for mitigation. The strategy will be to take that knowledge and evolve the research program accordingly. The challenge of integrating that knowledge into farm systems is not insignificant and will require continued collaboration with many organisations throughout the sector and Government. The Consortium believes that it is well placed after 10 years to continue to support delivery of excellence for pastoral based agricultural greenhouse gas mitigation research in New Zealand and potentially globally. We look forward to consolidating efforts to achieve this with NZAGRC and the Crown.



**Mark Leslie**  
CHAIRMAN PGGRC, 2002-2013

# BACKGROUND

## CLIMATE CHANGE, GREENHOUSE GASES AND PASTORAL AGRICULTURE

Agriculture is part of a complex social, economic and environmental global dynamic system. It feeds more than seven billion people, and underpins economic development and poverty alleviation over much of the world. It is a major source of greenhouse gases (GHG), yet stands to be impacted—both directly and indirectly—by climate change itself. With a growing global population to feed, it is vital that agricultural production grows but not its environmental impacts.

## LIVESTOCK AGRICULTURE AND CLIMATE CHANGE

There is robust scientific evidence that the climate is changing, and that most of the warming observed over the past 50 years is due to increasing GHG concentrations from human activities. Carbon dioxide (CO<sub>2</sub>) is the most important GHG produced by human activities, but other gases, such as methane (CH<sub>4</sub>), and nitrous oxide (N<sub>2</sub>O), also contribute substantially to the overall warming effect.

Since the industrial revolution, global average atmospheric concentrations of GHGs have increased by 150 per cent, from around 700 parts per billion by volume (ppbv) to 1745 ppbv in 1998. According to the IPCC, human activities are thought to be responsible for most of the global warming already observed over the past half century. Further increases in GHG concentrations are expected to result in even greater climate changes over the 21st century especially if no action is taken to reduce emissions. More climatic extremes, such as heat waves and drought, rising average temperatures and changes in rainfall patterns are to be expected. Scientists agree that GHG emissions (GHGe) need to be reduced significantly overall to meet the world's stated aim of constraining global warming to no more than 2°C.

The IPCC estimates that, as at 2005, agricultural emissions of CH<sub>4</sub> and N<sub>2</sub>O were responsible for around 12% of all human-sourced GHGe. The more we reduce emissions of all of these gases, the more we would expect to reduce the rate and magnitude of future climate change.

Agriculture emits CO<sub>2</sub> through land clearance, loss of soil carbon, feed crop production and energy use. Estimates by the United Nations food and Agriculture Organisation (FAO) indicate that these sources of CO<sub>2</sub> could account for about nine per cent of all human sourced CO<sub>2</sub> emissions, mainly from land clearance.

The livestock sector is also the single biggest contributor to human sourced CH<sub>4</sub> emissions at between 25% and 40%. Most of that comes from the stomachs of ruminant animals when bacteria break down cellulose in the absence of oxygen—a process called enteric fermentation. Some 10% of livestock CH<sub>4</sub> is produced from anaerobic manure storage, with still smaller emissions from animal manure deposited directly onto soils by grazing animals.

Nitrous oxide is emitted directly from soils when nitrogen (N) contained in synthetic fertilisers is broken down by soil bacteria. Almost six million tonnes of N<sub>2</sub>O are produced annually across the globe through urine from managed livestock and fertiliser use, constituting more than half of all human sourced N<sub>2</sub>O.



“A threefold challenge now faces the world: Match the rapidly changing demand for food from a larger and more affluent population to its supply; do so in ways that are environmentally and socially sustainable; and ensure that the world's poorest people are no longer hungry.”

Limiting GHG emissions from livestock is an important part of any international effort to limit GHG emissions overall. However, we live in a world where food security is already a major problem and predicted to become more so as our population expands. As of 2011, an estimated 925 million people—almost one in seven—were considered malnourished. The FAO found that a further two billion suffer what it calls ‘hidden hunger’. While not malnourished, they cannot access an optimum diet, and suffer adverse health effects from poor nutrition. By 2050 the FAO estimates that global food production will need to increase by some 70% to feed the predicted nine billion people on the planet.

While the population grows, rising wealth in the developing world is also driving increasing demand for higher protein food. Both meat and milk consumption is set to dramatically increase in the next 40 years with predictions that meat consumption will grow from 130 million tonnes in 2002 to over 300 million tonnes in 2050 and milk from 500 million tonnes today to almost 900 million tonnes in 2050. To fill this need livestock production will need to increase, driving up both CH<sub>4</sub> and N<sub>2</sub>O emissions from agriculture globally. These gases and their effect on the climate system must be controlled without compromising food security, knowing that at the same time reducing the rate and magnitude of climate change will help strengthen global food security and ease the risks to farmers' livelihoods from changing weather patterns.

Global climate change is predicted to drive agricultural productivity down and costs up, either directly through climatic effects, or indirectly through constraints on GHG emissions, nitrogen leaching and/or land clearance, and ultimately affecting international commodity prices. "A threefold challenge now faces the world: Match the rapidly changing demand for food from a larger and more affluent population to its supply; do so in ways that are environmentally and socially sustainable; and ensure that the world's poorest people are no longer hungry."

### THE IMPACT ON NEW ZEALAND

Agriculture plays a pivotal role in New Zealand's national economy. Our agricultural exports feed an estimated 20 million people and it is the industry's stated aim to double export production over the next decade. We have a unique emissions profile for a developed country, as agriculture is responsible for almost half of New Zealand's GHG emissions. In 2010 emission levels New Zealand were recorded at 33.7 million tonnes of carbon dioxide equivalent (Mt CO<sub>2</sub>-e), 95% of those come from the pastoral sector. This was an increase of 19.8 per cent over 1990 levels. On the back of an expanding national dairy herd and higher nitrogen fertiliser application, agricultural emissions are estimated to have increased by almost 10%—2.89 Mt CO<sub>2</sub>-e—above the 1990 level of 30.85 Mt CO<sub>2</sub>-e.

There is an urgent need to reduce livestock emissions if the country is to meet the goal of halving its GHG emissions by 2050. In addition, under current government plans, from 2015 New Zealand will be obliged to begin meeting the costs of some emissions under the Emissions Trading Scheme (ETS). This will result in reduced returns for New Zealand farmers, and impact their profitability and optimal management strategies.

As a result of global climate change, weather patterns in New Zealand are predicted to change. Modelling has indicated that, under the most likely scenario, western and southern regions of New Zealand will receive more rain and warmer temperatures while drought frequency is likely to double or even triple by 2040 in eastern and northern regions. Changes in temperature and rainfall patterns could alter the spread and distribution of existing pests and diseases, and enable the emergence of new diseases. Increased temperatures may also influence individual animal productivity. If climate patterns do shift it is likely that most primary industry sectors, meat and wool, dairy, arable, horticulture, viticulture and forestry, will experience changes in productivity and relative profitability. These changes will test the adaptability of farmers, and could shift production zones within New Zealand.

While climate change will affect productivity in New Zealand, increasing pressure on food production in other parts of the world, increasing demand for food, higher returns and competitive advantage could well provide New Zealand farmers with greater opportunities to leverage their already high efficiency and help to offset or even outweigh, domestic weather impacts and emissions mitigation costs. Quantifying the net effect of future international impacts of, and responses to, climate change is challenging and careful consideration will need to be given to the complex set of dynamics behind GHG control to reveal the best strategy for New Zealand, and how best to protect and enhance our environmental reputation.

### INTERNATIONAL CLIMATE CHANGE AGREEMENTS

On 9 May 1992, the governments of the world adopted the UN Framework Convention on Climate Change (UNFCCC), as the first step in addressing the environmental problems faced by mankind by climate change. The objective of the convention is to "achieve stabilization of GHG concentrations in the atmosphere at a level that would prevent dangerous anthropogenic (human sourced) interference with the climate system. Such a level should be achieved within a time-frame sufficient to allow ecosystems to adapt naturally to climate change, to ensure that food production is not threatened and to enable economic development to proceed in a sustainable manner."

The convention establishes the framework for intergovernmental efforts to address climate change. While climate change is a global issue, the balance of equity and responsibility in the convention for emissions reduction and resourcing lies with industrialized countries who have a greater ability to implement and finance changes.

The Kyoto Protocol, adopted in 1997 and which came into force in 2005, was the mechanism by which UNFCCC nations were required to implement emissions reductions. Under the protocol developed nations agree to legally binding limitations or reductions in GHG emissions in two commitments periods. The first commitment period was for emission levels in 2008-2012, and the second for emission levels in 2013-2020. New Zealand participated in Kyoto's first-period but has not taken on new targets in the second.

### MEASURING THE IMPACT OF GHG

The Kyoto protocol also established an international ETS where developed nations are able to trade emissions quotas between themselves and also receive credit for supporting emission reduction efforts in developing countries. The UNFCCC and the Kyoto Protocol don't prescribe emissions targets for individual gases, but instead set aggregate targets for a group of GHGs. This gives countries the flexibility to reduce emissions of a mix of gases in the most cost-effective way, including through international and domestic emissions trading.

The New Zealand emissions trading scheme (NZ-ETS) includes all GHGs, which means emissions in one industry sector can be traded against emissions reductions of another gas in a different sector. Agricultural emissions of CH<sub>4</sub> and N<sub>2</sub>O, mainly from sheep, beef and dairy, are in principle included in the NZ-ETS, although the Government announced in 2012 its intent to delay the entry of agriculture into the scheme.

In order to establish an emission's trading scheme a "common currency" upon which to trade is required. As different GHGs have different properties and therefore differing impacts on the environment, scientists have developed metrics that factor in the properties of gases and allow the impact of the emissions of different gases on the environment to be compared in an equivalent manner.

The UNFCCC has adopted Global Warming Potential (GWP) as the universal metric for reporting GHG emissions. The GWP uses CO<sub>2</sub> (the most important GHG) as a reference. It represents the amount of CO<sub>2</sub> that would have produced the same cumulative warming effect over a given period of time as the gas being emitted; or a 'carbon dioxide equivalent' ('CO<sub>2</sub>-eq'). There are however shortcomings to the use of this metric. The GWP of a gas depends on the

timespan. CH<sub>4</sub>, while a much more powerful GHG than CO<sub>2</sub>, has a short lifetime in the atmosphere, having its greatest warming effect within 50 years of its emission, while CO<sub>2</sub> produces a warming effect that lasts for many centuries. The longer into the future we chose to look, the greater CO<sub>2</sub> cumulative warming effect appears compared to CH<sub>4</sub>. An alternative metric, Global Temperature Change Potential (GTP) compares the warming predicted to occur in a given future year resulting from today's emissions. The 100-year GTP gives lower weight to CH<sub>4</sub> emissions than a 100-year GWP, because it focuses only on the more distant future and 'forgets' the warming that has occurred in between.

Countries are now in broad agreement that they wish to limit global warming to 2°C. There is strong consensus among scientists that, regardless of metric, achieving this goal requires reducing global CO<sub>2</sub> emissions to near zero before the end of the 21<sup>st</sup> century. Some scientists therefore argue that we should not use metrics at all as the overriding need is to reduce CO<sub>2</sub> emissions and reducing levels of other gases is not absolutely necessary to achieve this goal.

There is no simple scientific answer as to which is the 'correct' metric to use, because answers to these questions depend not just on science, but on the choices society wishes to make. Given that emissions trading is widely seen as a cornerstone of climate policy, it seems very likely that some form of metrics will be employed in future climate agreements, and therefore in domestic policy. Whatever measure is applied it is important to understand that the use of different metrics and time horizons could result in significantly different weights being assigned to different gases, which in turn could shift the balance of mitigation efforts. Changing the metric could affect not only overall emissions targets and abatement costs, but also the distribution of emissions costs between sectors and priorities for further research into developing cost-effective mitigation options.

Adapted (in-part) from "The Impact of Livestock Agriculture on Climate Change", "Impacts of Global Climate Change on New Zealand Agriculture" and "Economic and Policy Implications of Alternative GHG Metrics", fact sheets published by the NZAGRC which provide an accurate summary of the issues around GHG emissions in New Zealand in 2013.

The complete documents are available at [www.nzagrc.org.nz](http://www.nzagrc.org.nz)

# 10 YEARS OF SUCCESS

PGgRc was formed in 2002 to focus on pastoral based agricultural GHG mitigation research in New Zealand with the goal to reduce CH<sub>4</sub> and NO<sub>2</sub> emissions from ruminant animals. While being primarily focussed on mitigation for GHG the investment has an equal focus on enhancing ruminant productivity and also creating international opportunity for the sector. In the 10 years that have followed, the Consortium has successfully provided research outcomes and tools key to achieving these goals.

PGgRc programmes have supported targeted and strategic underpinning research, as well as applied research and technology development in order to better understand reasons for success or failure when developing and testing potential mitigation technologies. PGgRc works closely with the New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) to ensure better facilitation and alignment of direct GHG mitigation research. In reviews undertaken in 2006 and 2010, a panel of internationally recognised experts concluded that the PGgRc activity was “World leading research, with excellent productivity for money expended. The Consortium represents the biggest single integrated programme globally responding to issues around livestock greenhouse emissions”.

Greenhouse gas (GHG) mitigation research requires a high level of understanding of complex biological mechanisms and their interaction at the grazing system, whole animal, rumen, microbial community and population, cellular and sub-cellular levels. Advances will be, and have been based on a long history of development of the underpinning biological science. Our understanding of many of the important components and their interactions is still incomplete. However, good progress has been made in some specific areas that directly support future technology development.

PGgRc’s target in its first 10 years was to contribute technologies to the New Zealand pastoral sector to decrease total agricultural emissions of GHG by 10% per unit of output in 2013 relative to 2004 (estimated to be a 4Mt reduction in the agricultural GHG emissions (CO<sub>2</sub>-e) as identified in the National Inventory).

In 2004 the average GHG emission per unit of production across the pastoral sector was 13.4kg CO<sub>2</sub>e per kg product. By 2009-10, the latest year for which emissions data are available, this had fallen by 9.5% to 12.1kg CO<sub>2</sub>e per kg product. Extrapolating to 2013 suggests emissions would have fallen by a further 9% to 10.8 kg CO<sub>2</sub>e per unit product, representing a drop of 18% from 2004 levels. This data would suggest that the pastoral sector is achieving the target. PGgRc contributed to this in two ways:

## THE IDENTIFICATION OF MITIGATION TECHNOLOGIES

PGgRc has tested two new mitigation technologies that have been used in New Zealand—Dicyanamide (DCD) and forage brassicas. DCD was confirmed by the programme as a nitrification inhibitor reducing N<sub>2</sub>O and is now an accepted mitigation incorporated into the National Inventory. In the 2010 calendar year, DCD use mitigated 16300 tonnes of CO<sub>2</sub>-e. (MfE Nat GHG 1990-2010 pp155)\*. Work has also proven that some types of forage brassica reduce emissions by around 25%. It is estimated the quantity of forage brassica grown in New Zealand could be at between 250,000ha and 350,000ha. If half of this crop is sown to forage rape and swedes there is the potential for CO<sub>2</sub>e to be reduced by approximately 127,000 tonnes per annum. Taken together it is estimated that these two technologies could have reduced national emissions by approximately 0.5%, with an imputed value to the industry of in excess \$10m over the 2008-2012 period.

## RAISING AWARENESS

In the area of animal breeding the PGgRc program has clearly demonstrated that, in addition to potentially being able to reduce emissions directly through selecting low emitting animals, modifications to current selection indices can bring about reductions in emissions per unit of product without adversely affecting profitability.

\*DCD was voluntarily withdrawn from the New Zealand market in January 2013.

## Specific Achievements

**NITRIFICATION INHIBITORS** Working in conjunction with the fertiliser and dairy industries, PGgRc has played a pivotal role in demonstrating the ability of nitrification inhibitors to potentially reduce N<sub>2</sub>O emissions by up to 50% from urine patches (the main source of N<sub>2</sub>O) and up to 13% across the whole pasture area. Nitrification inhibitors have been used on approximately 100,000 ha of pastoral land (about 5% of dairy land) and are the only mitigation technology that is incorporated into the national agricultural emissions inventory. **Note:** DCD was voluntarily removed from the market in January 2013 until there is an international codex residue standard in place for rumen livestock products.

**STANDOFF PADS** PGgRc programmes have established experimentally that N<sub>2</sub>O emissions can be reduced by the strategic use of stand-off pads. These allow animals to be removed from grazing areas when soil conditions favour high rates of N<sub>2</sub>O emission.

**MAIZE SILAGE AND N<sub>2</sub>O EMISSIONS** PGgRc has demonstrated that use of maize silage as a supplement in winter-feeding systems has the potential to significantly reduce N<sub>2</sub>O emissions.

**METHANOGEN GENOME SEQUENCING** The first genome sequence of a rumen methanogen was generated and annotated in the PGgRc programme. The information from this and subsequent genome sequences of other major rumen methanogens is now being used to identify critical enzymes unique to methanogens that have become 'targets' for inhibition by vaccines and small molecule inhibitors. The PGgRc program has also generated an inventory of rumen methanogens in New Zealand ruminants, which focuses efforts to develop specific vaccines and inhibitors.

**GHG NATIONAL INVENTORY DEVELOPMENT** Work with Government and Industry partners has confirmed the efficacy of existing and new nitrification inhibitors in a range of New Zealand locations. This information provided the scientific justification for the acceptance of DCD as a mitigation technology and provided the quantitative data needed for national N<sub>2</sub>O emissions to be discounted to reflect the use of nitrification inhibitors on-farm.

**VACCINE DEVELOPMENT** PGgRc has demonstrated that animals can produce antibodies against rumen methanogens and that these antibodies can affect growth and CH<sub>4</sub> production of methanogens grown in pure cultures. It has also been shown that antibody responses in sheep are long-lived and can be boosted by re-vaccination. Two potential antigens found in rumen fluid and faeces are being tested in a prototype *in vivo* vaccine.

**ANIMAL VARIATION IN EMISSIONS** The programme has provided the first conclusive demonstration that individual animals receiving the same quantity and quality of food emit different amounts of CH<sub>4</sub> per unit of intake and that this is a heritable trait. The heritability in sheep (0.15) is similar to many production traits that are part of current animal breeding programmes. Two small flocks of sheep bred for differential emissions have been produced. Preliminary data suggest the low CH<sub>4</sub> trait is not associated with any negative effects on production traits.

**CHEMOGENOMICS AND POTENTIAL INHIBITORS** Genomic information was used to identify key enzymes that can be targeted for inhibition by small molecules. Crystal structures and screening procedures have been developed to identify 43 chemical compounds that can potentially target key methanogen enzymes. Of these 34 are inhibitory to methanogens in laboratory tests and 7 have inhibited CH<sub>4</sub> formation in *in vitro* tests using mixed rumen cultures. A routine methodology for rapid cost-effective screening of potential inhibitors is now available.

**FEED INFLUENCES ON CH<sub>4</sub> EMISSIONS** PGgRc measured CH<sub>4</sub> emissions from non-traditional feeds that are being increasingly used on-farm in New Zealand. Emissions from maize silage and palm kernel extract were similar to those from grass-clover diets. Feeding two brassica species reduced emissions by approximately 25% per unit of dry matter eaten when used as a sole feed. This will contribute to developing the evidence needed to get brassica accepted as a mitigation technology in the national CH<sub>4</sub> inventory.

**MITIGAS AND OTHER TESTING PROTOCOLS** Through a funding partnership with MPI's SLMACC program, PGgRc has developed, evaluated, and validated a standard testing protocol for measuring effects on CH<sub>4</sub> emissions. The MITIGAS protocol measures inhibitory effects on CH<sub>4</sub> formation using sophisticated *in vitro* systems, together with the use of standardised laboratory testing against methanogens and rumen microbial community profiling. CH<sub>4</sub> emissions from animals are quantified using validated and state of the art chamber-based techniques implemented in AgResearch's NZAGRC-funded New Zealand Ruminant Methane Measurement Centre. These protocols allow rigorous assessments of inhibition potential to be made.

**BUILDING RESEARCH CAPABILITY** The research team aligned with PGgRc has grown from 15 to more than 50 people, markedly increasing New Zealand capability in this important area. This team is now recognised internationally as having world leading expertise on ruminant methanogenesis and its management. This status gives New Zealand important credibility in international discussions/negotiations on climate change, GHG management and commitments to reduce national emissions.

**RESEARCH AND DEVELOPMENT COORDINATION** The PGgRc is one of a number of organisations funding research in the agricultural GHG space and it has played a major role in ensuring that the New Zealand research effort is well coordinated. The PGgRc signed an MOU with the Crown in 2003 and as part of this agreed to act as a focal point for coordination of industry funding into GHG mitigation work.

**INDUSTRY PARTNERSHIP** The PGgRc has provided a platform for industry to co-fund GHG mitigation research in partnership with the Government. Besides the tangible benefit of providing research funding, the partnership provides intangible benefits around the notion of industry and Government jointly resourcing research which will provide both industry and public benefits, and also demonstrates the willingness of industry to invest in developing mitigation technologies. The PGgRc partnership also provides industry with an opportunity to have an influence on the nature of research that is carried out, which is appropriate given the potential implications of legislation concerning agricultural GHGs.

# BEYOND 2012

## PGgRc's Strategy for Reducing GHG Emissions

Agriculture plays a critical role in the New Zealand economy and increasingly so as New Zealand's dairy and red meat sectors aim to increase their exports to match the world's growing population and affluence. While production efficiency gains over the past 20 years have reduced the pastoral-derived emission intensity of GHG (i.e. "emissions/unit product"), total emissions from this sector are increasing. If agriculture was to join the ETS in 2015 the sector will have an estimated 'liability' of \$272m. Unless technical solutions are found to reduce agricultural emissions, the cost to agriculture and the New Zealand economy will only increase.

PGgRc successfully renewed its contract with the crown in 2013 to further its programme of work to find practical solutions for the reduction of GHG emissions from the New Zealand pastoral sector. PGgRc industry partners and MBIE have committed more than \$35m to fund the activities of the Consortium for a further 7 years. The PGgRc programme works alongside aligned investment from the New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) and AgResearch.

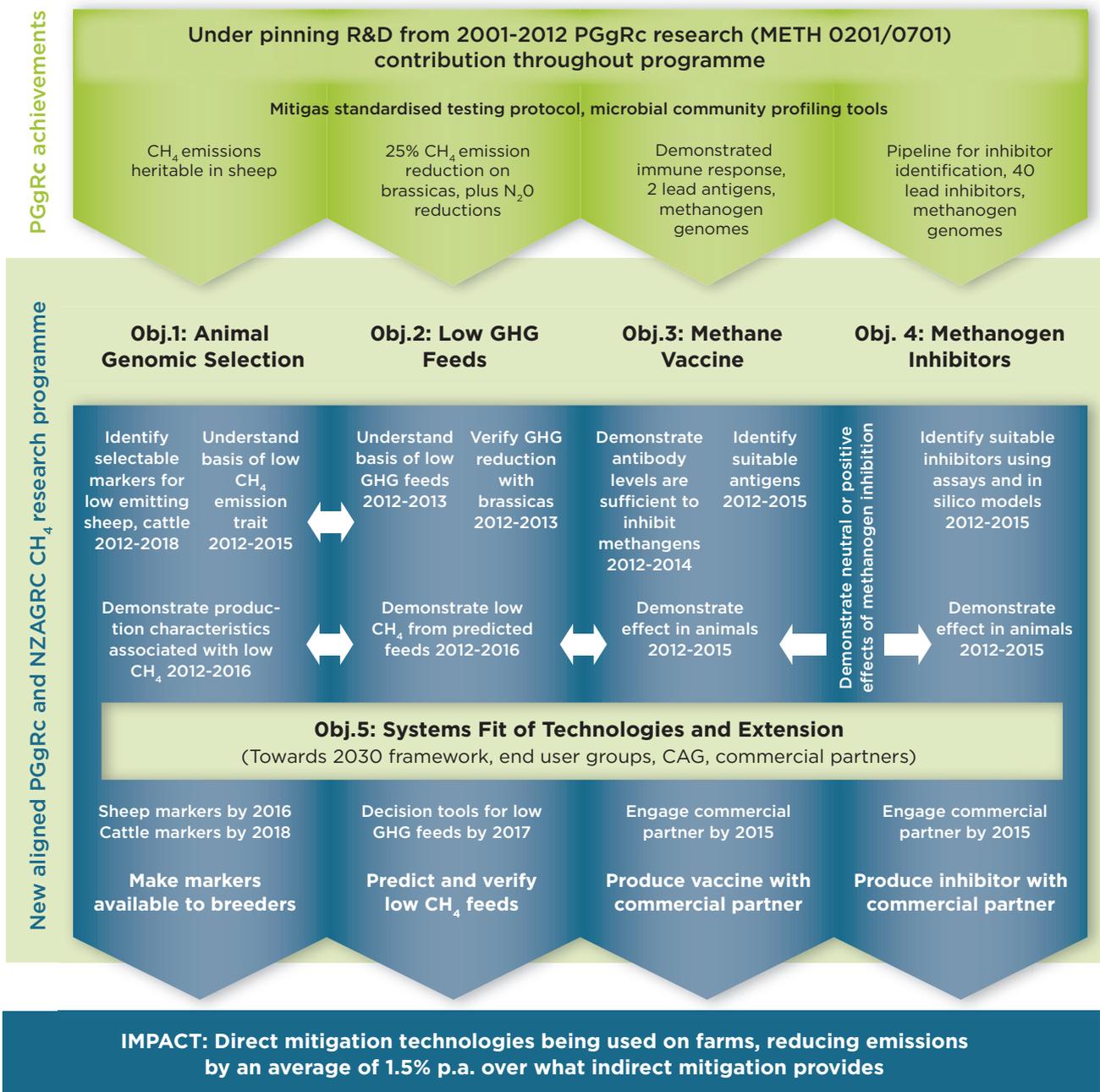
The 2013 PGgRc programme has five objectives which build on knowledge and research tools developed by PGgRc in the past 10 years (see Figure 1).

- Breed low-CH<sub>4</sub> emitting ruminants
- Identify low-GHG feeds
- Develop a vaccine to reduce ruminant CH<sub>4</sub> emissions
- Identify inhibitors that reduce ruminant CH<sub>4</sub> emissions
- Extension and enabling technologies.

The aim of the new programme is to develop a suite of implementation-ready, practical direct GHG mitigation technologies. PGgRc is the major funder of direct mitigation for methane R&D in New Zealand in collaboration with the NZAGRC.

The fifth objective 'extension and enabling technologies' will coordinate work in the other objectives to ensure the technologies developed are practical and readily adopted by farmers. This objective will also ensure that the technologies are developed to an implementation-ready stage that will attract commercialisation partners for further investment.

The PGgRc Governance Board will oversee and direct the programme and protect/manage its intellectual property (IP) under the PGgRc-NZAGRC collaboration agreement and joint R&D&E strategy. The Board will regularly seek advice from commercialisation, expert science and end-user advisory groups in collaboration with the NZAGRC Steering Group, in a consolidated operation with a New Zealand Inc. focus.



**Figure 1:** PGgRc-NZAGRC Methane Research Programme to develop new, commercialisation-ready direct GHG mitigation tools for pastoral farmers.

# TOWARD 2030 FRAMEWORK

The New Zealand R&D landscape for agricultural Climate Change is complex due to a large number of stakeholders (see page 16), however each group and research fund that form a part of this picture are strategic and provide an important contribution towards achieving real and tangible reduction in New Zealand's CO<sub>2</sub>-e.

In order to reduce complexity in the agricultural GHG mitigation research landscape, the PGgRc developed, in alignment with research funded by the NZAGRC, a coordinated "New Zealand Inc." approach to GHG reduction known as the "Toward 2030 Framework". The purpose of the framework is to optimise investment effectiveness, identify value-add opportunities and avoid any duplication of effort in the agricultural GHG mitigation research arena. This approach underpins the renewed partnership between the PGgRc and MBIE for the continued development of mitigation solutions, and the close cooperation with the NZAGRC and Ministry for Primary Industries (MPI).

The Toward 2030 Framework has a long term mission reaching out to 2050, but the more immediate stretch goal is to reduce agricultural GHG by 8% below 1990 levels by 2030, in line with Government aspirations, whilst maintaining a pastoral growth target of 2% p.a. To achieve this, separate R&D programmes funded by different stakeholders must link to one another, as well as engage and work in concert with the other activities across the pastoral economy to ensure valuable science resources are efficiently used, new knowledge is generated and that technologies are relevant, practical and adoptable into New Zealand's farming systems.

**Mission:** Through investment in innovation, adoption and collaboration ensure New Zealand agriculture continues to prosper through productivity gain and carbon emission reduction whilst supporting the national goal of reducing GHG emissions by 50% over a 1990 baseline by 2050.

There are four streams of activity in the Toward 2030 Framework:

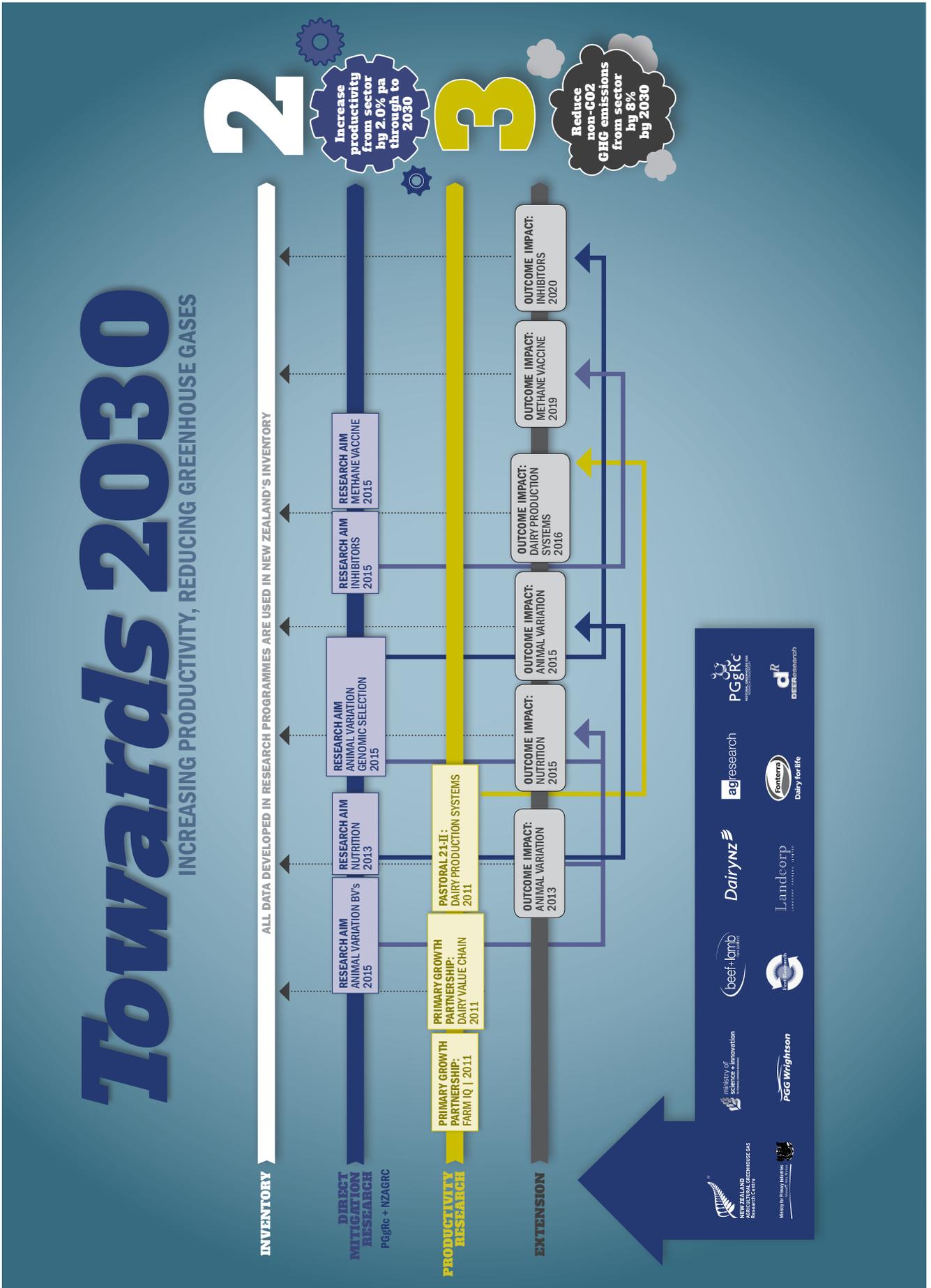
**1) Direct Mitigation research.** R&D into cost effective technologies and practices to directly reduce GHG emissions intensity ((CO<sub>2</sub>-e) /unit of input) solutions for livestock GHGs (e.g. modification of rumen processes, nitrification inhibitors, vaccines and dietary manipulations). The aim is to deliver a 1.5% reduction in GHG Intensity

**2) Productivity research (Indirect Mitigation).** R&D into technologies/practices that reduce CO<sub>2</sub>-e /unit product through improving farm system efficiency by enhancing livestock productivity and efficient use of resources across the sector (e.g. improved farming practices, conception rates in dairy cows). The aim is to maintain and deliver a 1% reduction in GHG intensity.

**3) Extension.** The use of Industry and Government initiatives to accelerate the uptake of new knowledge and use of cost-effective tools and products that contribute to best farming practices and also reduce CO<sub>2</sub>-e (e.g. farm system configuration, breeding selection and mating management programmes). This includes initiatives within Industry-good organisations, Primary Growth Partnerships (PGP) contracts and SLMACC technology transfer programmes.

**4) Inventory.** The inventory activity identifies our international GHG obligations, supports the ETS and identifies future opportunities and threats to allow appropriate responses. Government investment into inventory development ensures that mitigation actions are incorporated into the national inventory and count towards reducing New Zealand's international UNFCCC commitments.

Figure 2 shows the alignment of work streams and how outputs and outcomes link and flow through to extension and inventory. It will be critical to ensure that these alignments and linkages are well understood and maintained to achieve the greatest impact in the shortest time.



**Figure 2.** The conceptual 'New Zealand Inc. Towards 2030 Framework' developed by PGgRc, in alignment with research funded by the NZAGRC, illustrating the linkages needed between all programmes.

# AGRICULTURAL CLIMATE CHANGE R&D STRATEGY

The New Zealand Government's Climate change strategy has been formulated over a number of years. The aim of the strategy is to reduce total GHG emissions by 50% by the year 2050. Since 2002 the Government, in collaboration with industry, has founded new entities and established new funding pools for the support of climate change research in New Zealand (see Figure 3).

Through these initiatives the New Zealand Government provides funding for research projects to:

- Reduce agricultural GHGs,
- Encourage the establishment of forest sinks and the management of deforestation,
- Understand the impacts of a changing climate,
- Adapt and respond to changes in climate
- Capitalise on new business opportunities arising from the world's response to climate change.

Alongside Government partnered investments, Industry and research providers are also contributing to research, development and extension directly in order to accelerate the adoption of best practice.



**Figure 3:** Key agricultural climate change events in New Zealand R&D Landscape

## **SUSTAINABLE LAND MANAGEMENT AND CLIMATE CHANGE (SLMACC)**

In early 2007, the Government developed a Sustainable Land Management and Climate Change Plan of Action to address:

- the impacts of and adapting to climate change,
- reducing New Zealand's GHG emissions and enhancing carbon sinks, and
- exploring business opportunities arising from climate change

Joint work programmes have been developed in the plan with the cooperation of the agriculture, forestry, horticulture and arable farming sectors, Māori and local government to achieve a combined Sector-Government response to climate change. Since 2007 the Government has introduced a New Zealand Emissions Trading Scheme (ETS) that will cover all GHGs and all sectors of the economy. Different sectors will join the scheme at differing times to allow for a gradual adjustment to emission pricing. As of 2012, there the need for Agriculture to join the scheme has been delayed.

To assist in preparation for the ETS, the Government has allocated more than \$175 million in the Sustainable Land Management and Climate Change (SLMACC) fund and is investing in a range of programmes to help the sectors adapt to, mitigate and exploit the business opportunities of climate change.

### **NEW ZEALAND AGRICULTURAL GREENHOUSE GAS RESEARCH CENTRE (NZAGRC)**

The New Zealand Agricultural Greenhouse Gas Research Centre (NZAGRC) was opened March 2010 and is a partnership between the leading New Zealand research providers working in the agricultural GHG area and the Pastoral Greenhouse Gas Research Consortium (PGgRc). Other members are AgResearch Limited, DairyNZ, Landcare Research, Lincoln University, Massey University, NIWA, Plant and Food Research and Scion. The NZAGRC funds research that is carried out by researchers working in their own organisation. The NZAGRC is funded by the New Zealand Government through the PGP.

The NZAGRC works with its partner organisations, particularly the PGgRc, to deliver innovative, practical and credible science. It has three main areas of focus:

- Methane emissions from ruminant animals and waste systems
- Nitrous oxide emissions from ruminant animals and nitrogen fertiliser
- Soil carbon enhancement

The NZAGRC and its science programme cannot be viewed in isolation from those of other GHG science funders in New Zealand. NZAGRC investment works with and builds on existing PGgRc investment, particularly in the methane area, and also aligns with SLMACC-funded projects to ensure that New Zealand has a comprehensive New Zealand Inc. approach to its GHG research portfolio without major gaps or duplication.

NZAGRC staff and key NZAGRC funded researchers also work alongside MPI in the Global Research Alliance (GRA) and promote New Zealand's leadership in this area on the international stage.

Further information about the NZAGRC can be found at [www.nzagrc.org.nz](http://www.nzagrc.org.nz).

### **THE NEW ZEALAND FUND FOR GLOBAL PARTNERSHIPS IN LIVESTOCK EMISSIONS RESEARCH**

In 2011 the New Zealand Government established a contestable, international fund worth NZ\$25 million to support research on mitigating GHG emissions from pastoral farming. The fund is open to international scientists working in conjunction with New Zealand partners to develop cost-effective and sustainable solutions for livestock farmers in New Zealand.

### **NATIONAL GREENHOUSE GAS INVENTORY RESEARCH GRANTS**

New Zealand has a requirement to continuously improve the National Inventory in order to meet commitments to the UNFCCC. MPI funds research that provides information and assists with compiling New Zealand's National Inventory including research focused on:

- Improvement of agriculture activity data, including statistics
- Agriculture inventory "best practice"
- Agriculture mitigation technology incorporation and monitoring
- Agriculture projections and net position
- Measurement of emissions and sinks from soils.

# GLOSSARY

ACVM Act	Agricultural Compounds and Veterinary Medicines Act 1997; <a href="http://www.legislation.govt.nz/act/public/1997/0087/latest/DLM414577.html">www.legislation.govt.nz/act/public/1997/0087/latest/DLM414577.html</a>
Carbon units	The value assigned to reducing or offsetting emissions of GHG, usually equivalent to one tonne of carbon dioxide equivalent (CO <sub>2</sub> -e). Emitters of GHG must either reduce their emissions or purchase carbon units to pay for them
C	Carbon
CH <sub>4</sub>	Methane
CH <sub>4</sub> -em	Methane emissions
Clades	A group consisting of an ancestor and all its descendants
CO <sub>2</sub>	Carbon Dioxide
CO <sub>2</sub> -e	Carbon dioxide equivalency for a GHG.
CRC	Australian Cooperative Research Centre
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DCD	Dicyandiamide
Direct GHG Mitigation	Technique which reduces GHG emissions by reducing emissions per unit of substrate used (e.g. feed or nitrogen fertiliser)
DM	Dry Matter
DMI	Dry matter intake, referring to amount of feed eaten by an animal
DNA	Deoxyribonucleic Acid
DPIVic	Department of Primary Industries, Victoria, Australia; <a href="http://www.dpi.vic.gov.au">www.dpi.vic.gov.au</a>
ELISA	Enzyme-Linked Immune Sorbent Assay
ETS	Emission trading scheme
Genotype	An individual's set of genes or genome
gCH <sub>4</sub>	Grams of methane
GHG	Greenhouse gas
GPLER	New Zealand Fund for Global Partnership in Livestock Emissions Research; a NZ GRA funding mechanism; <a href="http://www.mpi.govt.nz/environment-natural-resources/climate-change/research-and-funded-projects/the-new-zealand-fund-for-global-partnerships-in-liH2">www.mpi.govt.nz/environment-natural-resources/climate-change/research-and-funded-projects/the-new-zealand-fund-for-global-partnerships-in-liH2</a>
GRA	Global Research Alliance on Agricultural Greenhouse Gases; <a href="http://www.globalresearchalliance.org">www.globalresearchalliance.org</a>
H <sub>2</sub>	Hydrogen
Hapu	Māori kin group
HCOOH	Formate
HD SNP	High-density chip for measuring single nucleotide polymorphisms in DNA samples (see 'SNP' below)

<i>in silico</i>	Performed on computer or via computer simulation
<i>in vitro</i>	Literally 'within the glass', refers to experimental systems in containers in contrast to the natural system
<i>in vivo</i>	Literally, 'within the living', refers to experiments using whole, living organism
Indirect GHG Mitigation	Technique which reduces GHG emissions by increasing production efficiency of animals so GHG emissions/unit product is reduced
IP	Intellectual property
IPCC	Intergovernmental Panel on Climate Change <a href="http://www.ipcc.ch/">http://www.ipcc.ch/</a>
ITS1	Internal transcribed spacer 1
Iwi	Māori tribe
Kaitiakitanga	The exercise of guardianship by the iwi of an area in accordance with Māori custom in relation to natural and physical resources, including the ethic of stewardship.
Kyoto Protocol(KP)	<a href="http://www.mfe.govt.nz/issues/climate/international/kyoto-protocol.html">www.mfe.govt.nz/issues/climate/international/kyoto-protocol.html</a>
LOPAC	Library of Pharmaceutically Active Compounds
MBIE	Ministry for Business, Innovation and Employment; <a href="http://www.mbie.govt.nz/">www.mbie.govt.nz/</a>
Methanogen(s)	Methane-producing microbe(s) in the rumen (in this programme)
MPI	Ministry for Primary Industries; <a href="http://www.mpi.govt.nz">www.mpi.govt.nz</a>
N	Nitrogen
NH <sub>3</sub>	Ammonia
NH <sub>4</sub> <sup>+</sup>	Ammonium ions
N <sub>2</sub> O	Nitrous oxide
NCIB	US National Center for Biotechnology Information, <a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>
NI	Nitrification inhibitors
NO <sub>3</sub> <sup>-</sup>	Nitrate
NZAGRC	New Zealand Agricultural Greenhouse Gas Research Centre; <a href="http://www.nzagrc.org.nz">www.nzagrc.org.nz</a>
p.a.	Per annum
PCR	Polymerase chain reaction technique
PGgRc	Pastoral Greenhouse Gas Research Consortium; <a href="http://www.pggrc.co.nz">www.pggrc.co.nz</a>
PGGRL	Pastoral Greenhouse Gas Research Company; the agent company for PGgRc
PGP	Primary Growth Partnerships
Phenotype	Observable traits that result from interactions between an individual's set of genes and the environment
Phylogenetic	Evolutionary relationships
Phylogenetic tree	Branching diagram showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and/or genetic characteristics. The entities joined together in the tree are implied to have descended from a common ancestor.
R&D	Research and development
R&D&E	Research and development and extension

RA	Research Aim
QD PI	Queensland Department of Primary Industries, <a href="http://www.daff.qld.gov.au/">http://www.daff.qld.gov.au/</a>
SCFA	Short chain Fatty Acids
SCOPUS h-indices	Research performance measure as reported in SCOPUS; <a href="http://www.en.wikipedia.org/wiki/H-index">www.en.wikipedia.org/wiki/H-index</a> , <a href="http://www.info.sciverse.com/scopus">http://www.info.sciverse.com/scopus</a>
SF <sub>6</sub>	Sulphur Hexafluoride
SIL	Sheep Improvement Ltd, <a href="http://www.sil.co.nz">www.sil.co.nz</a>
SLMACC	Sustainable Land Management and Climate Change
SNP	Single nucleotide polymorphism, genetic variations in the DNA of individuals that can be used to differentiate them and to identify variations in regions of their genomes
UI	Urease inhibitors
UNFCCC	United Nations Framework Convention on Climate Change (1994) <a href="http://www.mfe.govt.nz/issues/climate/international/unfccc.html">http://www.mfe.govt.nz/issues/climate/international/unfccc.html</a>
VFA	Volatile Fatty Acids
Vision Mātauranga	The MBIE framework for unlocking the science and innovation potential of Māori knowledge, people and resources
Whenua	Māori word for land
WFPS	Water filled pore spaces (of soil)

# RESEARCH PROGRESS 2007–2012

The following section gives a more detailed account of the major programmes of work funded through PGgRc and its funding partners over the period 2007-2012. We have endeavoured to combine related pieces of work and give a flavour of the research across the wide investment we have been involved in. As such this information has been drawn from some of the published papers and we have identified a lead person who has been integral to the work presented.

There is an explanation at the beginning of each research section that provides some context and relevance to the development of mitigation solutions.

## Co-funding acknowledgement

The PGgRc has been the major funder of the research that is profiled here, but not the only one and we wish to acknowledge the following Government organisations that have worked with the consortium and continue to be partners in this research. Their strategic and financial support is integral to the progress that has been documented here.



**MINISTRY OF BUSINESS,  
INNOVATION & EMPLOYMENT**  
HĪKINA WHAKATUTUKI

Matching our industry commitment across all of our research investments.

**Ministry for Primary Industries**  
Manatū Ahu Matua



Jointly funding research projects through three major initiatives:

- Sustainable Land Management and Climate Change funding (SLMACC)
- New Zealand Agricultural Greenhouse Research Centre (NZAGRC)
- Global Research Alliance (GRA).



## CAPABILITY DEVELOPMENT

The investment in research has also resulted in the advancement of several PhDs:

**Ben Vlaming (2008).** Thesis Title: *Quantifying variation in estimated methane emission from ruminants using the SF<sub>6</sub> tracer technique*

**Jeyamalar Jeyanathan (2010).** Thesis title: *Investigations of rumen methanogens in New Zealand livestock*

**Natasha Swainson (2010).** Thesis title: *Methane emissions and mitigation technologies in cattle, sheep and red deer*

**Kirsty Hammond (2011).** Thesis title: *Methane emissions from ruminants fed white clover and perennial ryegrass forages*

*Photographs and images used supplied by the New Zealand Agricultural Greenhouse Gas Research Centre, Beef + Lamb New Zealand Ltd., DairyNZ and Deer Industry New Zealand.*

# MICROBIAL ECOLOGY

## BACKGROUND

Feed eaten by ruminant animals is physically broken down by chewing when it is eaten, again by re-chewing during rumination, and finally by microbial degradation in the rumen of the animal. Plant material, saliva and drinking water enter the rumen as an animal feeds and different components of this mixture are broken down and flow out of the rumen at different rates. The microbes that cause degradation (fermentation) in the rumen are made up of many types of bacterial, fungal, protozoal and archaeal species. As the feed is broken down a number of products are formed including volatile fatty acids (VFAs) which are absorbed through the rumen wall and used as a major energy source by the animal; and microbial cells which exit the rumen and are digested further down the digestive tract along with residual plant material that has not been completely degraded. Hydrogen (H<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) gas are also produced during digestion.

The group of microbes known as archaea and more specifically a subgroup called methanogens, use these gases to produce energy for their own metabolism and in doing so they produce methane (CH<sub>4</sub>). It is important to remove these gases from the rumen to ensure that it functions normally. A high concentration of hydrogen inhibits the microbial breakdown of the feed and causes nutritionally unfavourable patterns of VFAs to form. Archaea, although relatively minor in number, are important as they maintain the H<sub>2</sub> concentration at a low level.

Methane formed by the archaea in the rumen is belched (eructated) to the atmosphere by animals. Sheep belch about 25-55 litres of methane per day and cattle about 150-420 litres. This loss of methane, although necessary for continued rumen function, does cause a significant loss of energy for the animal as well as contributing to greenhouse gas emissions. Therefore mitigation strategies that reduce methane emissions from animals have the potential not only to reduce green house gases

in the atmosphere but also increase the amount of energy available to an animal from its feed for use in production.

Knowledge of the types of methanogens found in the rumen is an important key to develop strategies to mitigate CH<sub>4</sub> production. From what is currently known it appears that only a few major groups of methanogens are potential targets for reducing methane emissions from ruminant animals. PGgRc work in this area has been focussed on understanding the structure of the methanogen community in order to identify how the rumen functions and key targets for mitigation strategies.

## WHY THIS IS IMPORTANT

The ability to accurately monitor the effects that any mitigation has on the rumen microbial populations is critical to making any progress toward reducing CH<sub>4</sub> from the rumen. The underlying risk in solely targeting methanogens is that fibre degrading microbes are also negatively impacted. This research provides us with the tools to be able to ensure that this doesn't occur. This section summarises technical papers developed and published over the last 5 years, including two reviews and a series of experiments that enhanced our ability to identify microbe species and their interactions and to understand the ecology of the total rumen microbial population. Without the tools and this understanding we would not be able to develop accurate high impact mitigation solutions for livestock.

## Reference

Peter H. Janssen. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics.

*Grasslands Research Centre, AgResearch, Private Bag 11008, Palmerston North, New Zealand.*



## Minireview: structure of the archaeal community of the rumen

Peter Janssen and Marek Kirs 2008. AgResearch.  
peter.janssen@agresearch.co.nz

### INTRODUCTION

Archaea are a very small component of the total rumen microbial community accounting for only between 0.3 and 3.3% of the microbial ribosomal nucleic acid (rRNA) found in the rumen. They are strictly anaerobic, and most of them are methanogens. Most methanogens grow using H<sub>2</sub> and often formate (HCOOH) as energy sources to convert CO<sub>2</sub> to CH<sub>4</sub>. A few species can grow with acetate and produce CH<sub>4</sub>, but this is not significant in the rumen.

The methanogens either exist free in the rumen fluid or attached to particulate material, the rumen epithelium or protozoa; or living within the protozoa (as “endosymbionts”). These are very different habitats and a large diversity of methanogens is found in the rumen, with varying characteristics which allow them to adapt to their environment.

### METHANOGENS CULTURED FROM THE RUMEN

Methanogens have been classified into 28 genera and 113 species, although probably many more occur in nature. However, very few methanogens have been isolated from the rumen, and only seven species have been cultured to date including *Methanobacterium formicicum*, *M. bryantii*, *Methanobrevibacter ruminantium*, *M. millerae*, *M. olleyae*, *Methanomicrobium mobile* and *Methanoculleus olentangyi*.

Small-scale cultivation studies of rumen microbes provide single species microbial isolates for further study. However, this approach usually does not uncover the full extent of microbial diversity because some species are more readily cultured than others. This approach gives little insight into community structure.

### CULTIVATION-INDEPENDENT SURVEYS OF RUMINAL METHANOGENS

Surveys of methanogens and total archaea in the rumen have been made by using PCR techniques (polymerase chain reaction) to identify components of rRNA in the genes of archaea. This methodology has been used to find the dominant groups of archaea in the rumen by comparing the gene sequences of the different species to determine their evolutionary relationships.

Information from 14 published studies using this approach and other screening techniques was analysed in this study to provide information about the abundance of different archaeal groups in the rumen. Nine studies reported data for total rumen archaea, free-living archaea, particle-associated archaea and archaea associated with other microbes; and five reported data for protozoan-associated archaea. The studies were from different research groups in various countries, using samples from a number of ruminant species fed different diets, and samples were collected and processed using a variety of methodologies. The studies were not strictly comparable but this “global” data set provides a means of gaining a valuable insight into the identity of the dominant archaeal groups in the rumen.

### ABUNDANT ARCHAEA IN THE RUMEN

Based on the global data set, 92% of the rumen archaea detected can be placed in three genus-level groups (Figure 1): *Methanobrevibacter* (61%), *Methanomicrobium* (15%) and a large group of uncultured archaea labelled as rumen cluster C or RCC (16%).

This supports the previous view that *Methanobrevibacter* is the dominant group in the rumen. Within this genus are two major subgroups or clades, which is a group of closely related strains or species. One contains *M. gottshalkii*, *M. thaueri*, and *M. millerae*, which account for 34% of the rumen archaea, and the other *M. ruminantium* and *M. olleyae* which is 27%. Members of other *Methanobrevibacter* species also occurred but were rare. *Methanobrevibacter* species seem to be early colonisers of the developing rumen in young animals.

It is not possible currently to further unravel the genetic diversity within these clades, but would be valuable to determine how many functionally different groups there are within the clades and what their biochemical and ecological difference are.

### COMPARISON WITH OTHER METHODS

A number of other methods have also been used to analyse the rumen archaeal community including RNA-targeted DNA probes, temporal temperature gradient gel electrophoretic separation and surveys of marker diversity.



### ABUNDANT ARCHAEA ASSOCIATED WITH PROTOZOA

Methanogens can be attached externally to protozoa or located inside them. Most of these (94%) are in the same three groups that dominate the total rumen archaea i.e. *Methanobrevibacter*, *Methanomicrobium* and the RCC clade. Further research would be needed to establish if this finding is consistent across the other types of species inside each clade but evidence suggests that it is unlikely that there are significant populations of uniquely protozoan-associated methanogens in the rumen.

### ROLES AND CO-OCCURRENCE IN THE RUMEN

Of the eleven clades of archaea detected in the global analysis nine were hydrogen-utilising methanogens. These belonged to five different genera. It is not clear how these genera manage to co-exist in the competitive rumen environment but there appear to be differences in abundance of each across studies due to a host or diet influence. There also may be an interaction between archaea and different hydrogen-producing organisms, i.e., protozoa, bacteria or fungi.

The roles of some of the less abundant archaea in the rumen are still not clear, e.g., there is only limited evidence that the RCC clade is comprised of methanogens.

Research to establish how archaeal groups co-exist in the rumen, and what determines their abundance, needs to be conducted.

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#### Reference

Janssen PH, Kirs M (2008) *Structure of the archaeal community of the rumen*. Applied and Environmental Microbiology 74: 3619-3625.





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

Jeyamalar Jeyanathan and Peter Janssen 2011. AgResearch.  
C/- peter.janssen@agresearch.co.nz

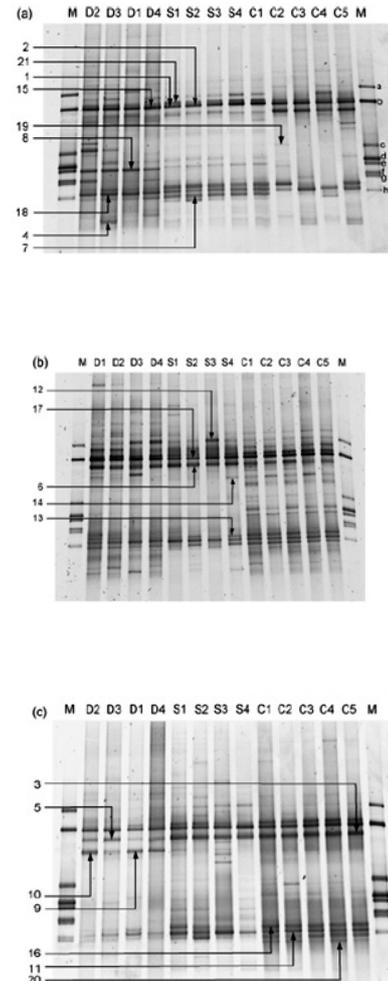
### INTRODUCTION

The varying proportions of the three major clades of archaea found in the rumen (*Methanobrevibacter*, *Methanomicrobium* and RCC) has been reported across a number of different studies. Analysis of the genome of *Methanobrevibacter ruminantium* has identified a number of proteins and pathways that could be targeted in CH<sub>4</sub> mitigation efforts. However, to be practically useful, such an intervention that involves the use of small-molecule inhibitors or vaccines, would need to be effective across the diversity of methanogens present in different ruminant species and different diets. This research was conducted to compare the diversity and relative proportions of methanogens present in rumen samples from New Zealand sheep, cattle and red deer fed different diets.

### METHODOLOGY

Four wether sheep, five non-lactating dairy cows, and four castrated red deer stags were grazed on perennial ryegrass-white clover pasture in summer, on perennial ryegrass-white clover pasture in winter; and were fed lucerne silage in winter. Pasture-grazed animals were grazed on the pasture diets for at least a month pre-sampling, and silage-fed animals were fed twice daily for 15 days before sample collection. Samples of whole rumen contents (fluid plus solids) were collected through rumen fistulae. Samples were taken 2, 4, 6 and 8 hours after feeding for the silage-fed animals. Rumen contents were also collected at slaughter from four wether sheep fed a concentrate-based diet; and from five ewe hoggets fed willow during autumn.

Total DNA was extracted from each rumen sample and analysed by PCR to determine the quantity of total archaea, total bacteria and the RCC group of archaea. Denaturing Gradient Gel Electrophoresis (DGGE) was used to generate DNA fingerprints.



**Figure 1.** DGGE fingerprints of ruminal archaea in red deer (D1-D4), cattle (C1-C5) and sheep (S1-S4) fed (a) summer pasture (b) winter pasture and (c) lucerne silage. The arrows indicate the bands that were sequenced. Reproduced from Jeyanathan *et al.* (2011) FEMS Microbiology Ecology 76: 311-326.

### Reference

Jeyanathan J, Kirs M, Ronimus RS, Hoskin SO, Janssen PH (2011) Methanogen community structure in the rumens of farmed sheep, cattle and red deer fed different diets. FEMS Microbiology Ecology 76: 311-326.

## RESULTS AND DISCUSSION

DNA fingerprinting showed that the archaeal communities in this study were similar across all of the animal species and diet treatments investigated. The rumen of sheep, cattle and deer grazing summer and winter pasture, and fed silage in winter (Figure 1); and also sheep fed willow and a concentrate-based diet all contained similar dominant archaeal populations. There appeared to be some treatment effects on minor parts of the archaeal community and in subgroups within major clades, particularly with changing diets in cattle and deer. Red deer had particularly high variation in banding patterns and this may have been associated with their strong seasonal patterns of digestive function. However, on the whole the archaeal communities of the different animals were remarkably similar, suggesting a common core set of ruminal methanogen species. This means that only a limited number of methanogen groups will need to be targeted by researchers in an attempt to control the majority of CH<sub>4</sub> producers. It is unclear, however, whether elimination of the major archaeal groups will allow the minor groups to increase in abundance as they occupy the niches vacated, or whether they are specialised to such a degree they have limited capacity to respond.

Sequencing of the dominant bands in the DNA fingerprints identified six clades of archaea: relatives of *Methanobrevibacter ruminantium*; relatives of *M. gottschalkii*; *Methanosphaera* species; *Methanosarcina* species; *Methanoculleus* species; and rumen cluster C (RCC). The first three clades were the dominant groupings. Analysis suggested that the RCC grouping formed about 25% of total archaeal grouping and that they are a relatively constant part of the community, although analysis of sequence types indicated that within group variability was large.

The degree of variation in the archaeal and bacterial communities was compared for sheep fed winter pasture and the concentrate-based diet. There was a greater species diversity in the bacterial community than that seen in the archaeal. This is to be expected as bacteria use feed directly as their energy source, whereas archaea utilise H<sub>2</sub> produced during fermentation.





## Characterisation of rumen ciliate community composition in domestic sheep, deer and cattle

Sandra Kittelmann and Peter Janssen 2011. AgResearch.  
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### INTRODUCTION

The rumen contains a complex mix of bacteria, fungi, ciliate protozoa and archaea that digest feed eaten by ruminants. The ciliates are a group of protozoa characterized by the presence of hair-like organelles called cilia that are used for swimming, crawling, attachment, feeding, and sensation. Bacteria, fungi and ciliate protozoa carry out the initial attack on the feed producing volatile fatty acids that are a major energy source for the host animal; they also produce  $H_2$  which is an energy source for the archaeal, which produce  $CH_4$ .

Ciliates are not essential for ruminant animal survival, but they benefit the microbe community and hence the animal for several reasons. They add complexity to the community and increase community resilience, some scavenge oxygen and so help maintain anaerobic conditions, and they also may assist in transfer of bacterial nitrogen to the ruminant animal. Large amounts of  $H_2$  are produced by ciliates and this is utilised by methanogens in the rumen, some of which are externally attached to or contained within the protozoa. Up to 37% of rumen-derived  $CH_4$  can be produced by ciliate-associated methanogens, so eliminating or manipulating the ciliate community is a potential  $CH_4$  mitigation strategy.

The rumen ciliates are subdivided into the orders Entodiniomorpha and Vestibuliferida and these comprise at least 25 genera. Factors influencing the nature of the rumen ciliate community are not known.

Ciliates have been classified into four different community types, each characterised by one or more key species: A (*Polyplastron multivesiculatum*), B (*Epidinium ecaudatum* or *Eudiplodinium magii*), O (*Entodinium* spp., *Dasytricha* spp. and *Isotricha* spp.) and K (cattle specific; *Elytroplastron bubali*). There is some evidence that the A-type leads to less  $CH_4$  production than B-type, and, if this is the case, then targeting suppression of the more productive types might reduce  $CH_4$  production.

Traditionally ciliate community studies have involved tedious and time-consuming microscopy-based methodologies. In this research the use of more modern and powerful molecular methodologies was explored. If successful, these methodologies would assist in shedding light on the structure and variation of ciliate communities in New Zealand ruminants.



## METHODOLOGIES

Samples of whole rumen contents were taken from four rumen fistulated wether sheep, five non-lactating dairy cows and four castrated red deer stags. These were kept in separate groups and fed lucerne silage twice daily (at 1.2x maintenance requirements) for at least 15 days in winter prior to sampling; samples were taken two hours after the morning feed. The animals were then grazed continuously on perennial ryegrass-white clover pasture during the following winter and summer and samples were taken during these periods. Whole rumen samples were also collected at slaughter from four sheep fed a concentrate-based diet, from five sheep fed ryegrass-white clover in autumn, and from five sheep fed willow.

Total DNA was extracted from freeze-dried samples, molecular fingerprinting was carried out using DGGE, and quantitative PCR and phylogenetic analysis of almost full-length 18S rRNA gene clone libraries was conducted.

## RESULTS AND DISCUSSION

DGGE analysis was found to provide a cheap and easy pre-screening of ciliate communities and a way of identifying interesting samples that could be subjected to a more thorough phylogenetic analysis via clone libraries.

Quantitative PCR analysis showed animal species or diet had very little influence on ciliate abundances in rumen samples, suggesting that their main effect, if any, would be in terms of community composition rather than total community size.

There was a high degree of animal to animal variation detected within treatment groups which influenced the ability to detect differences between groups. Variability was highest for sheep, slightly lower in deer and least in cattle. While this might be explained by a sheep's ability to graze selectively, the variability was high when fed a fixed diet (silage and concentrate-based) so selective feeding was unlikely to be the cause. A further possibility is that differences in rumen volume of individual animals may lead to differences in rumen function and physiology and so an influence on the ciliate community composition.

Analysis of DGGE banding patterns showed that there were significant effects of diet on community composition for cattle, but no clear effects for deer or sheep. Cattle had stable ciliate communities that consistently changed with diet, whereas ciliated community composition in sheep and deer was variable.

Libraries of 18S rRNA genes were prepared from selected rumen samples that spanned the overall diversity of ciliates present. These samples will be used as a reference database for future projects.

Sequences from the clone libraries were assigned to nine genera: *Epidinium*, *Eudiplodinium*, *Ostracodinium*, *Anoplodinium* - *Diplodinium*, *Entodinium*, *Polyplastron*, *Dasytricha* and *Isotricha*. These may represent the dominant ciliate protozoa in New Zealand ruminants. There was virtually no new genus-level diversity detected, which contrasts with studies on rumen bacteria and archaea where novel genus-level groups are usually reported.

Four distinct types of ciliate communities were described in a classification scheme outlined in the introduction. Almost all sheep samples and the deer samples contained B-type communities, but the cattle samples (and sheep fed silage) harboured A-type communities. It has been suggested that ciliate communities possess varying stabilities depending on the host, e.g., for sheep, once a B-type community has been invaded by an A-type community it will not switch back, whereas a switch back can occur for cattle. In the research described here, however, sheep switched from A-type on silage, to B-type on winter pasture and stayed with the B-type on summer pasture.

This work shows that different ruminants in New Zealand are colonised by distinct ciliate communities. Previous work detected only B-type communities in New Zealand sheep and cattle, but in this research cattle contained A-type communities. It has been suggested that A-type communities lead to less CH<sub>4</sub> production than B-type communities, particularly when dominated by *P. multivesiculatum*. This species is heavily colonised by intra-cellular bacteria, but only associates with a few methanogenic archaea.

Further work is needed to better understand the possibilities such knowledge offers in terms of potential CH<sub>4</sub> mitigation strategies, and to determine how it can be utilised for development of practical technologies and practices.

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## Reference

Kittelmann S, Janssen PH (2011) *Characterisation of rumen ciliate community composition in domestic sheep, deer, and cattle, feeding on varying diets, by means of PCR-DGGE and clone libraries*. FEMS Microbiology Ecology 75: 468-481.



## A proposed taxonomy of anaerobic fungi (class *Neocallimastigomycetes*)

Sandra Kittelmann and Peter Janssen 2012. AgResearch.  
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### INTRODUCTION

Anaerobic fungi of the class *Neocallimastigomycetes* play a key role in the rumen by physically and enzymatically attacking fibrous feed and breaking down the tough cell walls of the ingested plant material. During these processes they produce volatile fatty acids for their ruminant hosts, and also large amounts of hydrogen which can be utilised by *methanogenic archaea* to form the greenhouse gas methane (CH<sub>4</sub>). Six genera of fungi have so far been described, based mainly on their morphological characteristics: *Anaeromyces*, *Caecomyces*, *Cyllomyces*, *Neocallimastix*, *Orpinomyces* and *Piromyces*. The role of these different fungi in CH<sub>4</sub> formation and their potential niche-specificity are not yet clear. Through their penetration and degradation of plant tissue in the rumen, fungi may influence the structure of other members of the microbial community in the rumen, i.e. the bacteria, protozoa and methanogenic archaea, and therefore fermentation pathways and CH<sub>4</sub> emissions by the host animal.

The microscopic and microbiological methods which have previously been used to identify anaerobic fungi in the rumen are tedious and time consuming. Modern molecular monitoring tools provide an opportunity for more effective experimentation on the fungal communities and for simultaneously analysing larger numbers of animals.

The internal transcribed spacer 1 (ITS1) region is widely accepted as a molecular marker for fungi and has proved useful for community structure comparisons. In this research ITS1 clone libraries were constructed from 11 rumen samples selected from different species of New Zealand ruminants (cattle, red deer and sheep). The 401 sequences obtained from these, together with 16 sequences from excised DGGE bands and 342 sequences obtained from public sources, were used to build an improved taxonomic framework for anaerobic fungi. This allowed a detailed assessment of anaerobic fungal community structure and diversity in New Zealand ruminants.

### METHODOLOGY

Rumen samples were collected through the rumen fistula from four wether sheep, five non-lactating cows, and four castrated red deer stags. The animals were fed perennial ryegrass-white clover pasture during winter and summer and lucerne silage twice daily in a different period during the winter. One sample was taken per animal per season 15 days after feeding commenced. Rumen samples were also collected at slaughter from three other groups of sheep: four wethers fed a concentrate-based diet, five ewe hoggets grazed on perennial ryegrass-white clover pasture during the autumn, and five ewe hoggets fed on willow. Anaerobic fungal community structure in rumen samples was analysed using denaturing gradient gel electrophoresis (DGGE) fingerprinting and clone libraries were constructed from 11 representative samples.

### RESULTS AND DISCUSSION

DGGE fingerprinting and clone libraries were successfully applied to gain insights into the diversity and structure of anaerobic fungal communities in the rumen, the degree of animal-to-animal variation, and the potential influence of host species and diet.

DGGE patterns for the same groups of sheep and cattle fed the three diets (summer and winter pasture, and silage) suggested that there were diet and ruminant species effects on the fungal communities and there was a diet/species interaction. These effects were detectable despite animal-to-animal variations which were found to be highest in sheep and smallest in cattle. The additional groups of sheep also harboured different fungal communities suggesting that anaerobic fungal communities do not randomly assemble in the rumen, but that different species occupy distinct environmental niches influenced by diet and/or the ruminant host.

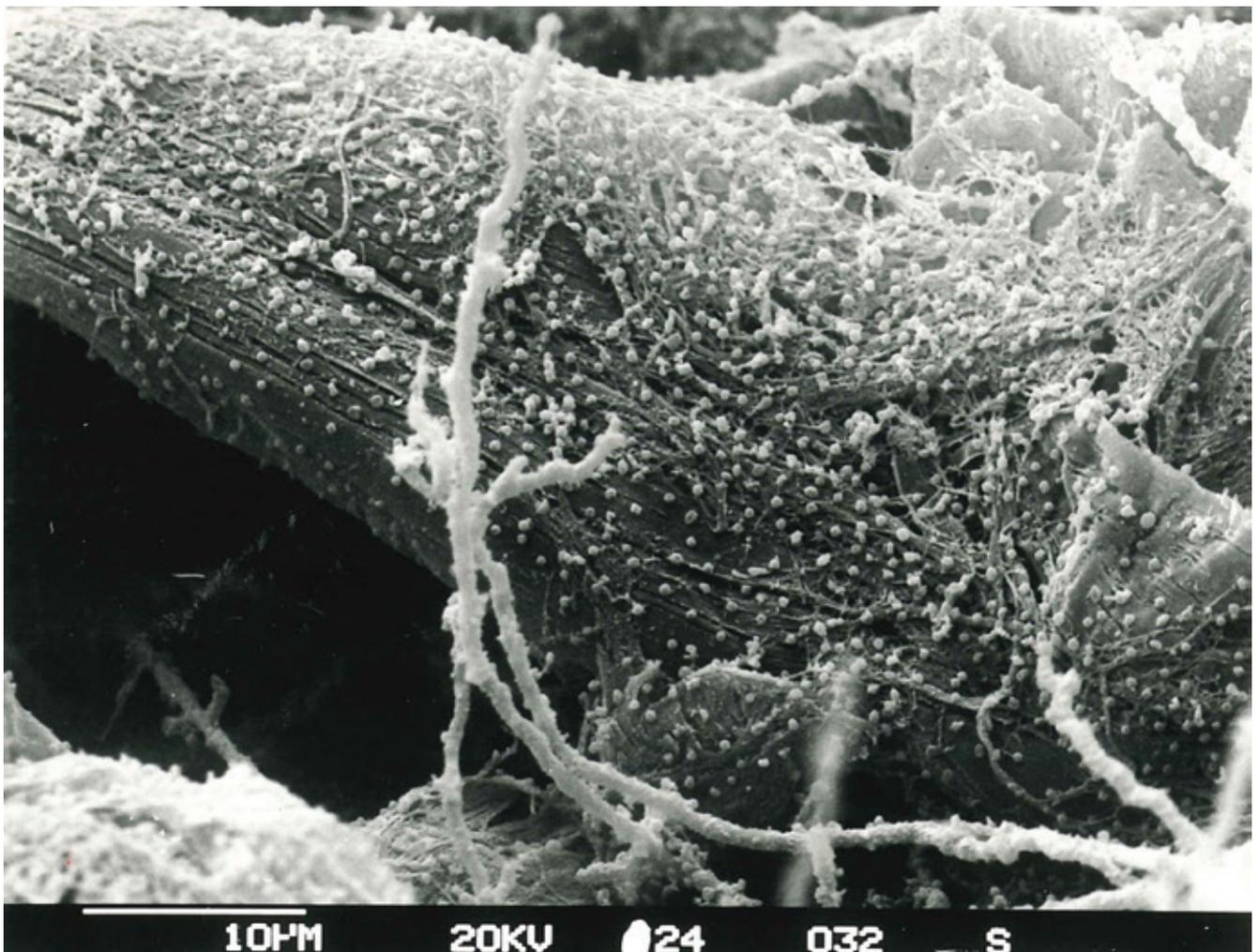
The ITS1 region is commonly used as molecular marker for diversity studies of fungi in diverse environments. However, its highly variable nature has so far hindered the calculation of a robust phylogenetic framework. Therefore, assigning anaerobic fungal

sequence data to taxonomic groups has been problematic. This research took a pragmatic approach by constructing a phylogenetic tree and a finer-scale nomenclature scheme which uses established taxonomic assignments for known genera and species and temporary assignments where the true affiliation is so far unknown. The phylogenetic analysis led to 37 distinct clusters. By comparing this revised taxonomy with the taxonomic assignment of sequences deposited in the GenBank database, it appears that more than 29% of ITS1 sequences from anaerobic fungi had so far been misnamed at the genus level.

The revised taxonomic framework was used to analyse anaerobic fungal community structure in 11 rumen samples from New Zealand ruminants. The analysis suggested that anaerobic fungal diversity was similar in sheep and cattle, and lower in deer. Overall, *Piromyces* and *Neocallimastix* were the most

dominant genera in all samples analysed, followed by species belonging to the genera *Caecomyces* and *Orpinomyces*. In addition, four novel groups of sequence types were found in the samples which may represent so far undetected genera or species of anaerobic fungi.

This work shows that the diversity of anaerobic fungi in the rumen is greater than previously reported and that community structure is influenced by ruminant host species and diet. Anaerobic fungi should be included in the molecular monitoring of microbial communities of animals showing different phenotypes, particularly with respect to CH<sub>4</sub> emissions or productivity. In future studies, the taxonomic framework proposed here will serve for reliable, fine-scale taxonomic assignment of data obtained from hundreds of rumen samples using high-throughput next generation sequencing technologies.



Scanning electron micrograph showing hyphal growth of the anaerobic fungus *Neocallimastix frontalis* on a blade of grass in the rumen. Photograph taken by Graham Naylor.

## Reference

Kittelmann S, Naylor GE, Koolaard JP, Janssen PH (2012) A proposed taxonomy of anaerobic fungi (Class Neocallimastigomycetes) suitable for large-scale sequence-based community structure analysis. PLoS ONE 7(5): e36866.



## Exploring interaction patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities

Sandra Kittelmann and Peter Janssen (2013). AgResearch.  
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### INTRODUCTION

Sequencing studies of the rumen using phylogenetic markers have mostly concentrated on bacteria and archaea. Protozoa and fungi are far less abundant than bacteria and archaea in cell numbers, but can make up half of the total rumen microbial biomass. Until recently, species identification and physiological characteristics of fungal and ciliate protozoan communities in the rumen have been measured using microscopy and cultivation-based techniques.

In this research, barcoded pyrosequencing of phylogenetic marker genes was used to simultaneously characterise diversity of bacteria, archaea, ciliate protozoa and fungi in the rumen. A method was developed that allows adjustment for species diversity, abundance and amplicon length of the different microbial groups, but also to minimise the effect of DNA from plant material in rumen samples and endophytic fungi. The approach maximises sample throughput while giving satisfactory coverage of diversity and thus increases the ability to compare effects of experimental treatments.

### METHODOLOGY

Eleven rumen samples were collected through rumen fistula from sheep, cattle and deer feeding on six different diets typically used in New Zealand.

DNA was extracted from freeze-dried and ground samples and PCR amplification of bacterial and archaeal 16S rRNA genes, ciliate 18S rRNA genes and fungal ITS1 genes was carried out. Amplicons from all samples were pooled by microbial group, purified and mixed in different ratios. Pyrosequencing was conducted and phylogenetic analysis of pyrosequencing reads was carried out.

### RESULTS AND DISCUSSION

Estimates were made of the average number of sequences of each microbial group that had to be sampled to describe phylogenetic diversity across all samples; this appeared to be 1000 for archaea, fungi and ciliate protozoa and 5000 for bacteria. An amplicon mixing ratio of 5:1:1:0.2 for bacteria, archaea, ciliate protozoa and anaerobic fungi gave the required number of sequence reads from DNA samples analysed in this study, allowing for up to 100 rumen samples to be processed in a single 454 pyrosequencing run and yielding sufficient sequence information to analyse diversity of all four microbial groups per sample.

Bacterial communities in the rumen samples, analysed at the family level had similar diversity, and bacterial community composition was very similar. The largest group of bacteria were classified into the family *Lachnospiraceae*, constituting 14-40% of all bacterial 16S rRNA gene sequences amplified; and the family *Prevotellaceae* which constituted 12-45%. Presumably these play a large role in feed degradation in the rumen. Other abundant bacteria were in the families *Ruminococcaceae* (mean 8%), *Fibrobacteraceae* (mean 6%), *Clostridiales*, unidentified family (mean 9%) and an unidentified *Bacteroidales* family (14%). These bacterial groups accounted for an average 84% of sequences in all samples, and were present in all cases. However, their relative abundances varied by up to eight-fold, illustrating differences in community structure across animal species, diets and/or herds/flocks. There appeared to be a trade-off between abundance of members of the order *Clostridiales* and *Bacteroidales*. This limited diversity reflects that found by other researchers.

The diversity of methanogenic archaea at 'operational clade' level was similar across the samples analysed and lower than bacterial diversity. Community composition in the analysed samples was also similar. Sequences clustering within the *Methanobrevibacter ruminantium* clade (38%), *M. gottschalkii* clade (32%), Rumen Cluster C (18%) and the genus *Methanosphaera* (9%) were most abundant. One deer sample contained a large number of sequences from *Methanosarcina*, usually rare in the rumen because of their low growth rate. This species was detected in the same animal grazing summer pasture in other work, and its high abundance in deer typically showing slow passage of rumen contents in the summer is consistent with the physiology of this organism.

Ciliate protozoa communities showed a broad range of diversity at the genus level and community structure was very different across samples. Species of the genus *Entodinium* were the most abundant (40%). Four of the 11 rumen samples showed A-type communities (characterised by the presence of *Polyplastron multivesiculatum*) and these were dominated by *Ostracodinium*, *Dasytricha* and *Entodinium* species. B-type communities were observed in six rumen samples, and characterised by the presence of *Epidinium* and *Eudiplodinium* species. The other two samples harboured type-O ciliate communities consisting almost entirely (>94%) of *Entodinium* species.

These results suggest that this molecular approach can easily detect and distinguish between the distinct ciliate community types previously established using microscopy.

The diversity of anaerobic fungal communities, compared at cluster level, was also relatively broad and particularly variable. Using the newly developed taxonomic framework for anaerobic fungi, it was found that major phylogenetic groups belonged to the genera *Neocallimastix* (28%) and *Piromyces* (20%), the novel clades SK1, SK3 and SK4 (16%), *Orpinomyces* (12%), BlackRhino (8%) *Caecomyces* (8%) and *Cyllamyces* (5%).

This work allows the identification of patterns of co-occurrence in the microbial community (Figure 1). Of particular interest was a general negative correlation between the H<sub>2</sub> utilising methanogen clades *M. ruminantium* and *M. gottschalkii*. It is thought that these two major clades directly compete within the rumen. Other groupings were positively correlated, for example, the methanogens in the *M. ruminantium* clade and bacteria in the family *Fibrobacteraceae* are typically found together. In contrast, methanogens in the *M. gottschalkii* clade were typically found associated with bacteria in the family *Ruminococcaceae*. *Ruminococcus* species and *Fibrobacter* species both degrade cellulose in the rumen, thereby producing substrates for methanogens. While *Ruminococcus* species produce large amounts of H<sub>2</sub>, *Fibrobacter* species produce formate (HCOOH). This suggests that the two methanogens may be adapted to different rumen conditions. There is some evidence *M. ruminantium* might be specialised to low rumen H<sub>2</sub> concentrations.

The correlations seen may result from highly-specific interactions between certain methanogens and bacteria which might, for example, facilitate a better transfer of H<sub>2</sub> between the partners. There was also a positive association between members of the archaeal Rumen Cluster C and members of the genus *Methanosphaera*. This might indicate cooperation between or coexistence of the two groups. Both produce CH<sub>4</sub> from H<sub>2</sub> and methanol and it seems likely they both increase in numbers when methanol availability is high.

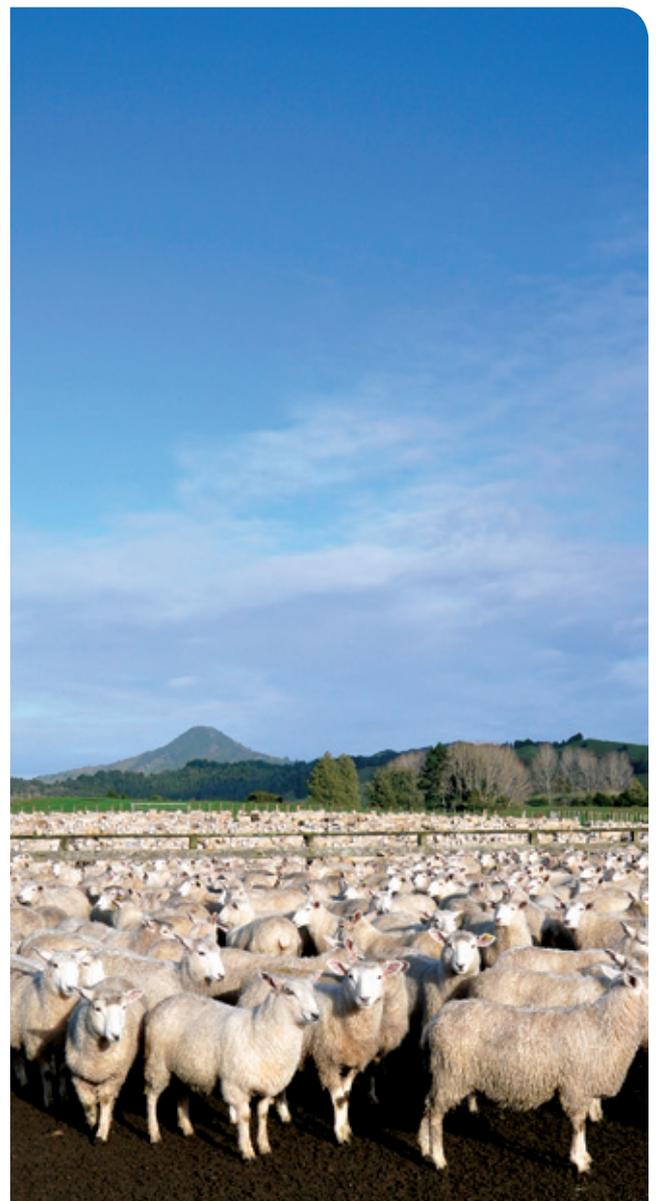
There was a strong positive association between the ciliate protozoa genera *Polyplastron* and *Ostracodinium* members of A-type ciliate communities in New Zealand ruminants. Similarly, members of the *Eudiplodinium* genus and the *Anoplodinium-Diplodinium* cluster, and of the genera *Eudiplodinium* and *Epidinium*, all members of B-type communities, were generally found together. Ciliates of the genus *Isotricha* were negatively associated with anaerobic fungi of the BlackRhino group, whereas ciliates of the genus *Entodinium* were strongly positively associated with fungi of the genus *Neocallimastix*.

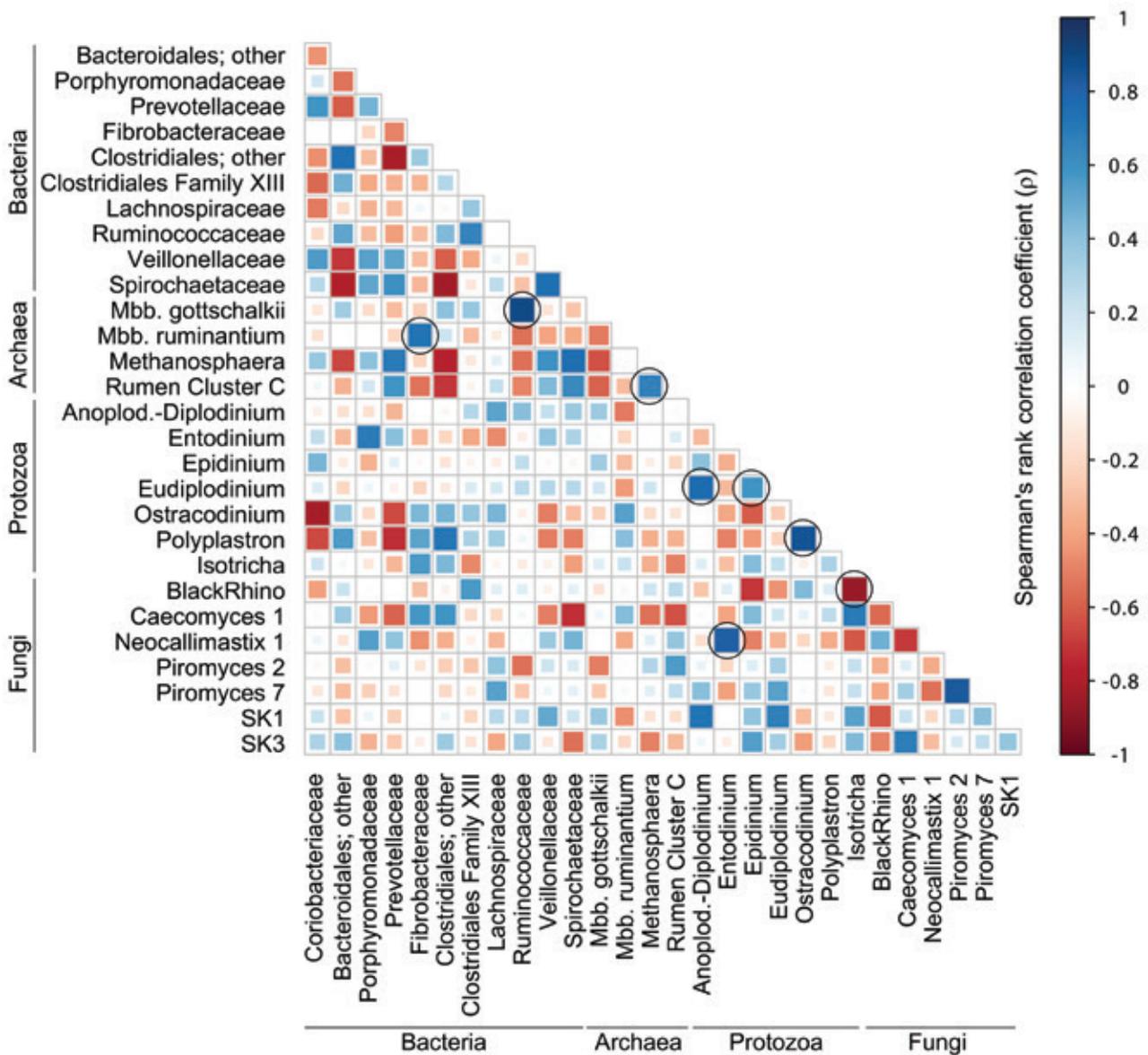
## Future work

The simultaneous analysis of the structure of bacterial, archaeal, protozoal and fungal communities in the rumen has potentially broad applications. Further investigation of the patterns of co-occurrence may provide new clues about metabolic networks between rumen inhabiting microbial groups and help define their contributions to function of the rumen.

Information found using this approach can help in design of potential mitigation interventions, including small-molecule inhibitors or vaccines, to reduce rumen CH<sub>4</sub> production whilst maintaining or even enhancing animal productivity.

The future focus will be on screening sera and inhibitors for effects on rumen microorganisms; determining which methanogens are inhibited in animal trials; provision of cell material for the genomics and vaccine programme; and testing microbiology hypotheses in high/low emitting animals.





**Figure 1.** Correlation matrix of the dominant microbial populations across the four groups studied in rumen samples. Strong correlations are represented by large squares and weak correlations by small squares. The colours show the nature of the correlation between any two microbial taxa. Red is a negative correlation, Blue is positive. Correlations marked with circles are discussed in the text. Reproduced from Kittelmann *et al.* (2013) PLoS ONE 8(2): e47879.

## Reference

Kittelmann S, Seedorf H, Walters WA, Clemente JC, Knight R, Gordon JI, Janssen PH (2013) *Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities.* PLoS ONE 8(2): e47879.



## Presence of novel potentially homoacetogenic bacteria in the rumen

Gemma Henderson and Peter Janssen 2010. AgResearch.  
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### INTRODUCTION

As outlined previously digestion of forages results in H<sub>2</sub> release in the rumen. Methanogens utilise H<sub>2</sub> as part of their specialised metabolic pathway, and in so doing maintain rumen H<sub>2</sub> concentrations at the low levels that are presumed necessary for rapid feed digestion. Homoacetogens, bacteria which convert H<sub>2</sub> to acetate, are of particular interest for reducing CH<sub>4</sub> emissions because they potentially present an alternative route for maintaining reasonably low H<sub>2</sub> concentrations in the rumen without the need to produce CH<sub>4</sub>.

Several homoacetogens have been isolated from ruminants, and their prevalence is influenced by diet, animal age and time of sampling. Traditional 16S rRNA gene-based survey approaches for identifying bacteria within, e.g., a rumen fluid sample, are not suitable for estimating the prevalence of homoacetogens, so an alternative methodology is required. Access to such a methodology is necessary before the incidence of resident homoacetogens and identification of factors influencing their prevalence can be researched.

The formyltetrahydrofolate synthetase (FTHFS) gene has been used as a functional marker for homoacetogens because, as well indicating their presence, it also plays a functional role as an enzyme which catalyses a key step in the metabolic pathway leading to acetate formation from H<sub>2</sub>.

In this research, the diversity of FTHFS gene sequences in rumen samples was assessed, novel rumen bacterial isolates were screened for the presence of FTHFS genes, and the novel bacterial isolates were tested for their ability to grow as homoacetogens.

### METHODOLOGY

Rumen isolates selected on the basis of PCR analysis were grown in a medium containing clarified rumen fluid, and with H<sub>2</sub> or glucose as an energy source. Growth was determined by measurements of optical density and acetate formation was measured.

A total of 275 partial sequences of genes encoding FTHFS were PCR-amplified from the rumen contents of a cow, two sheep and a deer. A phylogenetic tree was constructed using these sequences and the translated amino acid sequences, together with other sequences from public databases and from novel non-homoacetogenic bacteria isolated from the rumen. Amino acids that were present in sequences across a range of known homoacetogens were identified and their presence in sequences from rumen samples was used to calculate a homoacetogen similarity score (HS).

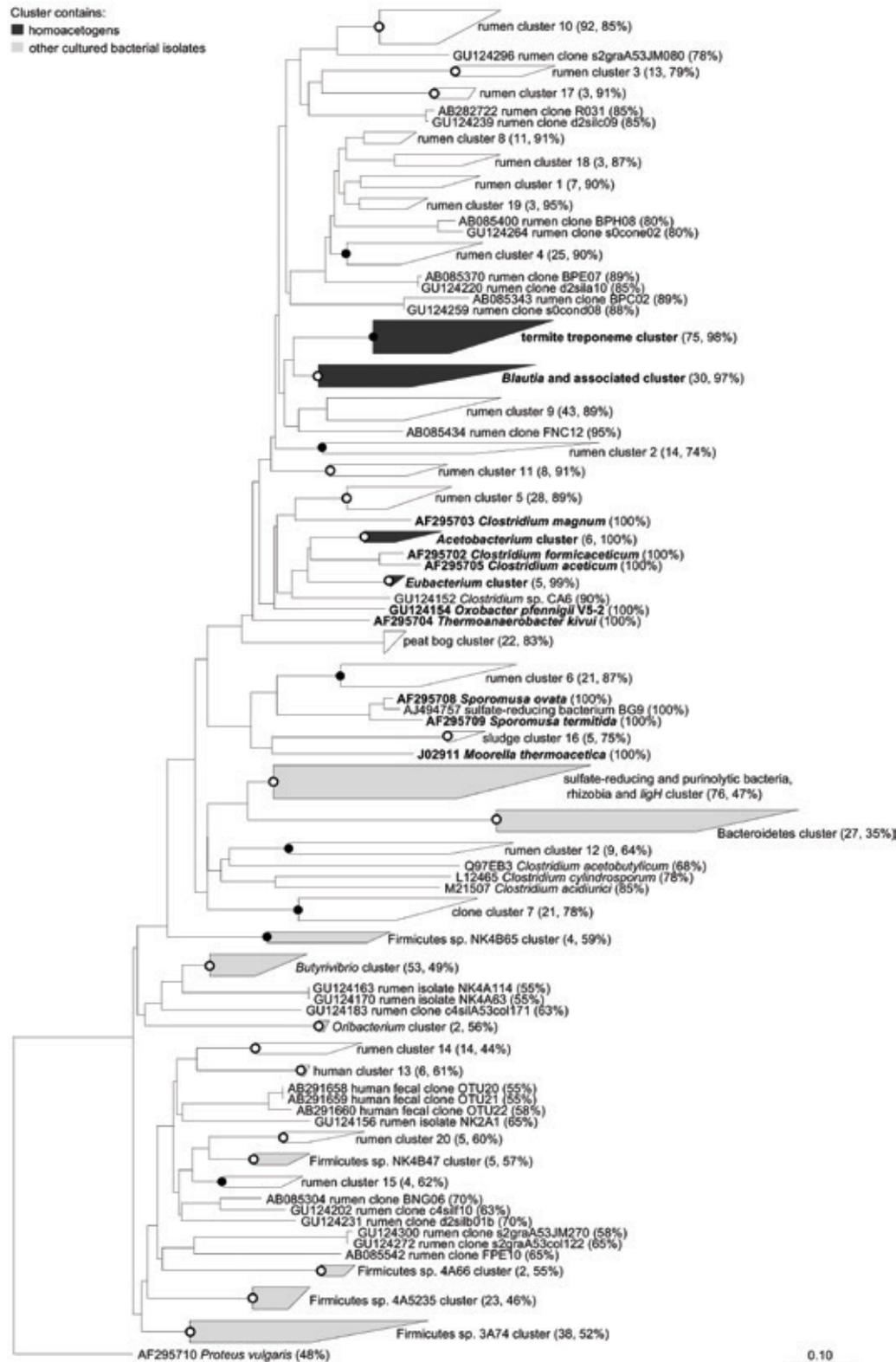
A modelling approach (homoacetogen FTHFS profile hidden Markov model 'HoF-HMM') was used to assess the homology or evolutionary similarity between rumen and homoacetogen FTHFS sequences.

### RESULTS AND DISCUSSION

Of the new rumen bacterial isolates derived from the rumen samples, 21 of a total of 51 were identified as having FTHFS sequences and were tested for their ability to produce acetate from H<sub>2</sub> and CO<sub>2</sub> or glucose. None of these grew on H<sub>2</sub> and CO<sub>2</sub> or produced acetate, suggesting they were not homoacetogens. FTHFS is not used exclusively in the relevant acetate producing pathway, so it is likely that in these organisms it was used for a different purpose.

Over 90% of the FTHFS sequences used fell into 34 clusters on the phylogenetic tree, but few of the rumen-derived sequences fell into clusters containing sequences of known homoacetogens. Many of the clusters had low homoacetogen similarity (HS) scores and poor HoF-HMM matches, suggesting the sequences grouped there did not stem from homoacetogens. This conclusion was strengthened by FTHFS sequences from non-homoacetogens being grouped in clusters with low HS scores. However, 15% of rumen-derived FTHFS sequences had both high HS scores, and strong HoF-HMM matches, and so could represent novel and as yet unidentified, homoacetogens.

Future work investigating the characteristics of novel rumen organisms with high HS scores and HoF-HMM matches would be valuable.



**Figure 1.** Phylogenetic tree based on 740 FTHFS sequences from public databases and rumen-derived samples. The percentage value in brackets for each cluster represents its similarity score (HS) to FTHFS of homoacetogens. Copyright © 2010, American Society for Microbiology. All Rights Reserved. Reproduced from Henderson *et al.* (2008) *Applied and Environmental Microbiology* 76: 2058-2066.

## Reference

Henderson G, Naylor GE, Leahy SC, Jansen PH. Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by analysis of formyltetrahydrofolate synthetase sequences from ruminants. *Applied and Environmental Microbiology* 76: 2058-2066.

# METHANOGEN GENOMICS

## BACKGROUND

Attempts have been made to reduce CH<sub>4</sub> production from ruminants using a variety of approaches. One promising avenue involves inhibition of the action of methanogens in the rumen using a variety of interventions. However most of these have failed or been only partially successful due to low efficacy of the inhibitor, poor selectivity, toxicity of the inhibitor towards the host ruminant, or build-up of resistance to anti-methanogenic compounds. Currently there are few practical CH<sub>4</sub> reduction technologies for housed animals and none for pasture-grazed animals, the feeding system used by the vast majority of farmers in New Zealand.

New DNA sequencing technologies are revolutionising the biological sciences via the cost-effective provision of large amounts of sequence data. Genome sequencing is particularly useful for rumen methanogens as they are difficult to culture and genetic manipulation systems are not available to allow determination of gene function. A methanogen's genome sequence can be used to gain a better understanding of its metabolism, physiology and role in the ecosystem and to identify targets for CH<sub>4</sub> mitigation technologies. Ideal targets are genes and proteins that are present in all methanogens and not in rumen bacteria, protozoa and bacteria, or if present they do not have a critical function. This is to ensure broad efficacy of the mitigation against all methanogen types in the rumen while not compromising the digestive function of other microbes. Broad efficacy will prevent the inhibition of one methanogen just leading to another methanogen multiplying and filling the vacant niche, and will also mean one or a few generally applicable technologies can be used rather than a multitude of specific technologies.

## How Methanogen Genomics is important to the PGgRcProgramme

The genome of a species is a blueprint showing all its details, but you need to be able to read and understand the blueprint; which is where the AgResearch team is carrying out unique science for PGgRc. Application of these technologies has refined the Consortium investment and enabled a focus on the specific attributes of methanogens which make them unique and therefore offer an opportunity to target them amongst rumen microbes. The technical papers included in this section detail specific aspects that have been investigated as a result of a Consortium world first—the complete gene sequencing of the rumen methanogen *Methanobrevibacter ruminantium* M1. These findings are a product of a world leading team that has gone on to explore more rumen methanogens and continue to be integral to the progress we have made toward mitigating rumen methane emissions.



## The genome sequence of *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions

Sinead Leahy and Bill Kelly 2010. AgResearch.  
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### INTRODUCTION

New Zealand researchers have embarked on a programme to sequence the genomes of the main rumen methanogen groups, where representatives can be cultured, to better understand the nature and function of these organisms and to define their specific and conserved features that can serve as targets for CH<sub>4</sub> mitigation technologies. Analyses of small subunit ribosomal RNA genes from rumen samples suggest the dominant archaeal species belong to the *Methanobrevibacter* genus, constituting about 62% of total rumen archaea. *M. ruminantium*, a major species within this grouping was the first rumen methanogen to ever be completely sequenced and this research was completed by the PGgRc programme.

### METHODOLOGIES

A strain of *M. ruminantium* known as M1 and stored in a German microbiological collection was obtained and cultured. The M1 genome was sequenced and subjected to comparative genomic and metabolic pathway analyses.

Detailed methodologies are outlined in the source paper by Leahy *et al.* 2010.

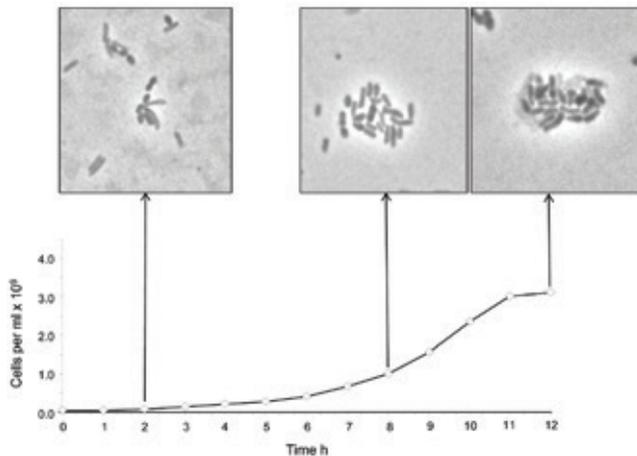
### RESULTS AND DISCUSSION

The genome sequence of M1 consists of a single circular chromosome. M1 has the largest genome size of the members of the *Methanobacteriales* order sequenced to date. This is due in part to the large number of genes encoding surface adhesin-like proteins. Adhesins are bacterial products that enable the products to adhere to and colonize a host. Also, a prophage is present within the M1 genome. A prophage is a viral genome inserted and integrated into the host chromosome; upon detection of host cell damage viral replication may begin via the lytic cycle, where the virus commandeers the cell's reproductive machinery and the cell may fill with new viruses until it lyses or bursts, or it may release the new viruses one at a time. Prophages are known to be important agents of horizontal gene transfer. The M1 genome also contains a variety of unique genes. Analysis of potential horizontal gene transfer events in M1 identified a number of genes that are similar to those in non-methanogens.

Many of the enzymes involved in the methanogenesis pathway are highly conserved (i.e. present across the range of organisms of interest) and found only among methanogens. The M1 genome shows for the first time details of this pathway for a rumen methanogen. Surprisingly, M1 has a number of alcohol *dehydrogenase* genes. In some methanogens these enzymes allow growth on ethanol or isopropanol, but previous research has suggested that M1 could not grow on ethanol or methanol. In this work it was found that M1 would grow on ethanol and methanol in the presence of limited amounts of H<sub>2</sub> and CO<sub>2</sub>, but not when H<sub>2</sub> is absent. Methanogens that use CO<sub>2</sub> as a source of carbon and H<sub>2</sub> as a reducing agent are known as Hydrogenotrophic methanogens and these usually code for an enzyme (mrclI) which is associated with CH<sub>4</sub> formation under conditions of high H<sub>2</sub> concentration; in contrast M1 appears to be adapted to grow at the low levels of H<sub>2</sub> in the rumen and codes only for the enzyme mrcl.

To examine the expression of genes involved in methanogenesis in the presence of H<sub>2</sub>-forming bacteria (as in the rumen), M1 was grown in co-culture with *Butyrivibrio proteoclasticus* in a medium containing just xylan as a carbon source. Several genes involved in the production of CH<sub>4</sub> from H<sub>2</sub> were 'upregulated' (increased gene expression) in the co-culture compared to the M1 monoculture grown just with H<sub>2</sub> and CO<sub>2</sub>. Interestingly, genes for formate (HCOOH) utilisation were also upregulated suggesting formate formed by *B. proteoclasticus* was transferred as part of a symbiotic association between the two organisms. It is also interesting as the use of formate as a donor for hydrogen ions (as opposed to H<sub>2</sub>) has previously been assumed not to be important in the metabolism of methanogens. Analysis of the M1 genome has also assisted in explaining the growth requirements of M1 for acetate, a volatile fatty acid formed in rumen fermentation and major energy source for ruminants.

The methanogen cell envelope is the physical interface between methanogens and the rumen environment and so represents a key area for identification of vaccine and drug targets. The main structural component of the M1 envelope is pseudomurein and the genomic analysis in this research has identified several genes encoding enzymes involved in pseudomurein biosynthesis and M1 cell wall construction. Polysaccharides and glycosylated molecules are the major component of the cell envelope and because they are accessible at the cell surface they are viable mitigation targets.



**Figure 1.** Interactions between *M. ruminantium* M1 and *B. proteoelasticus*. The graph shows the growth rate of M1 in co-culture and the microscopic images taken at 2, 8 and 12 hours post inoculation with *B. proteoelasticus* show co-aggregation of cells of the two organisms.

In the co-culturing experiment where adhesin-like proteins were upregulated, microscopic examination showed co-aggregation of M1 and *B. proteoelasticus* cells (Figure 1). It also showed immune sera produced by small peptides synthesised to correspond to M1 adhesin-like proteins were able to bind specifically to M1 cells. These proteins are a significant component of the M1 cell wall, and identifying highly conserved methanogen-specific features of them may present a pathway to vaccine development.

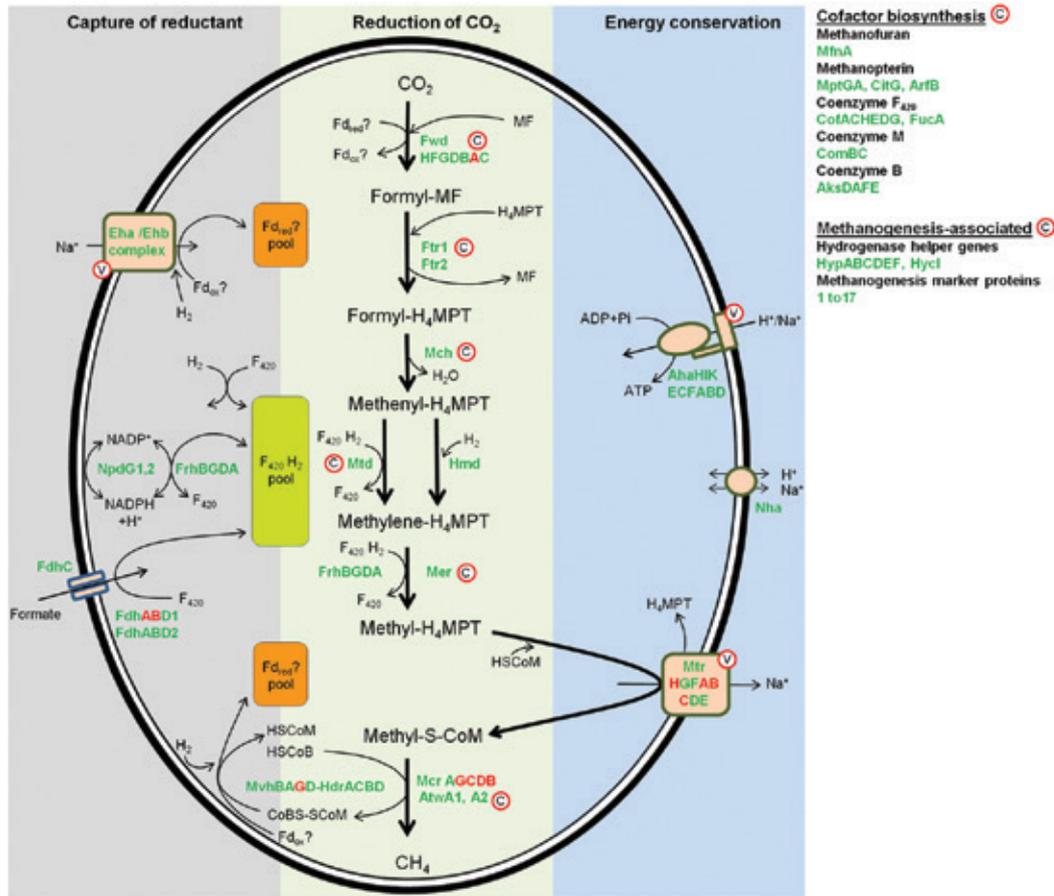
Phage exert a significant ecological impact on microbial populations in the rumen and have been suggested as biocontrol agents for methanogens. One region of the M1 genome encodes a prophage genome, partitioned into distinct modules encoding integration, DNA replication, DNA packaging, phage capsid (encapsulation), lysis and lysogenic (fusion of phage and host DNA) functions. Within the lysis module a gene encoding for a lytic enzyme, endo-isopeptidase PeiR, was identified. This is a novel enzyme as it is not similar to any sequence currently in public databases.

An additional novel feature of M1 is the presence of two large proteins showing the distinctive architecture of non-ribosomal peptide synthetases (NRPS). NRPSs produce a wide variety of small molecule natural products with industrial applications e.g. as antibiotics or immunosuppressants. The products of the M1 NRPSs are not known, however analysis of amino acid sequences suggests they may be important in substrate binding. The NRPS genes may be bacterial in origin.

Several approaches were used to define potential M1 gene targets for CH<sub>4</sub> mitigation. These fall broadly into two categories; chemogenomics and vaccines. Chemogenomics is the systematic study of the biological effects of a wide array of small molecular weight compounds on large macromolecular targets. The amount of existing data and new data generated is so large, information technologies play a crucial role in planning, analysing and predicting chemogenomic data. Genes suitable as chemogenomic targets were identified using metabolic profiling, review of the relevant literature, and comparative genomics (study of the relationship of genome structure and function across different species or strains, to understand the function and evolutionary processes that act on genomes). Thirty three candidate genes were identified. Most of these are involved in energy metabolism and mainly in the methanogenesis pathway (Figure 2), and included several methanogenesis marker proteins currently without defined function. Most of these methanogenesis enzymes are located within the cell cytoplasm and have been tagged as key targets for inhibitor discovery via a chemogenomics approach.

Vaccine approaches involve inducing the ruminant immune system to produce salivary antibodies against conserved features of rumen methanogens. To be effective the antibodies must bind to surface features on the methanogen which either leads to their inactivation or clearance from the rumen. Vaccines are typically composed of proteins or polysaccharides from killed or attenuated (i.e. with reduced virulence) cells or cell components present on the outside of cells e.g. flagella or cell walls. For rumen methanogens the most likely vaccine targets are surface-exposed or membrane-associated proteins that are conserved among methanogens and encode functions vital to methanogen function. *In silico* (computer-based) analysis identified an initial pool of 572 candidates, which was refined to 71 of future interest, and specifically 24 most suitable for vaccine development using synthetic peptides.

Most of these vaccine targets correspond to hypothetical proteins with unknown influence on M1 growth and development, so they are of lower priority as vaccine candidates. Of the remainder those involved in energy metabolism are again of most interest and particularly the Mtr enzyme complex (Figure 2) which has a known catalytic function in the methanogenesis pathway and has peptide loops located outside the methanogen cell membrane. These loops are potential antibody binding sites; to test this, peptides corresponding to loop regions were synthesised, coupled with a carrier protein and used as antigens to vaccinate sheep. The resulting immune sera bound specifically to immobilised M1 demonstrating the feasibility of this approach for vaccine target selection.



**Figure 2.** The predicted pathway of methane formation in M1. The diagram is in three parts: capture of reductant, reduction of CO<sub>2</sub> and conservation of energy at the methyl transfer step. The main reactions are shown by thick arrows, and enzymes catalysing each step are coloured green. Protein sub-units coloured red signify the corresponding genes were up-regulated during co-culture with *B. proteoclasticus*. This arrows show cofactor participation. Membrane-located proteins are in brown boxes and potential chemogenomic or vaccine targets are labelled with a circled C or V respectively.

## Future work

The wealth of biological information provided by the M1 genome provides a platform for development of mitigation technologies with broad efficacy. However work on other important rumen methanogen genomes will be essential to verify that the selected vaccine and chemogenomics targets are broadly applicable, and to ensure practical long-term mitigation technologies for CH<sub>4</sub> emissions from the rumen are developed.

## Reference

Leahy SC, Kelly WJ, Altermann E, Ronimus RS, Yeoman CJ, *et al.* (2010) *The Genome Sequence of the Rumen Methanogen Methanobrevibacter ruminantium Reveals New Possibilities for Controlling Ruminant Methane Emissions.* PLoS ONE 5(1): e8926. doi:10.1371/journal.pone.0008926.



## Exploring rumen methanogen genes to identify targets for methane mitigation strategies

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### INTRODUCTION

Publication of the first methanogen genome for *Methanobrevibacter ruminantium* M1 has opened avenues to a large scale approach to identifying CH<sub>4</sub> mitigation targets for development of vaccines or small-molecule inhibitors. However a detailed knowledge of the diversity and physiology of the range of rumen methanogens is required to identify conserved features that can be targeted for CH<sub>4</sub> inhibition. Hence, an ongoing second-generation programme in New Zealand and Australia (the Beef CRC) is sequencing eight additional methanogen genomes. It would be desirable to genome sequence all methanogen species inhabiting the rumen, but current techniques do not allow this. Therefore the approach is to direct efforts at the main methanogenic groupings in the rumen. There are only seven rumen methanogen species well-described among the 114 recorded in the US National Center for Biotechnology Information (NCIB). Also, slow growth of most rumen methanogens *in vitro*, low cell yields and difficulty in lysing cells for DNA extraction are impediments to conducting these studies. New developments in pyrosequencing and sequence-by-sequence technologies has greatly increased the speed

and capacity of DNA sequencing and dramatically reduced costs. Also, slow growth of most rumen methanogens *in vitro*, low cell yields and difficulty in lysing cells for DNA extraction are impediments to conducting these studies. New developments in pyrosequencing and sequence-by-sequence technologies has greatly increased the speed and capacity of DNA sequencing and dramatically reduced costs. These new techniques also allow assembly of individuals from less genomic DNA of lower average molecular weight, which is make sequencing easier and faster.

The draft sequences for these major rumen methanogen species, along with the completed genome for M1, are beginning to allow identification of underlying cellular processes that define these organisms and their lifestyle within the rumen. Two major types of opportunities exist to utilise this information for development of practical CH<sub>4</sub> mitigation technologies: screening for or designing small-molecule inhibitors via a chemogenomics approach, and identifying surface proteins common to a range of rumen methanogens that can be used as antigens in an anti-methanogen vaccine.

### METHODOLOGY

The methanogens being sequenced involve the following:

Genus	Species/(clade or closest relative) <sup>1</sup>	Strain	Funding organisation	Status
<i>Methanobrevibacter</i>	<i>ruminantium</i>	M1	PGGRC	Complete
<i>Methanobrevibacter</i>	( <i>ruminantium</i> )	YLM1	PGGRC-MPI	Draft
<i>Methanobrevibacter</i>	( <i>gottschalkii</i> )	SM9	PGGRC	Closed
<i>Methanobrevibacter</i>	( <i>wolinii</i> )	ABM4	NZAGRC	Draft
<i>Methanobrevibacter</i>	( <i>gottschalkii</i> )	YE286	CSIRO, QDPI, CRC	Draft
<i>Methanobacterium</i>	<i>bryantii</i>	YE299	CSIRO, QDPI, CRC	Draft
<i>Methanobacterium</i>	( <i>formicum</i> )	BRM9	PGGRC-MPI	Draft
<i>Methanosphaera</i>	( <i>stadtmanae</i> )	3F5	NZAGRC	Draft
<i>Methanosarcina</i>	( <i>barkeri</i> )	CM1	PGGRC-MPI	Draft
Rumen Cluster C	( <i>Thermoplasma</i> sp.)		CSIRO	In progress

<sup>1</sup> These isolates have been identified as belonging to individual species, or if in brackets as belonging to a clade or to the closest known cultured relative based on RNA sequence similarity.

Similarity between M1 sequences, those currently being sequenced, and the NCIB database were analysed.

## RESULTS AND DISCUSSION

Comparison of *Methanobrevibacter ruminantium* M1 and *Methanobrevibacter* sp. strain YE286 showed there is a very high degree of gene synteny between the two (Figure 1). Synteny means possessing common chromosome sequences; generally organisms of relatively recent evolutionary divergence show similar blocks of genes in the same relative positions in the genome. The study of synteny can show how the genome was cut and pasted during evolution. The strong similarity between the two occurred particularly for those genes encoding critical metabolic and regulatory functions. All the genes involved in CH<sub>4</sub> formation and substrate uptake are shared between the two strains.

An initial broad comparative analysis across the nine methanogens already sequenced or with sequencing in progress (and excluding Rumen Cluster C) compared to the NCIB database demonstrated several important points:

- Methanogen-specific genes are not always found in methanogen specific pathways
- Potential already exists to identify methanogen specific genes
- Gene synteny is maintained only in very similar organisms, but gene content is conserved to a much higher degree

These comparisons provide further information for specifically targeting and inhibiting methanogens through small-molecule inhibitors.

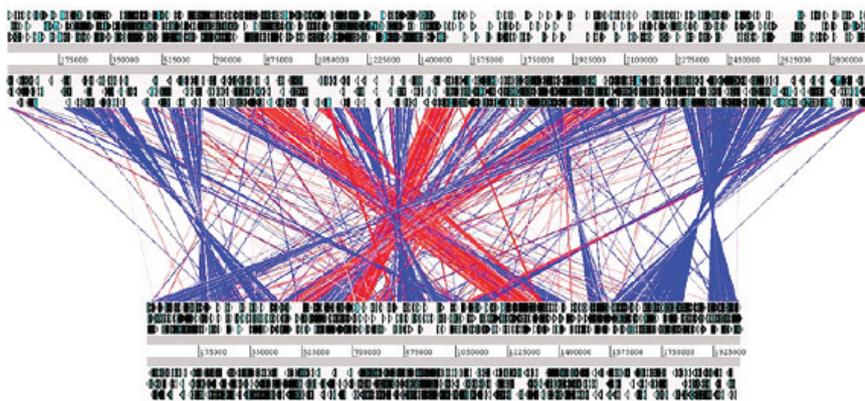
Evolutionary time has resulted in fermentation schemes for rumen microbial communities that are thermodynamically favourable and resilient. This suggests reducing CH<sub>4</sub> emissions will not be easy. Altering this process will require a change in rumen metabolism to fermentation schemes that still use H<sub>2</sub> and allow effective digestive functions of remaining rumen organisms. One possibility involves a more prominent role for acetogens which use H<sub>2</sub> and CO<sub>2</sub> to produce acetate rather than CH<sub>4</sub>, but insufficient is known at this stage to gauge if and how this might occur.

Genome sequencing projects are providing a wealth of information about rumen methanogens and their metabolisms, and are providing insights into methanogen interactions with other rumen microbes. This information is of great value in the search for CH<sub>4</sub> mitigation technologies, in particular via small-molecule inhibitors and vaccine-based interventions. Advances in DNA sequencing technologies are decreasing the cost and increasing the rate at which analyses can be conducted.

## Future work

The diversity of methanogen types in the rumen means more genome sequences will be required to assist with selection of targets and to ensure interventions that are developed are generally effective. This sequencing work is progressing.

This programme provides an essential platform for advancing the Consortium's vaccine and chemogenomics programmes.



**Figure 1.** Sequence alignment of the genomes of *Methanobrevibacter ruminantium* strain M1 (upper two grey lines) and the draft sequence of *Methanobrevibacter* sp. strain YE286 (lower 2 grey lines). Regions of high gene frequency identity are shown by the red and blue lines. This demonstrates a large amount of sequence similarity between the two strains.

## Reference

G.T. Attwood, E. Altermann, W.J.Kelly, S.C.Leahy, L.Zhang, M. Morrison. *Exploring rumen methanogen genomes to identify targets for methane mitigation strategies*, Animal Feed Science and Technology, volume 166, 65-75, 23 June 2011.

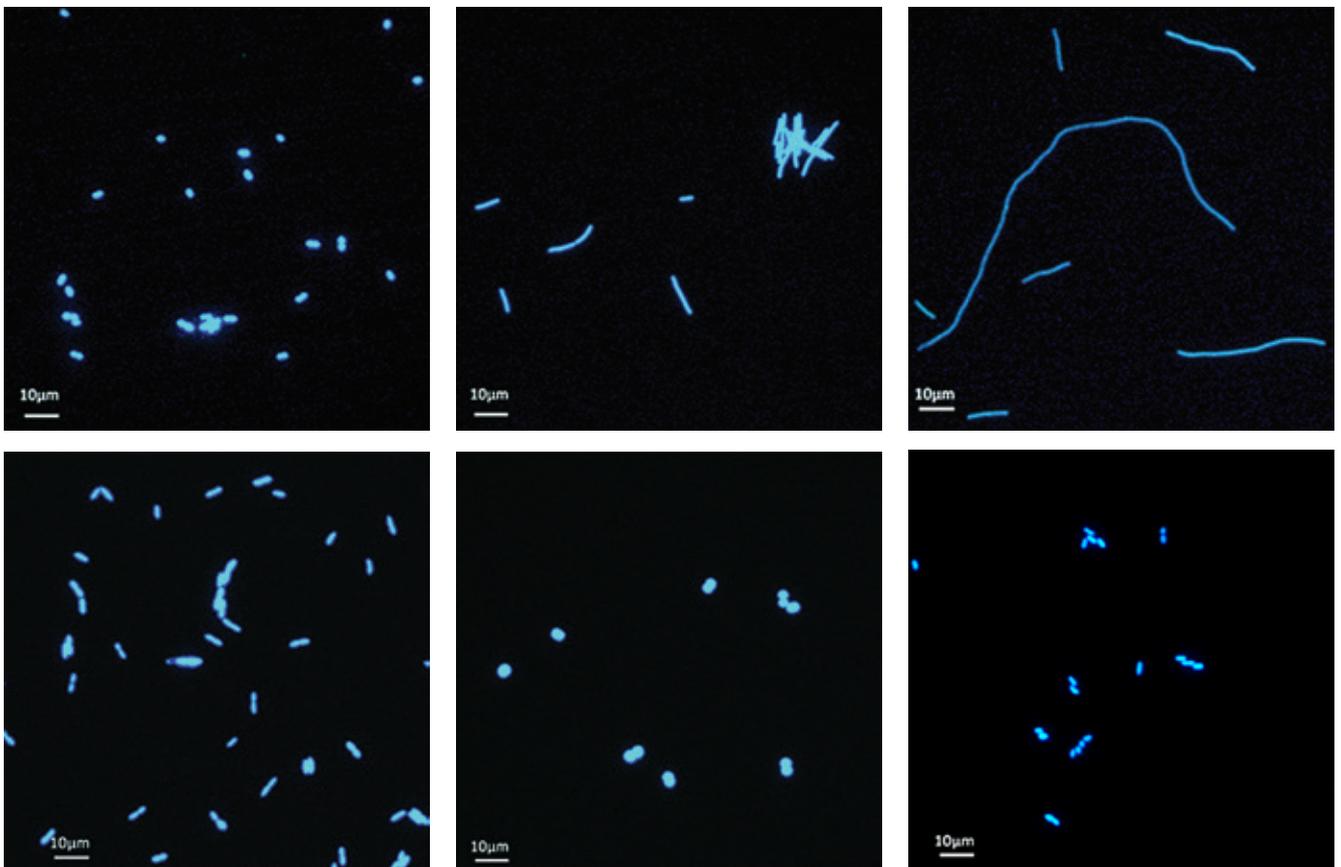
# UNIQUE PHAGES AND NON-RIBOSOMAL PEPTIDE SYNTHETASE

## BACKGROUND

Publication of the *Methanobrevibacter ruminantium* M1 genome revealed two surprising features; the presence of a prophage and identification of non-ribosomal peptide synthetases NRPS genes. Phages, viruses that infect and replicate in bacteria and archaea, are present in all biological systems. Their relative simplicity and modular structure make them important agents for genetic exchange between various microbial hosts. Also, the ability to penetrate, replicate within and break open (i.e. lyse) their host cells makes phages and their genes potential sources of mitigation technologies.

There have been nearly 300 phage genomes reported, but only six archaeal phages have been sequenced and described, only two of which are from methanogens, *Methanobacterium* and *Methanothermobacter*. More archaeal phages have been reported and examined by electron microscopy and *in vitro* techniques but there is little information on their genetic blueprint and gene functionality.

Non-ribosomal peptide synthetases (NRPS) and phosphopantetheinyl transferase (PPTase) are proteins manufactured by microbial cells that produce a wide variety of small and unusual natural peptides with industrial applications, such as antibiotics.



Six different species of New Zealand rumen methanogens. Photos © Caroline Kim and Peter Janssen.



## $\phi$ mr<sub>u</sub>: a prophage of the rumen methanogen *Methanobrevibacter ruminantium*

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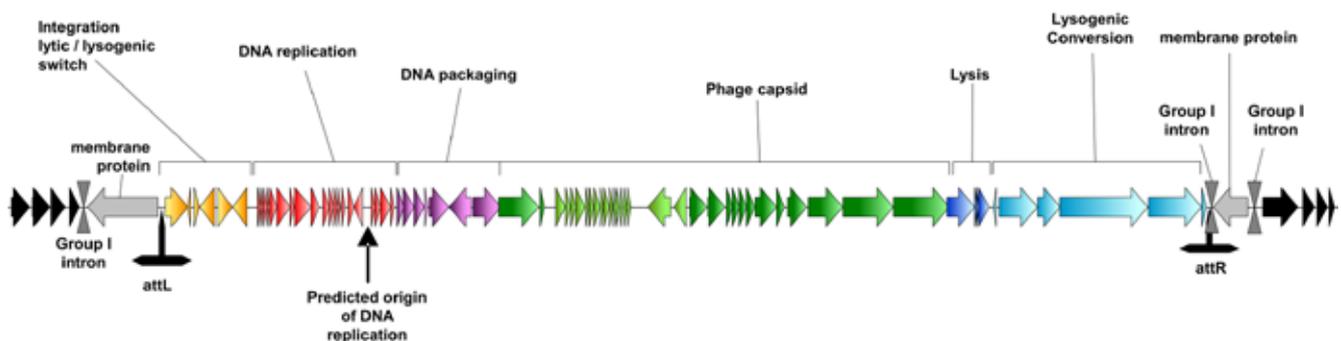
Sequencing of the first rumen methanogen genome *Methanobrevibacter ruminantium* has improved understanding of methanogen phage. Further whole genome sequencing of other rumen methanogen strains is expected to reveal the presence of embedded prophages and phage-like elements which would normally remain unnoticed using traditional *in vitro* techniques. Prophages are viruses that are present in bacteria without causing disruption to the cell. Their genomes are integrated into the host's chromosome and remain dormant until a specific activation signal—often a response of the host to an external stress factor—is detected. Once induced (“activated”) they multiply in the cell using the cells own reproductive machinery and subsequently lyse the host cell to find new, susceptible cells to continue the replication cycle.

Phages are specific for host species or even strains, and this specificity could be used to target individual methanogen groups without affecting other non-targeted microbes. However, phage and their respective hosts are involved in what could be described as an evolutionary “arms race” as the host changes to avoid infection and the phage changes to maintain infectivity. In combination with the application of other phage enzymes and structural components, a rotation system can be envisaged which might overcome the rapid adaptation mechanisms to phage challenges. More methanogen phages need to be identified, sequenced and characterised to capture the potential of phage-based technologies effectively. The specificity of phages may however limit their effectiveness against the wide diversity of methanogens in the rumen.

In this study the prophage  $\phi$ mr<sub>u</sub> was found to be divided into two sections (Figure 1), the first containing modules that give instructions for integrating into the host chromosome, DNA replication, DNA packaging, phage structure (phage capsid and tail) and lysis; and a second smaller section that harbours genetic information originating from an earlier host microbe DNA (lysogenic conversion) indicating that previous horizontal gene transfer as mentioned above has occurred. Computer-based (*in silico*) analysis of the second region suggests the lysogenic conversion region may be part of a system mediating changes to the DNA by adding sulphur to its backbone. The typical sequence and orientation of genes commonly found in phage of a similar type to  $\phi$ mr<sub>u</sub> may be disrupted by a number of antisense genes, which initially pointed to phage domestication by the host. However further analysis showed that  $\phi$ mr<sub>u</sub> DNA was present in relatively greater abundance than chromosomal DNA which suggested the  $\phi$ mr<sub>u</sub> prophage can be activated and replicate within its host cell. Furthermore, phage-like particles in extracts from *M. ruminantium* cultures suggest  $\phi$ mr<sub>u</sub> is able to produce viral particles and lyse its host (Figure 2).

### PROOF OF PHAGE-LIKE PARTICLES IN GROWTH SUPERNATANT

If  $\phi$ mr<sub>u</sub> is functional rather than just a piece of DNA, particles containing packaged DNA should be present in the supernatant of *M. ruminantium* cultures, and be detectable via PCR (polymerase chain reaction technique). Primers, short pieces of DNA, specific to *M. ruminantium* and  $\phi$ mr<sub>u</sub> were designed and used



**Figure 1.** Gene model of  $\phi$ mr<sub>u</sub>. The phage genome is divided into two subsections consisting of the bacteriophage genes (integration, replication, packaging, capsid and lysis genes) and a lysogenic conversion module.

in PCR amplifications. This technique allows the DNA to be amplified and analyzed to determine whether it's from the phage or the host. The *M. ruminantium* specific primer sets did not produce amplified DNA in the culture supernatant, whereas the phage-specific primer sets did, supporting the existence of  $\phi$ mr<sub>u</sub> phage-like particles in the supernatant.

In the experiment, free DNA and RNA in the supernatant was degraded using special enzymes (DNase and RNase). Only DNA that is protected from those enzymes (e.g. within a virus particle) remains intact. Absence of *M. ruminantium* amplicons of genomic regions indicated that no free DNA remains in the sample preparation, possibly leading to false positive results. The presence of  $\phi$ mr<sub>u</sub> specific amplicons (the viral particles were degraded after the DNase treatment to liberate packaged DNA) indicates that viral particles were present capable of protecting the DNA. However, that does not yet prove if  $\phi$ mr<sub>u</sub> is indeed fully functional, as we would need to develop/find a sensitive new host strain to re-infect.

### PHAGE-LIKE PARTICLES

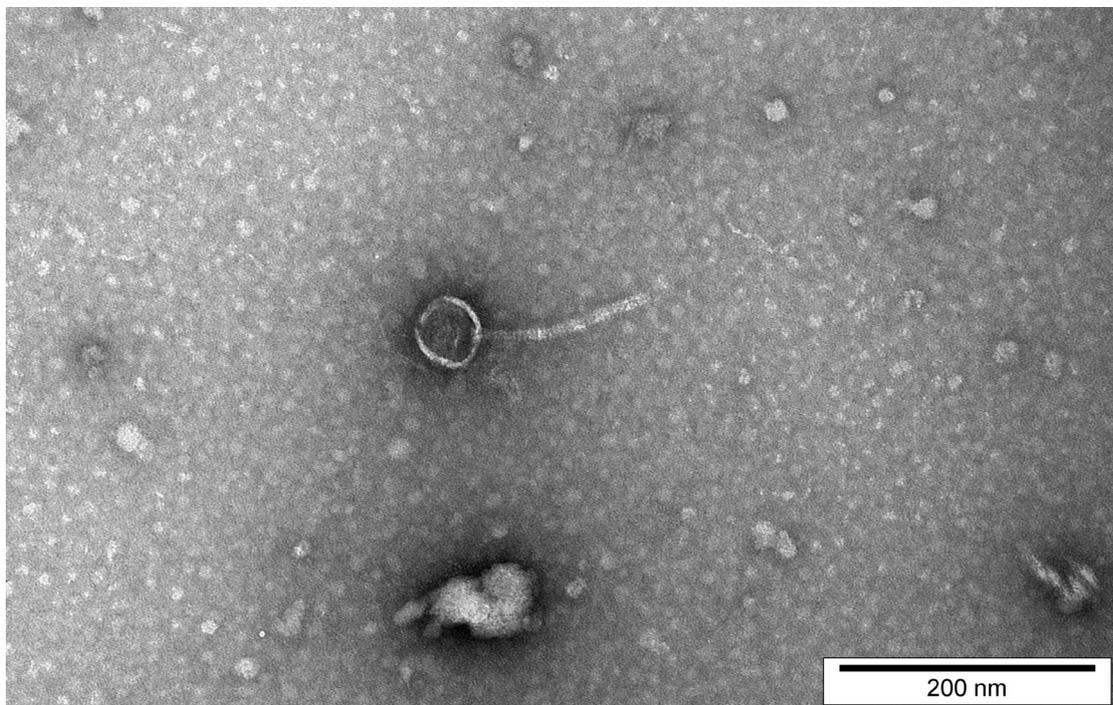
The phage-like particles were isolated and produced protein band patterns similar to those observed in other phage. Transmission electron microscopy, also

confirmed their presence in *M. ruminantium* culture supernatants (Figure 2). However, the phage-like structures showed a surprising lack of fine structure and had a large amount of variability in morphology. Further analysis is proceeding to elucidate these observations.

### APPLICATION OF PHAGE TO INHIBIT METHANOGENS

Phage-like particles present in the supernatant of *M. ruminantium* M1 cultures strongly indicate that  $\phi$ mr<sub>u</sub> is able to replicate and lyse its host. To utilise the ability of  $\phi$ mr<sub>u</sub> to destabilise M1 cell walls we are investigating a number of approaches including:

- Determining the factors that are needed to initiate the phage to replicate and cause collapse of the sensitive population.
- Investigating a variation to the traditional phage therapy strategy using just the tail protein genes of  $\phi$ mr<sub>u</sub>. Phage tails can bind onto host cells and tail proteins may therefore be a useful tool for damaging methanogen cells or facilitating the uptake of inhibitors.
- Applying the lytic enzymes of the phage via other mechanisms to lyse the cell from outside the methanogen.



**Figure 2.** Electron microscope picture of phage-like structures showing possible head and tail structures and variability in tail length.

### References

- Altermann *et al.* 2011. Initial characterisation of the novel *Methanobrevibacter ruminantium* virus  $\phi$ mr<sub>u</sub>.  
 Lambie *et al.* 2011. Methanogen phage tail protein synthesis.  
 Lambie *et al.* 2009.  $\phi$ mr<sub>u</sub>: a prophage of the rumen methanogen *Methanobrevibacter ruminantium*.



## Non ribosomal peptide synthetases (NRPS) from rumen methanogen isolates

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### INTRODUCTION

Genomic analysis of the rumen methanogens *Methanobrevibacter ruminantium* M1 and *Methanobrevibacter* sp. SM9 revealed the first NRPS and PPTase gene sequences identified in archaea. NRPSs have been characterised in bacteria and fungi but they have not previously been reported for archaea.

The products of the methanogen NRPS are not known. The identified roles of NRPS proteins for their respective microbial/fungal producers have been activities like (e.g., siderophores for iron uptake by the cell, cell-cell communication, defense, etc.), which may provide an insight to the possible applications in the rumen. The M1 and SM9 NRPSs each have one gene similar to a gene from *Syntrophomonas wolfei*, an anaerobic bacterium that oxidises fatty acids to acetate and propionate. These genes are predicted to code for NRPS proteins that produce dipeptides (i.e., two linked amino acids) as their initial peptide product. M1 and SM9 also each contain a second and unique NRPS gene, and computer based (*in silico*) analyses predict that these NRPSs would initially produce a tetrapeptide or dipeptide, for M1 and SM9 respectively. This work investigated the ability to synthesise these peptides for further research.

### METHODOLOGY

All four of the NRPS genes and the PPTase genes from M1 and SM9 were cloned and gene products were expressed in *E. coli*. The expressed proteins were purified and analysed using gel electrophoresis.

### RESULTS AND DISCUSSION

The six proteins were confirmed as being the expected NRPS's and PPTases from M1 and SM9. The next step in this work involves structural characterisation, elucidation of their function in the rumen, and investigation of the potential use of the proteins and their products to manipulate rumen function.

Those genes are much longer than the average microbial gene. Their presence in an intact form (not inactivated or removed from the chromosome) implies an important function for those methanogens, as their incorporation in the genome represents a significant energy burden on the cell. The burden comes from the energy required to maintain these genes in the chromosome and, when induced, to produce those very long proteins.

### Future work

The NRPS work described above offers potential to assist in the development of technologies for rumen manipulation and CH<sub>4</sub> mitigation. However, research is still in the early stages of understanding and requires considerable resources to advance to a stage where its applicability could be reliably assessed. Given the need to focus on development of practical technologies within a constrained timeframe, the decision has been made not to proceed with this line of research within the PGGRC methane mitigation programme in the immediate future. However the Consortium will look to collaborate with other research groups, making available the knowledge already developed to enhance this understanding and advance its application.

# INHIBITOR DISCOVERY

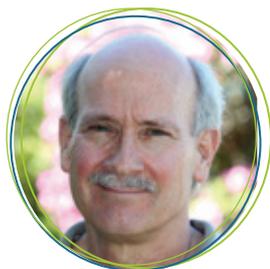
The Consortium has long recognised the role that inhibitors could have in mitigating methane in intensive farming systems where daily dosing and delivery of inhibitors in feed or water supply may offer opportunity. Their formulation into a bolus or some other slow release form of delivery may also extend inhibitor applicability to more extensive farming systems. The deep investment into understanding rumen methanogen genomics undertaken by the PGgRc has presented an opportunity to take a chemogenomic approach in this field and ensure that we target high impact solutions for methanogens whilst avoiding impacts on fibre-degrading microbes in the rumen. This section contains a number of selected papers identifying some of the key aspects of the inhibitor discovery programme and the process that we have developed for identifying lead targets and compounds.

## BACKGROUND

Anti-methanogen agents or “inhibitors” offer significant potential to reduce methane (CH<sub>4</sub>) production in the rumen. A wide variety of potential inhibitors has been researched by many parties internationally including chloroform, fats, oils, plant extracts and small-molecule compounds. These compounds can impact methanogen activity in the rumen, leading to marked reductions in methanogen numbers, increased H<sub>2</sub> concentrations and reductions in (and in some cases almost total elimination of) CH<sub>4</sub> production. However, inhibitor use has animal welfare and regulatory implications. In addition, past inhibitor effects in some cases have not been long lasting and the necessary frequency of delivery to the animal has been impractical, furthermore there have been observed small negative effects on animal production through reduced feed digestion or changes in rumen fermentation balance. Along with this will be the need to future proof the approach by having a number of targets and inhibitors developed to ensure the strategy can endure.

Chemogenomics is the systematic screening of small-molecule compounds for activity against individual biological targets. Small-molecule inhibitors are low molecular weight (<500 Daltons) organic compounds that can regulate biological processes by binding to biopolymers (large molecular compounds) such as proteins or nucleic acids, and alter their activity or function; they can rapidly diffuse across cell membranes. Chemogenomic targets are typically enzymes that control essential functions of the target organism that can be inhibited through small-molecule inhibitors. Knowledge of the genome of *M. ruminantium* has allowed identification of potential targets involved in energy metabolism, many of them within the methanogenesis pathway or cofactor pathways that support methanogenesis.

Modelling of enzymes is a useful tool for designing and identifying inhibitors. Structural information can be used to design compounds that specifically bind at the catalytic site of an enzyme to inhibit the function of that enzyme. The amino acid sequence information of an enzyme target can be used to predict the shape (or conformation) of the catalytic site on the enzyme to which the inhibitor must bind. Crystal structures of already-published characterised proteins can act as templates for modelling the targets. A better approach is to use protein expression and crystallisation to make the enzyme and then define the catalytic site precisely by solving the enzyme structure. If a suitable bioassay exists the expressed enzyme can also be used in high throughput screens of libraries of chemical compounds to find suitable inhibitors.



## Utilising chemogenomics techniques to rapidly find novel rumen methanogen control agents

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### INTRODUCTION

This programme aims to develop novel, non-toxic and environmentally friendly inhibitors of rumen methanogens. These inhibitors will be small-molecule compounds and the approach used involves elements of genomics, biochemistry and structural biology. It involves cloning genes of rumen methanogens for expression in *Escherichia coli* and obtaining purified enzymes that can be used for developing high through-put compatible enzyme assays and determining new structures; and modelling existing enzyme structures. Crystals, structures and models have been obtained representing a number of metabolic pathways including methanogenesis, energy generation, and coenzyme synthesis.

### METHODOLOGY

#### Selection of targets

*M. ruminantium* genome analysis revealed a number of enzymes with archaeal and methanogen-specific features, which were involved in CH<sub>4</sub> production, energy metabolism and protein, lipid, cofactor, and cell wall synthesis. Sixty three targets were prioritised based on how essential each is to methanogen function; number of subunits; size; effects of oxygen on assays; presence of existing inhibitor data; whether there is already a published assay; and the presence of published structures.

#### Cloning in E.coli

Genes were amplified (multiplied) and cloned into plasmids which were used to genetically modify *E. coli* cells. Plasmids are small DNA molecules designed for protein expression in cells. The plasmid is used to introduce a specific gene into a target cell, and can commandeer the cell's mechanism for protein synthesis to produce the protein encoded by the gene.

#### Expression of recombinant protein

*E. coli* cells were grown and the enzymes were extracted and purified using nickel-affinity chromatography.

#### Assay development

A number of assays have been developed to assist the research program. For example, an assay has been developed for ArfA (GTP cyclohydrolase III) which is involved in the coenzyme F420 synthesis pathway and MptA (GTP cyclohydrolase IV) which is involved in the methanopterin biosynthesis pathway.

#### Biochemical characterisation

Researchers have worked on characterising the biochemical processes enzymes take part in. For example, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) oxidises 3-hydroxy-3-methylglutaryl-CoA to mevalonate which is crucial for production of archaeal membrane precursors. Enzyme kinetics ( $K_m$ ,  $k_{cat}$ ) for HMGR were defined. Drugs known as statins have been shown to inhibit growth of methanogens, and simvastatin and lovastatin were found to inhibit HMGR.

#### High throughput screening for inhibitors

High throughput screening is used to screen target compounds for activity. For example, purified ComC (Coenzyme C, which has a role in the methanogenesis pathway) from *Methanobrevibacter* sp. SM9 was used in a high throughput assay to screen 1280 compounds from the Library of Pharmacologically Active Compounds (LOPAC). Five compounds were found to be inhibitory at 20  $\mu$ M and three of these were validated in standard assays, with one showing 50% inhibition at 1  $\mu$ M.

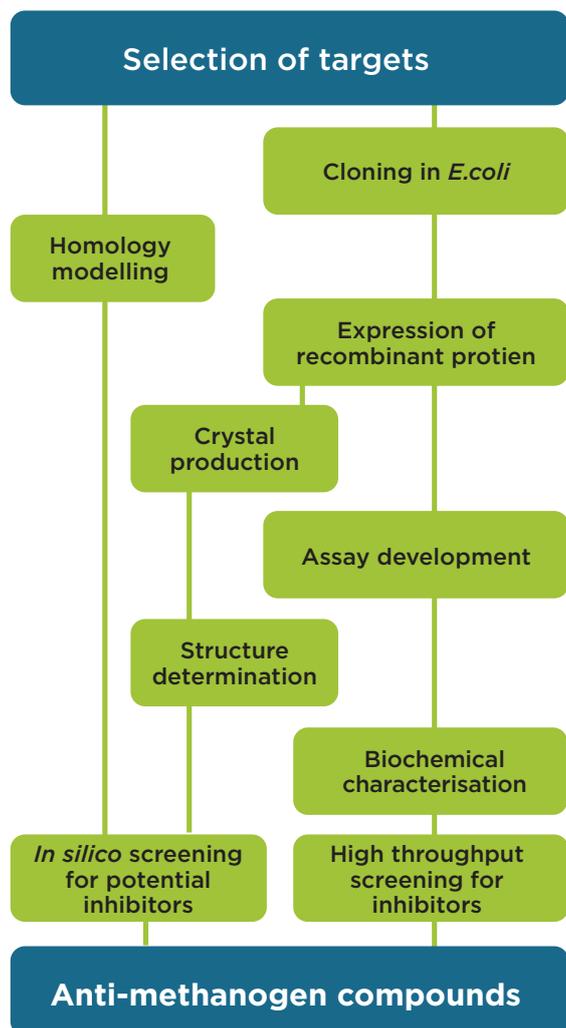
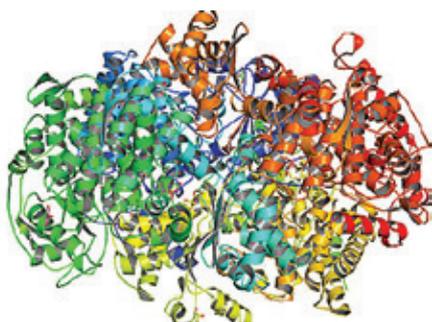


Figure 1. Inhibitor discovery approach.



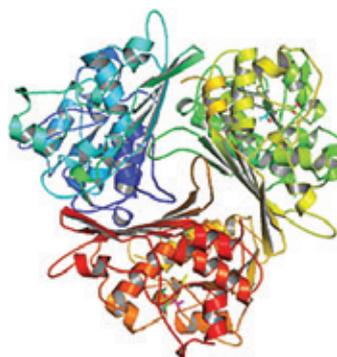
**Figure 2.** Structure of the enzyme Mcr.

#### Homology modelling

Computer based homology modelling has been used to determine the structures of key enzymes based on the consideration of evolutionary links. For example, the structure of Methyl-coenzyme M reductase (Mcr) from *Methanothermobacter marburgensis*, which is essential for the production of CH<sub>4</sub> in the final step of the methanogenesis process, was modelled *in silico* (Figure 2).

#### *In silico* screening for potential inhibitors

Results from homology modelling and from knowledge of enzyme structure determined from commercial libraries and in-house results have led to screening of compounds against four *M. ruminantium* models. For example, the structures of 42 compounds were checked for inhibition of Mcr, with eight being significantly inhibitory (at 1 mM) in pure culture and several being completely inhibitory at lower (25-100 μM) concentrations (Figure 4). One compound targeting Mcr gave complete inhibition at 0.5 mM in a rumen fluid trial.



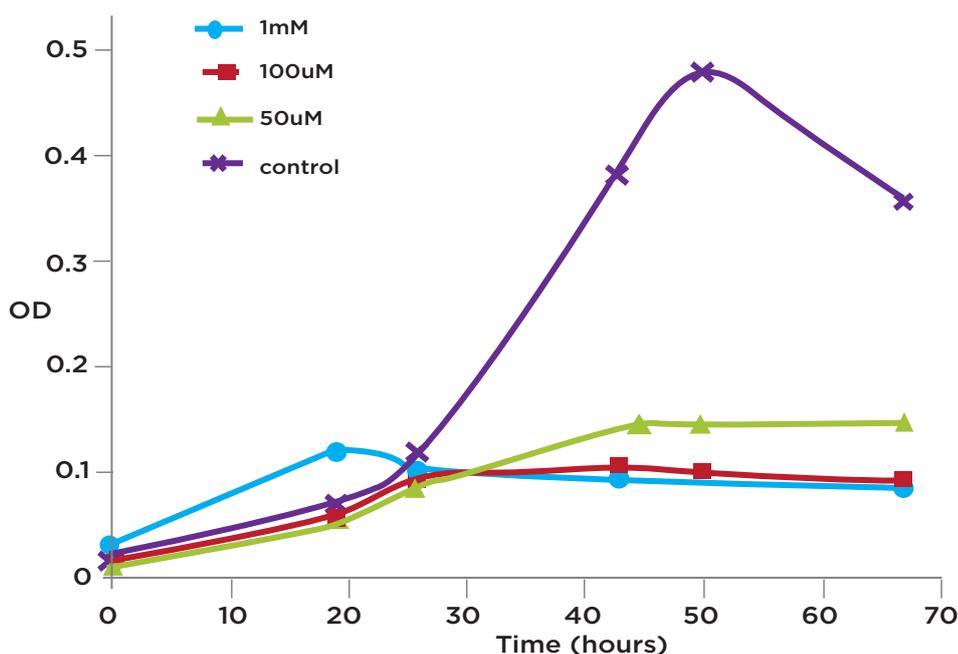
**Figure 3.** Structure of Mch.

#### Crystal structure and structure determination

Twenty nine enzymes have been screened so far and seven were crystallised and their crystal structures determined, e.g. Methenyltetrahydromethanopterin cyclohydrolase (Mch) which is involved in the methanogenesis pathway (Figure 3).

#### Anti-methanogen compounds

Consideration of high-throughput and *in silico* screening results has led to identification of small-molecule inhibitors for further testing.



**Figure 4.** Targeted inhibition of the methanogen enzyme Mcr in a pure methanogen culture by a candidate inhibitor at four concentrations.



## The crystal structures of Mch and GTP cyclohydrolase from *Methanobrevibacter ruminantium*

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### INTRODUCTION

Mch (Methenyltetrahydromethanopterin cyclohydrase) and GTP cyclohydrolase (guanosine triphosphate cyclohydrolase) are important enzymes involved in the process of methanogenesis in the rumen.

Mch is involved in the conversion of N<sup>5</sup>-formyl-5,6,7,8-tetrahydromethanopterin to methenyltetrahydromethanopterin and GTP (guanosine triphosphate) is a common biosynthetic sorting point for a large array of purine-based molecules. GTP transformations are carried out by four structurally different GTP cyclohydrolases (GCH) within different biosynthetic pathways. GCH I is involved in folic acid synthesis; GCH II in synthesis of flavo-coenzymes; GCH III is involved in GTP conversion and in riboflavin and coenzyme synthesis, is unique to Archaea, requires Mg<sup>2+</sup> for catalytic activity and is activated by monovalent cations e.g. K<sup>+</sup>; GCH IV catalyses GTP conversion in the pathway for pterin synthesis in archaea.

The structures of Mch and GCH III and their modes of docking with substrates were determined.

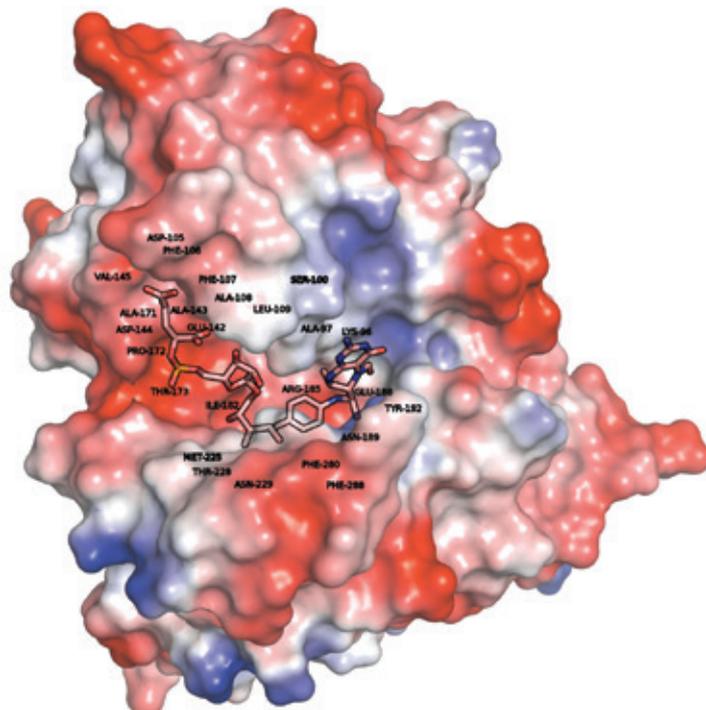
### METHODOLOGY

Mch and GCH III proteins from *M. ruminantium* were expressed and purified from *E. coli* cells. The proteins were crystallised, diffraction data collected using micro crystallography and crystal structures were determined. A model of substrate binding was produced using a molecular docking software programme.

### RESULTS AND DISCUSSION

The three Mch molecules (see page 48, Figure 3) are asymmetric trimers or molecules formed by combining three identical smaller molecules, with multiple salt bridges to amino acids. This network of contacts stabilises the formation of the trimer, limiting the exposure of hydrophobic patches to the cytoplasm. It is believed that the catalytic function of Mch is optimal when it forms this stable structure. Comparison of sequences across several methanogen

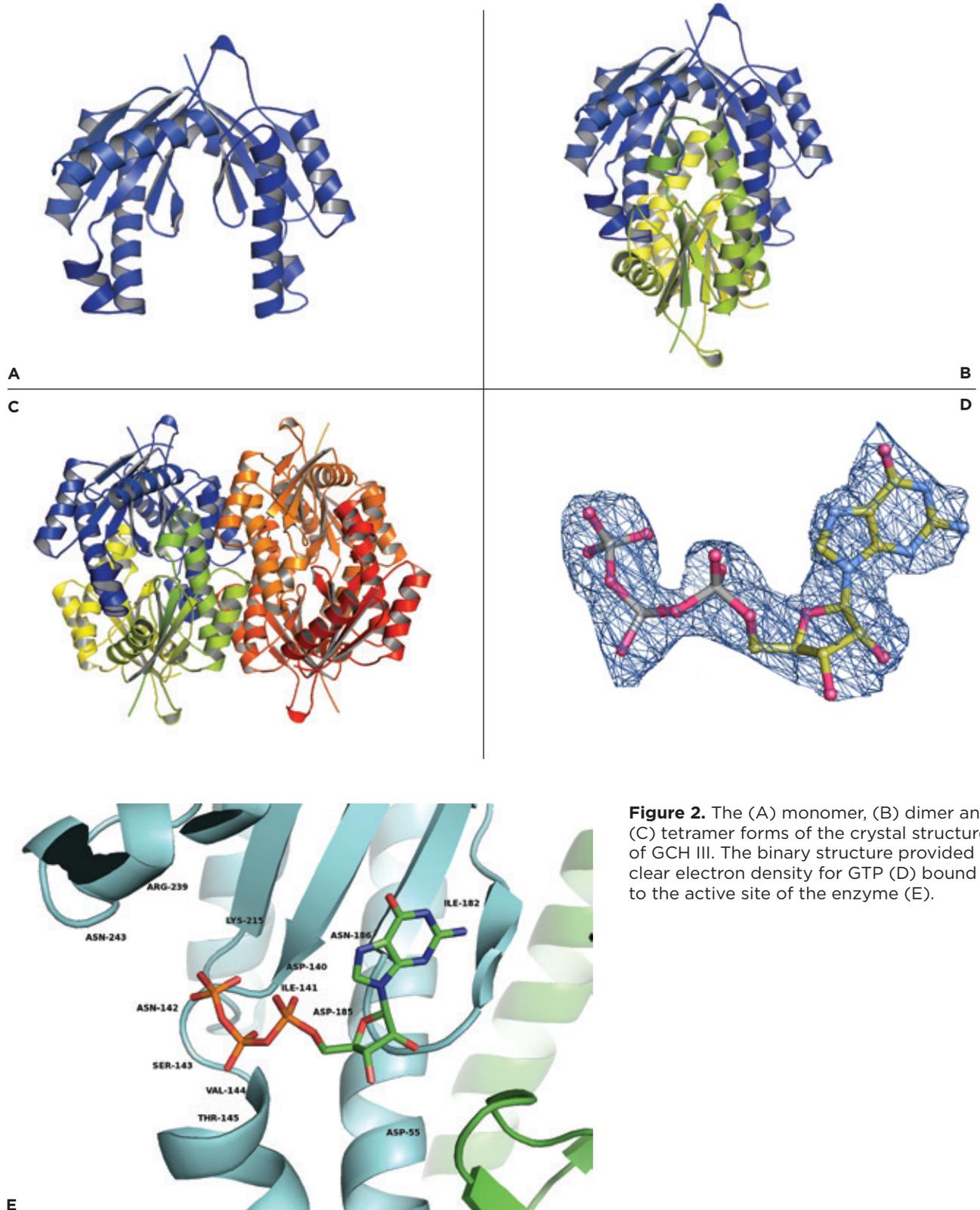
species suggests that this structure is common in methanogens. Three specific amino acid residues (lysine, arginine and glutamine) are common to several methanogens and may be part of a “catalytic triad” for the Mch hydrolysis reaction. Computer modelling using a model substrate suggested the triad would be located within a deep cavity and expanded the range of possible active sites to include several other amino acid residues (Figure 1). Online analysis of these additional residues found that most of them are close to or 100% common across methanogens, confirming their possible generic role as active sites within the enzyme.



**Figure 1.** The Mch monomer with the docked substrate molecule.

GCH III is made up of four identical subunits or a tetramer (Figure 2). It has monomer (Figure 2A), dimer (Figure 2B) and tetrameric forms (Figure 2C). Crystals of GCH III displayed clear evidence of GTP binding in all four subunits of the tetramer (Figure 2D).

The analysis revealed the possibility of metal binding sites and specific interactions with residues contributed by both protein chains of the dimer.



**Figure 2.** The (A) monomer, (B) dimer and (C) tetramer forms of the crystal structure of GCH III. The binary structure provided clear electron density for GTP (D) bound to the active site of the enzyme (E).



## Expression, purification and characterisation of L-sulfolactate dehydrogenase (ComC) from *Methanobrevibacter* species SM9

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### INTRODUCTION

ComC (L-sulfolactate dehydrogenase) is an enzyme that is involved in coenzyme M synthesis, an essential part of the methanogenesis pathway. It catalyses the conversion of 2-hydroxy acids into their corresponding 2-oxyacids; disruption of ComC synthesis is a potential target for small-molecule inhibitors intended to reduce methanogenesis in the rumen.

### METHODOLOGY

The ComC gene from *Methanobrevibacter* species SM9 was amplified and cloned into an expression vector. This plasmid was used to transform *E. coli* cells and ComC was purified from cell cultures using nickel affinity chromatography. Conditions that maximised ComC activity were defined and activity was measured using an enzyme assay, involving spectrophotometric measurement of NADH consumption at 37°C.

Inhibition by 25 compounds at 1 mM concentration was investigated.

### RESULTS AND DISCUSSION

The optimum conditions for ComC activity were defined and seven compounds were tested as ComC substrates. The activity of ComC was affected by potassium chloride (KCl) concentration. ComC has very low activity in the absence of KCl, and which increases as the concentration of KCl increases, reaching an optimum at 100 mM KCl.

No inhibition was measured when each of the 25 chosen potential inhibitors was added directly to the enzyme assay. However inhibition did occur for seven inhibitors when they were pre-incubated with ComC and NADH at room temperature overnight.

Screening of compounds to identify potent inhibitors that are non-toxic to animals and have very little environmental effect is continuing. Progress in this search is occurring; however the work will need to be extended to check inhibitors against methanogen cultures and in animals before inhibitors for use on-farm are developed.



Figure 1. Structural model of ComC



## Evaluating $A_1A_0$ -ATP synthetase of *Methanobrevibacter ruminantium* as a target for small compound inhibitors

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### INTRODUCTION

Adenosine triphosphate (ATP) is used within cells as a coenzyme and transports chemical energy for metabolism. Microorganisms contain a number of membrane-bound enzymes (ATP synthases) that are used to synthesise ATP via a proton ( $H^+$ ) or sodium ( $Na^+$ ) ion gradient. Methanogens produce  $CH_4$  from a limited group of substrates including  $H_2$  and  $CO_2$ , formate (HCOOH) and alcohols, by a pathway involving unique coenzymes. Methanogens produce both  $H^+$  or  $Na^+$  gradients during methanogenesis, and these gradients are used to synthesis ATP by a mechanism involving a membrane-bound  $A_1A_0$ -ATP synthetase. It is not clear however how this occurs. The sequencing of the *Methanobrevibacter ruminantium* M1 genome provided a molecular platform to investigate the mechanisms involved.

### METHODOLOGY

To study the role of different electrochemical gradients in the growth of M1, cells were cultured and the effects of inhibitors on M1 growth were tested.

To study the role of  $H^+$  and  $Na^+$  gradients in membrane bioenergetics,  $A_1A_0$ -ATP synthetase from M1 was purified and characterised. M1 cells were disrupted, membrane vesicle preparations were derived and effects of inhibitors on ATPase activity were assayed.

The M1 genome harbours nine genes in a functioning unit of DNA (operon) that encodes  $A_1A_0$ -ATP synthetase. The operon was cloned into an expression plasmid which was used to induce ATP synthase production in *E. coli*. M1 ATP synthetase was extracted from *E. coli* membranes, purified and concentrated. The properties of the  $A_1A_0$ -ATP synthetase were assayed by measuring the liberation of inorganic phosphorus (Pi) from ATP hydrolysis.

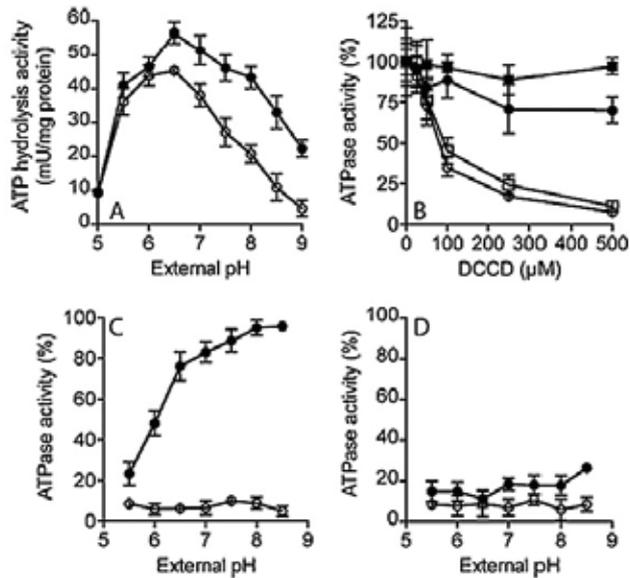
### RESULTS AND DISCUSSION

M1 growth was inhibited by the protonophores to varying degrees. Protonophores are lipid-soluble molecules that transport protons across lipid bilayers. Monensin, which breaks down  $Na^+$  ion gradients, was a potent M1 growth inhibitor. Amiloride and EIPA (ethyl isopropyl amiloride) are blockers of sodium channels and of antiporter activity and both inhibited M1 growth. Antiporters are proteins involved in transporting  $Na^+$  and  $H^+$  across membranes in opposite directions. The ATP synthase inhibitors DCCD (dicyclohexylcarbodiimide) and TBT-Cl (tributyltin chloride) also partially or fully arrested M1 growth. These results confirmed that M1 requires both  $H^+$  and  $Na^+$  gradients to grow and that classical ATP synthase inhibitors slow growth of M1.

The ATPase activity of M1 membranes was sensitive to TBT-Cl and amiloride but not to DCCD.

The optimum pH range for ATP hydrolysis activity of the purified M1 ATP synthase was broad (Figure 1), with highest levels of activity between pH 5.5 and 9.0. Activity was stimulated by  $Na^+$ . DCCD inhibition could be prevented by addition of  $Na^+$ , but this was pH dependent and protection greatest at pH 8.5.

ATP synthesis in *E. coli* membrane vesicles expressing M1 ATP synthase was induced by potassium ion gradients ( $K^+$ ) diffusion potential, but was eliminated by preincubation with TBT-Cl, DCCD or CCCP. However monensin had no effect on ATP synthesis under these conditions indicating ATP synthesis was not dependent on a  $Na^+$  gradient. Membrane vesicles did not synthesise ATP when a  $Na^+$  gradient was created; and if a  $K^+$  gradient was established in addition ATP was synthesised and this was inhibited by monensin, TBT-Cl or DCCD, but not by CCCP.



**Figure 1.** Effect of pH, NaCl and DCCD on A1A0-ATP synthetase activity.

A. Effect of pH with (closed circles) or without (open circles) NaCl.

B. Effect of DCCD at pH 6 (open circles) and 8.8 (open squares) with (open symbols) and without (closed symbols) NaCl.

C. Effect of pH on DCCD inhibition with (closed circles) or without (open circles) NaCl

D. Effect of pH on TBT-Cl inhibition with (closed circles) or without (open circles) NaCl.

Analysis of the operon structure suggested that there were two Na<sup>+</sup> binding sites within duplicated subunits which both had two fused helical hairpins. Modelling of inhibitor docking with A<sub>1</sub>A<sub>0</sub>-ATP synthetase suggested that both EIPA and amiloride should be effective inhibitors due to the promotion of hydrogen bond interactions, and that EIPA should be the most effective due to its nonpolar-hydrophobic components.

Recent work suggests M1 grows by using H<sub>2</sub> to reduce CO<sub>2</sub> to CH<sub>4</sub> and couples this metabolism to the generation of electrochemical gradients using H<sup>+</sup> and Na<sup>+</sup> pumps. The sensitivity of M1 to uncouplers and sodium ionophores shows both gradients are formed during, and are essential for growth. M1 also contains genes for a Na<sup>+</sup>/H<sup>+</sup> antiporter, providing the ability to interconvert and H<sup>+</sup> gradients. Methanogens are the only microorganisms reported to produce two primary (i.e. using chemical energy) ion gradients. How this chemical energy is used to synthesise ATP is as yet not known. *Methanobrevibacter ruminantium* M1 grows in the rumen over a pH range of 6.5 to 7.7 at high concentrations of Na<sup>+</sup>. This suggests Na<sup>+</sup> energetics would be favoured over proton-coupled processes at higher pH.

## Future work

Screen potential inhibitors in rumen *in vitro* tests, and assess their degree of toxicity if any.

Develop hits into lead inhibitors using chemical refinement and synthesis.

Prove ability of leading inhibitor candidates to reduce CH<sub>4</sub> in animal trials and produce data as a platform for commercial development of on-farm mitigation technologies.

Maintain the chemogenomics pipeline to produce further potential inhibitors: develop assays for “new” high-priority targets identified in comparative genomics and other work streams, and continue discovery of novel inhibitors by *in vitro* and *in silico* screening.

## References

Expression, purification, crystallisation, X-ray structure determination and *in silico* modelling of *Methanobrevibacter ruminantium* enzymes for the discovery of novel inhibitors.

Crystallisation, X-ray structure determination, and *in silico* modelling of *Methanobrevibacter ruminantium* enzymes for the discovery of novel inhibitors.

Mitigating ruminant methane emissions: searching for specific inhibitors of essential enzymes from rumen methanogens.

Carbone V, Schofield LR, Beattie AK, Sutherland-Smith AJ, Ronimus RS. The crystal structure of methenyltetrahydromethanopterin cyclohydrolase from *Methanobrevibacter ruminantium* Proteins: Structure, Function, and Bioinformatics 81 (11), 2064-2070.

# VACCINES AND IMMUNOLOGY

## BACKGROUND

Vaccination against rumen methanogens could reduce CH<sub>4</sub> emissions by reducing the number or activity of methanogens in the rumen of grazing animals. The major advantages of this approach to reducing GHG is that vaccination is cheap and simple to carry out, and would only have to be done infrequently. It would be particularly valuable for use in extensive grazing situations where animals are handled relatively infrequently e.g. hill country sheep and beef farming. Vaccination is already used routinely for disease control in many farming systems, and so its use as a mitigation tool could be rapidly adopted on farm.

The approach would involve vaccinating animals to generate a substantial salivary antibody response that delivers a high yield of antibodies to the rumen. The volume of saliva generated daily by sheep is 6-16 litres or 1 to 3 times the volume of the rumen, and 100-160 litres for cattle or 1.5 to 2.5 times the volume of the rumen. The antibodies would be antagonistic to the methanogens which produce CH<sub>4</sub> using H<sub>2</sub> as a substrate, reducing their numbers and/or their activity and so CH<sub>4</sub> production would be reduced. The general approach has been shown to work in recently published research for a rumen-dwelling bacterium (*Streptococcus bovis*).

Antigens are the component of a vaccine that stimulates the animal to produce specific antibodies. Australian research has found that use of whole cells of methanogens could reduce CH<sub>4</sub> yield from sheep by up to 8%, but use of a vaccine containing five methanogens as antigens in New Zealand research has not been successful. The use of sub-cellular antigens is now the current focus. Two approaches are being pursued by the PGgRc research team to identify effective antigens.

The first approach is to use information from the sequencing of the genome of *Methanobrevibacter ruminantium*, to identify proteins that are located on the surface or in the membrane of the methanogen (see "The genome sequence of *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions", page 45, for further details). The reason for focusing on these proteins is the antibodies generated in response to a vaccine will need to bind to methanogens to reduce their activity, and so proteins located at the surface of methanogens are a logical choice as potential antigens. These proteins will be tested as potential

antigens for use in an anti-methanogen vaccine. Sheep are injected with the vaccine containing the antigens. The immune system produces antibodies in response to the proteins that are antigenic. Antisera, blood serum containing the antibodies, can be extracted from the blood of the sheep and tested for their ability to inhibit methanogen activity. Screening of the antigens from sequencing is normally carried out in the laboratory rather than in animals.

The second approach is to screen antisera that have been developed in sheep in response to injection of sub-cellular fractions extracted from *M. ruminantium* and test these for anti-methanogenic activity in the laboratory.

Research is also being carried out to identify suitable vaccine adjuvants; these are another component of vaccines, whose role is to facilitate a strong antibody response to the antigen in the animal when it is vaccinated.

Finally the optimum delivery method will need to be identified, most farm vaccinations are delivered by injection. Once antigens and adjuvants have been confirmed the research will establish the method or novel methods for vaccinating livestock to get the optimum effect delivered to the rumen





## Vaccination of sheep with methanogen fractions induces antibodies that block methane production *in vitro*

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### INTRODUCTION

In this work sheep were vaccinated with whole cells and several cell fractions from *M. ruminantium* to see if antibodies were developed in response to the vaccination, and then to see whether those antibodies affected growth and CH<sub>4</sub> production of *M. ruminantium*.

### METHODOLOGY

Whole cells and four sub-cellular fractions of *M. ruminantium* M1 were prepared from a laboratory culture of the organism, and each used to vaccinate four 9-11 month old sheep, which were then re-vaccinated ('boosted') after three weeks.

The sub-cellular fractions were:

1. From the cytoplasm (cytoplasm)
2. A cell wall fraction obtained after further processing of the cytoplasmic fraction (cell wall + trypsin)
3. A cell wall fraction obtained as for the previous fraction but excluding one enzyme degradation step (cell wall-trypsin)
4. A cell wall protein fraction obtained by further processing of the cell wall-trypsin fraction (cell wall proteins).

Antisera were collected from 'pre-immune' new-born lambs, from the sheep before vaccination, and then again from the sheep two weeks after re-vaccination. Saliva samples were also collected from the re-vaccinated sheep.

Antibody concentrations in the serum and saliva were measured using ELISA (Enzyme-Linked Immune Sorbent Assay). The effect of these antisera on growth of rumen methanogens and CH<sub>4</sub> production was measured in the laboratory. *Methanobrevibacter ruminantium* grown in the laboratory was added to clarified rumen fluid taken from pasture-hay fed rumen-fistulated cows. The antisera were each added to tubes containing rumen fluid and *M. ruminantium*, and growth rate of the cultures measured. At the end of the measurement period the presence or absence of clumping of cells (agglutination) was noted. Methane production was estimated by analysing CH<sub>4</sub> concentration in gas samples from the tubes.

### RESULTS AND DISCUSSION

All of the sub-cellular fractions contained a complex mixture of proteins with widely varying molecular sizes.

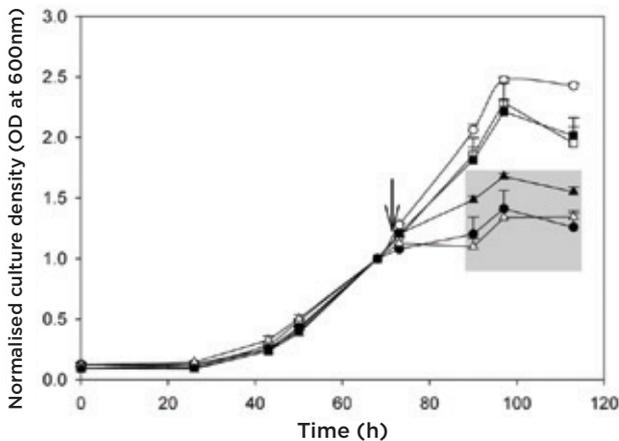
There were no antibodies to methanogens detected in the serum of new-born lambs. However there were significant levels of IgG (Immunoglobulin G) antibodies in the serum of the older sheep before vaccination and also in the saliva although only at low levels (Table 1). This demonstrates that natural exposure to methanogens leads to development of antibodies in young sheep. These methanogens would not have to be rumen-dwelling organisms, as they are also present in the lower digestive tract. Serum and saliva levels were elevated at the sampling after re-vaccination (Table 1).

**Table 1.** Mean IgG levels in serum and saliva of sheep vaccinated with five *M. ruminantium* M1 antigenic fractions, pre- and post-vaccination (results are expressed as titres measured using serial dilution).

	Serum		Saliva	
	Pre-vaccination	Post-vaccination	Pre-vaccination	Post-vaccination
Whole cells	250	4,200	1.0	6.5
Cytoplasm	200	4,000	1.3	2.3
Cell walls + trypsin	300	11,200	1.8	26.0
Cell walls - trypsin	450	5,600	2.3	13.0
Cell wall proteins	500	44,800	2.0	56.0

There were also low levels of IgA (Immunoglobulin A) antibodies in the saliva. The CH<sub>4</sub> mitigation technology being targeted in this work is suppression of rumen methanogens by antibodies delivered in the saliva and so salivary antibody level is critical. Previous work suggests the level of antibodies in the saliva (which are derived through movement from blood to saliva in the salivary gland) is strongly influenced by choice of adjuvant. Also, oral delivery, rather than through injection may lead to enhanced salivary levels.

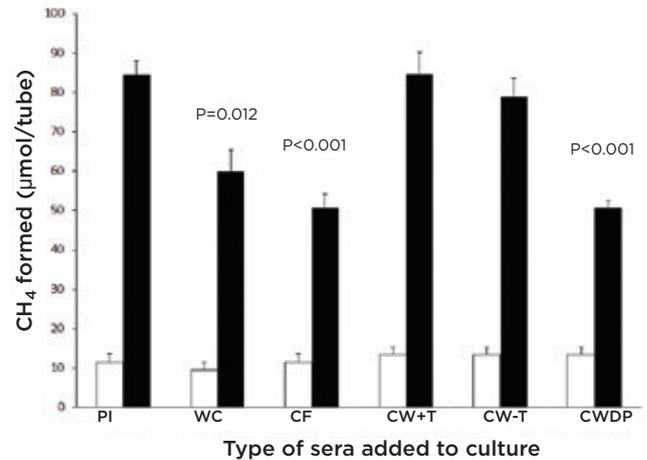
Growth of methanogens was lower when antisera from sheep vaccinated with the five antigenic fractions were added, as compared with growth when pre-immune sera was added (Figure 1).



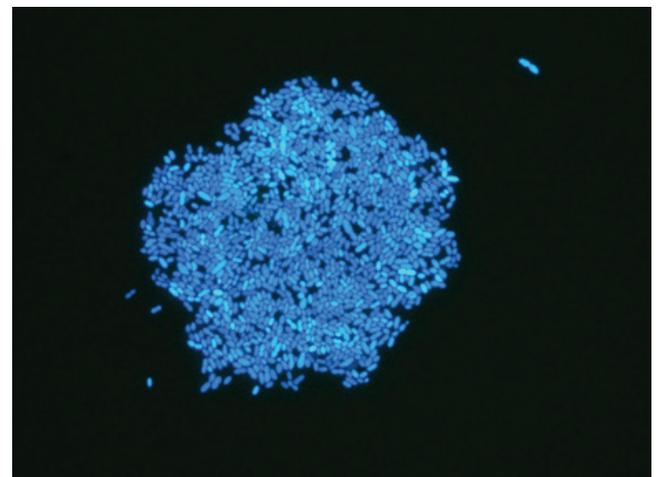
**Figure 1.** Growth of *M. ruminantium* M1 cultures (expressed as optical density) treated with antisera from sheep vaccinated with whole cells (closed circles), cytoplasm (open triangles), cell wall + trypsin (closed squares), cell wall - trypsin (open squares), and cell wall proteins (closed triangles) or with pre-immune sera (open circles). The arrow shows when sera were added, and the values in the shaded square are statistically less than the pre-immune values.

Antisera produced against whole cells, cytoplasmic fractions and cell wall proteins reduced CH<sub>4</sub> production (Figure 2) but there was no effect for the two cell wall fractions. Antisera for all the five antigenic fractions caused a high degree of clumping of methanogen cells in comparison with less clumping seen with pre-immune sera.

The clumping of methanogen cells included by all post-vaccination antisera (Figure 3) could in itself reduce CH<sub>4</sub> production. However not all of the antisera caused reductions in measured CH<sub>4</sub> production.



**Figure 2.** Methane production by *M. ruminantium* M1 cultures before (white bars) and after (black bars) addition of sera: PI = pre-immune, CW-T = cell wall - trypsin, CW+T = cell wall + trypsin, WC = whole cells, CF = cytoplasm, CWDP = cytoplasm proteins; statistical significance levels are relative to the effect of pre-immune sera.



**Figure 3.** Agglutination of *M. ruminantium* M1 cells by antisera produced against a cell wall fraction.

## Reference

Wedlock *et al.* 2010. Development of a vaccine to mitigate greenhouse gas emissions in agriculture: Vaccination of sheep with methanogen fractions induces antibodies that block methane production *in vitro*. *NZ Vet. J.* 58:29-36.



## Immunological tools to reduce greenhouse gas emissions

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### INTRODUCTION

Following on from the work above, specific proteins present in the cytoplasm and cell wall protein fractions used in those trials were identified. In addition antigens present in methanogens other than *M. ruminantium* M1 were investigated, because commercial vaccines will need to be effective against more than one organism. Also, potential adjuvants were tested to identify candidates that stimulate high antibody responses in sheep sera and saliva for inclusion in prototype vaccines

### METHODOLOGY

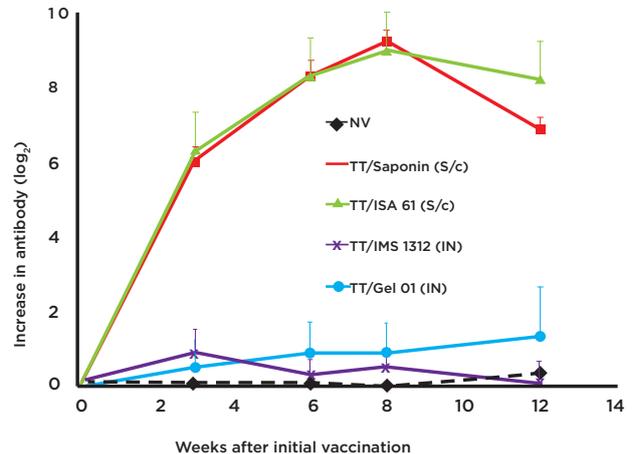
Proteins in the cytoplasm and cell wall protein fractions of *M. ruminantium* were separated by gel electrophoresis and 'blotted' with antisera, using the Western blot technique. Sub-cellular fractions were prepared from *M. ruminantium* M1 and also prepared from two other methanogens *Methanobrevibacter gottschalkii* SM9 and *Methanosarcina barkeri* CM1. Antisera were produced against these fractions (by the process described in the above work) and Western blotting and mass spectrometry was used to identify immunogenic proteins.

The ability to stimulate antibody responses in sheep serum and saliva was assessed for a range of potential adjuvants included in a model vaccine using tetanus toxoid (TT) as antigen. In two trials groups of sheep (5 or 6 animals per group) were vaccinated with variants of the model vaccine each containing one of the candidate adjuvants.

### RESULTS AND DISCUSSION

Western blotting together with mass spectrometry identified more than 40 highly immunogenic proteins and some of these proteins have key roles in methanogenesis. A small number of proteins were found to be 'cross-reactive' for both *M. ruminantium* M1 and *M. barkeri* CM1.

Two of the candidate adjuvants (saponin and ISA 61) stimulated strong serum (Figure 1) and moderate saliva antibody responses against TT.



**Figure 1.** Increases in anti-TT antibody levels in serum after vaccination with a model vaccine containing different adjuvants and the model antigen, TT.

### Future work

The ability of antisera to inhibit the growth and production of CH<sub>4</sub> of rumen methanogens, suggests antibodies against methanogens could reduce CH<sub>4</sub> production in the rumen. However, only some of the antisera produced in response to vaccination with the various cell fractions were effective.

In the future it is intended that defined protein antigens that induce the production of antibodies which limit methanogen growth and CH<sub>4</sub> production will be identified. Research described in the Methanogen genomics and Inhibitors discovery programmes will under-pin discovery of candidate proteins.

Adjuvants that stimulate optimal antibody levels in saliva will also be identified.

Prototype vaccines containing selected antigens and adjuvants will be tested in sheep, and effects on rumen microbial populations (i.e. not just methanogens) will be measured. CH<sub>4</sub> emissions will be measured in respiration chambers.

The best timing of vaccination in the animal's life will be investigated e.g. early in its life or when it is an adult?

Successful vaccines will be tested in cattle.

### Reference

Wedlock *et al.* 2010. Immunological tools to reduce greenhouse gas emissions. *Greenhouse Gases and Animal Agricultural Conference, Banff, Canada, 3-8th October, 2010.*

# LOW METHANE RUMEN

## BACKGROUND

Feed eaten by ruminants is fermented in the rumen by a complex microbial community. One product of this fermentation is hydrogen gas ( $H_2$ ) and this is in turn used by hydrogenotrophic organisms (i.e. organisms that convert hydrogen to other compounds as part of their metabolism) called methanogens, to reduce  $CO_2$  and produce  $CH_4$  and water. Methanogens are not bacteria, fungi or protozoa; they are classified as archaea.

Rumen-based technologies to reduce  $CH_4$  emissions fall into three broad categories: use of alternative electron acceptors (i.e. other than  $CH_4$ ); manipulation of rumen fermentation to reduce  $H_2$  production; and use of direct anti-methanogen agents.

Methanogens maintain rumen  $H_2$  at a low level, which allows fermentation of feed to proceed more rapidly. If removal of  $H_2$  from the rumen is decreased through mitigation practices that reduce methanogen activity, there could be a negative feedback on other aspects of rumen function and hence on feed digestion and animal production. Alternative hydrogenotrophic microbes that use  $H_2$  without producing  $CH_4$  could provide a means of maintaining  $H_2$  at a level that allows normal digestive function to occur. This alternative pathway for utilisation of  $H_2$  would also reduce feed energy losses associated with  $CH_4$  emissions and hence could potentially increase animal productivity. Rumen bacteria classified as homoacetogens present one interesting possibility; these utilise  $CO_2$  and an electron donor such as  $H_2$  to produce acetate rather than  $CH_4$ . Unfortunately homoacetogens cannot compete for  $H_2$  with methanogens at the low concentrations present in the rumen, as they require higher  $H_2$  concentrations to grow at the same rate as methanogens.

## WHY THIS IS IMPORTANT

Understanding what happens to rumen function when methanogens are inhibited or impacted is critical to our progress. The risk we are challenged with is that in targeting methanogens we also impact fibre degrading organisms and reduce digestive efficiency. The solution we seek to develop is one that reduces methane emissions and enhances the production of the ruminant.

The knowledge, tools and techniques developed in this programme will be used to determine what the true effects of methane mitigation are. Put simply the risk that we reduce the efficiency in which our ruminant animals convert hard to digest feed into meat milk and fibre is one that we can't ignore and it is through this study into the fundamental biology of a methane inhibited rumen that we will be able to ensure the right robust solution is found.





## Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle

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### INTRODUCTION

For direct anti-methanogen interventions in the rumen to be practically effective their effects need to last for a sustained period of time. For example bromoethanesulfonate (BES) is a potent inhibitor of methanogenesis and its activity in the rumen appears to be methanogen-specific. However methanogens adapt to its presence in as little as four days. It has been suggested that methanogens are less able to rapidly adapt to another potent inhibitor i.e. chloroform, which means it could be used as an experimental tool to study the influence of lowered rumen methanogen activity on rumen function over a more extended period.

In this research small daily doses of chloroform were administered to cattle to inhibit rumen methanogens, over a 42 day period. Methanogen populations and key rumen function indicators were monitored.

### METHODOLOGY

During February and March 2007 six non-lactating rumen-fistulated cows were systematically allocated to experimental groups of three cows each in such a way that average  $\text{CH}_4$  yields ( $\text{CH}_4/\text{kg}$  feed DM) per group were similar. This allocation was based on previously obtained  $\text{CH}_4$  emission values. During the trial the cows were managed as a single group in the same pen most of the time, but were housed in individual stalls for feeding. The cows were fed a lucerne silage:concentrate mix twice a day, and were dosed before the morning feed for 42 days with either sunflower oil, or sunflower oil containing chloroform.

Rumen fluid samples were collected through the rumen fistula two hours after the morning feeding, commencing one day before treatments started and ending seven days after they finished. Methanogen, acetogen and protozoa populations were estimated using a combination of DNA and microbiology methodologies and utilising phylogenetic and diversity analytical software. Volatile fatty acid (VFA) and ammonia concentrations in the rumen fluid were also measured.

Feed digestibility was estimated during days 18-22 of the treatment period using titanium oxide as an indigestible marker. The marker was administered twice daily as a capsule through the rumen fistula, and commenced five days before each intake run. Faecal samples collected twice daily were analysed for marker concentration; faecal output was calculated and when combined with feed intake measurements allowed calculation of feed digestibility.

At the conclusion of each digestibility run rumen contents were totally removed, weighed, mixed and sampled for dry matter (DM) contents, pH was measured, and fluid was collected for VFA analysis. Rumen fill was calculated as a proportion of cow liveweight, and rumen retention time was calculated based on estimates of rumen fill and feed intake measurements.

Methane emissions were estimated multiple times (two days before treatments started, six times during the treatment period, then daily for a week after treatments stopped) for each cow using the  $\text{SF}_6$  tracer technique. Using this technique a permeation tube with a known rate of  $\text{SF}_6$  release was administered down the throat to the rumen seven days before the study commenced. And at each measurement run samples of expired air were collected above the nostrils for 24 hours using collection yokes. The samples were analysed for  $\text{SF}_6$  and  $\text{CH}_4$  concentration and daily  $\text{CH}_4$  emissions were calculated.



Dairy cattle fitted with  $\text{SF}_6$  apparatus for Methane measurements

## RESULTS AND DISCUSSION

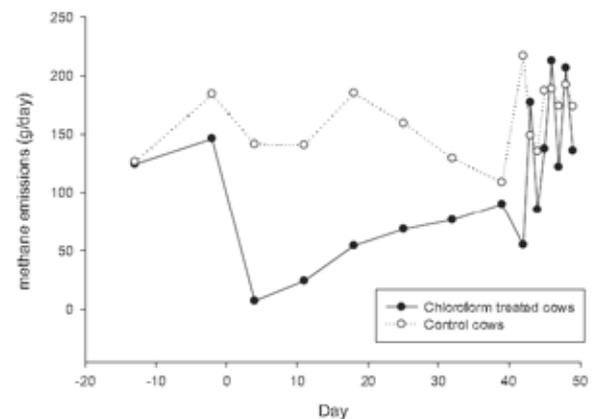
Rumen methanogen, acetogen and protozoa populations were similar for the two groups of cows before the treatments were started. The methanogen community was dominated by *Methanobrevibacter gottschalkii* and *M. ruminantium*. This remained the case with the control cows, but for the chloroform-treated cows there was an immediate and dramatic reduction in methanogens after chloroform administration, being most pronounced after one week. Methanogen numbers had recovered by day 28. However diversity of the *M. gottschalkii* and *M. ruminantium* groupings was reduced late in the treatment period suggesting some strains within the groups had adapted better to chloroform than others. The third dominant group of methanogens, named RCC (an uncultured group known as rumen cluster C) tended to increase during the chloroform treatment period. Acetogen numbers were reduced to about 1% of pre-treatment number throughout the treatment period so it appears chloroform may have directly inhibited acetogen activity while protozoa numbers tended to increase. Reduction in methanogen activity presumably would have increased H<sub>2</sub> concentrations in the rumen which should have led to enhanced acetogen activity.

Methane emissions were significantly lower for the chloroform treated cows from day 4 until day 32 at which stage CH<sub>4</sub> emissions had rebounded to close to their pre-treatment levels (Figure 1).

Feed digestibility was 66% and similar for the two treatments. Also, cow liveweight was similar for the two treatments, as was rumen fill, daily feed intake (8.4 kg DM) and retention time of DM in the rumen (19-23 hours).

Rumen pH and ammonia and total VFA concentrations did not differ between treatments. However during the treatment period the chloroform animals had higher propionic acid and lower acetic acid concentrations than the control cows. This effect may have been a result of reduced methanogen activity, as this effect has been reported in other work. The probable increased H<sub>2</sub> concentration could be expected to move the balance away from acetate towards propionate formation.

Other research has suggested that the likelihood of methanogens developing resistance to chloroform would be relatively low. However in this research although CH<sub>4</sub> emissions were initially reduced by 95% within 4-5 days, there was a slow recovery to about 62% of pre-treatment levels. The mechanisms driving this recovery are not completely clear, but could include adaptation by the methanogens or increased metabolism and transformation of the chloroform by other rumen microbes. The DNA and microbiological analyses on the rumen samples suggested the initial decrease in methanogens may have been caused by washout of susceptible species from the rumen as part of the normal turnover process. The RCC methanogen grouping tended to increase during the treatment period although it appears the surviving community was less diverse than it was pre-treatment; and *M. ruminantium* after initial depression rebounded to a degree.



**Figure 1.** CH<sub>4</sub> emissions for the control and chloroform treated cows during the study. Chloroform treatment started on day 4 and ceased on day 42.

Medium and long-term studies are required to study the development of resistance in the methanogen population to anti-methanogen agents. These agents will need to be specifically antagonistic to methanogens and not disrupt normal rumen function. This work suggests chloroform has value as a research tool in this general area. It directly influenced methanogen activity and CH<sub>4</sub> production for a relatively lengthy period, but appeared not to disrupt normal rumen function.

## Reference

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# ANIMAL VARIATION AND GHG EMISSIONS

## BACKGROUND

Selection and breeding of animals that produce less CH<sub>4</sub> per unit of feed eaten has large potential for reducing GHG emissions from grazing systems. Use of breeding technologies has already been used to increase productivity from New Zealand's farmed animals. Genetic improvement of sheep, dairy and beef cattle and deer is an ongoing, cost-effective and permanent technology in our flocks and herds, targeting such productivity traits as lambing percentage, growth rate and milk production.

Methane emission measurements within groups of animals of the same species characteristically show very large animal-to-animal variation. The database of CH<sub>4</sub> measurements on sheep in the respiration chambers at AgResearch Grasslands in Palmerston North show a 50% difference in CH<sub>4</sub> yield from animal to animal when measured as per unit of dry matter (DM) intake.

If at least some of this animal-to-animal variation could be passed on from one generation to the next (heritable), then it would be possible to breed for lower CH<sub>4</sub> emissions. At the same time it would be necessary to check the extent of any negative or positive correlations between a low CH<sub>4</sub> emission trait and other desirable production characteristics.

Although the processes in the animal that result in CH<sub>4</sub> emissions are understood, the mechanisms leading to differences in emissions between individual animals are not known. In order to investigate this variation it is necessary to have an accurate and repeatable method of experimentally measuring CH<sub>4</sub> emissions. The SF<sub>6</sub> technique used for measuring emissions outdoors has proven to have levels of variability that are too large for this purpose. Housing animals in respiration chambers provides the most accurate method of measuring CH<sub>4</sub> emissions, and an eight chamber facility was built at AgResearch Grasslands in 2007.

PGgRc research has been focussed on determining if variation in CH<sub>4</sub> emissions is heritable, and whether differences among individual animals are maintained when animals are grazed on different feeds. The research has also investigated the possible physiological mechanisms that might lead to individual animal variation.

## WHY THIS IS IMPORTANT

Animal selection to reduce GHG offers a well understood and applied option for livestock farmers to use. The challenge is that this trait is likely to be considered alongside more directly important traits such as meat or milk production, therefore progress in reducing methane emissions may be small but will accumulate over time. Therefore in assessing the value of whether breeding can offer a realistic mitigation solution we also need to gain an understanding of what the interactions with other production traits are and ensure that by selecting for them, we are not inadvertently increasing emissions.

Measuring emissions in production livestock is difficult and a goal of the research is to identify low cost methods that can be applied easily and allow selection to be an option that can be applied. This is the focus of the on-going programme of work; confirming and delivering the tools for selection in ruminants.



Cattle wearing neck harness for measuring CH<sub>4</sub> at grazing using the SF<sub>6</sub> technique



## Repeatability of methane emissions from sheep

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### INTRODUCTION

Trials were carried out on a large number of sheep in respiration chambers to estimate the repeatability and heritability of CH<sub>4</sub> emissions. Sheep with consistently high and consistently low emissions were selected and re-measured on two contrasting diets to assess whether the type of feed affected the consistency of the high/low ranking for individual animals.

### METHODOLOGY

A flock of 105 10 month old ewe lambs were screened in the respiration chambers for their CH<sub>4</sub> yields. These lambs came from a progeny testing programme, which meant the genetic background of each animal was known. If it could be demonstrated that individual animals had repeatable high or low CH<sub>4</sub> emissions across experiments, then knowledge of animals' genetic background would allow calculation of whether that repeatable characteristic was genetically linked and so would be heritable.

In the first trial (screening phase), in July to September 2008, animals were acclimatised to a molasses containing grass silage diet for more than 3 weeks then CH<sub>4</sub> emissions were measured for one day in the respiration chambers, four times for each animal, with two weeks between each measurement. Animal were fed at 1.3 times their maintenance requirements.

The 10 animals with the lowest and the 10 with the highest emissions were then selected for further study and grazed outdoors for a period. These animals were then acclimatised to fresh perennial ryegrass indoors, and each animal was measured for two days in a respiration chamber during May 2009. The same animals were then acclimatised to a pelleted diet for three weeks during June 2009 and measurements made in the respiration chambers repeated. The pelleted diet was a 40:60 mix of concentrate: forage (wheat grain and lucerne hay). When the pelleted diet was fed animals also had access to a small amount of hay to ensure adequate fibre was present in their diet.

All feeds were given to animals at levels of 1.3x maintenance. Fresh air was circulated through the respiration chambers at a known flow rate, and samples of air entering and leaving the chambers was analysed for CH<sub>4</sub> and H<sub>2</sub> concentration. The amount of feed eaten and its chemical composition was measured.

### RESULTS AND DISCUSSION

In the screening trial mean CH<sub>4</sub> yield was 18.4g/kg DM intake, and estimates for repeatability and heritability were 0.15 and 0.30 respectively. The repeatability estimates suggest that individual animals can have consistently higher or lower CH<sub>4</sub> emissions; and the heritability estimates suggest that at least in part these differences are inherited.

These were the first repeatability and heritability estimates published for sheep and further work is required to verify these results as the standard errors for these estimates were high ( $\pm 0.10$  and  $\pm 0.26$  respectively). Methane yield averaged 19.2 and 17.8 g/kg DM for the 10 highest and 10 lowest emitting sheep respectively i.e. emissions were 8% higher in the high group than the low group.

When the effects of the two different diets were compared, the high and low rankings were maintained. With the fresh ryegrass diet CH<sub>4</sub> emissions from the high and low groups averaged 24.9 and 22.1 g/kg DM intake; and with the pelleted diet CH<sub>4</sub> emissions were 24.2 and 17.8g/kg DM. There was a group x diet interaction, because the difference between high and low groups was 13% for the ryegrass diet and much higher at 36% for the pelleted diet.

Sheep fed ryegrass had no measurable H<sub>2</sub> emissions but sheep fed the pelleted feed had significant H<sub>2</sub> emissions. This did not differ across the high and low groups which was unexpected since CH<sub>4</sub> formation requires H<sub>2</sub>. Hydrogen emissions from the sheep fed pellets suggests that full conversion of excess H<sub>2</sub> into CH<sub>4</sub> does not always occur in the rumen.

The lower CH<sub>4</sub> emissions from the pelleted vs. the ryegrass diet were as expected, because it is well known that increasing concentrate levels in the diet leads to reduced CH<sub>4</sub> emissions.

### Future work

Further work with a larger number of sheep from diverse genetic backgrounds is required to verify these results as the standard errors of the repeatability and heritability estimates were high ( $0.16 \pm 0.10$  and  $0.30 \pm 0.26$  respectively). There is also value in studying stable selected lines flock to identify what makes these sheep differ in emissions and extending the study to determine heritability for emissions in cattle and deer.



## Investigating whether rumen retention time causes differences in methane emissions in sheep

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### INTRODUCTION

Recent research has shown that CH<sub>4</sub> yield (emissions per unit feed eaten) is repeatable for individual animals and heritable. This suggests that animal breeding may provide practical, cost-effective and lasting mitigation technologies. However, the mechanisms responsible for the measured animal-to-animal differences in emissions remain mostly unknown. Knowledge of these mechanisms would provide valuable information for development of mitigation technologies, including animal breeding.

Methane production in the rumen is carried out by archaea microbes. The relationship between retention time of feed particles in the rumen and rate of CH<sub>4</sub> emissions is commonly accepted, based on the fact that the longer fibre spends in the rumen, the more it is broken down, producing more H<sub>2</sub> and ultimately CH<sub>4</sub>.

However, very little research has been undertaken examining this relationship. Rumen retention time has been shown to be repeatable and heritable, and to be responsible for a large proportion of the animal-to-animal variation in CH<sub>4</sub> emissions. This study was carried out to see if rumen retention time is associated in differences in CH<sub>4</sub> emissions per unit feed eaten for groups of sheep known to be high or low CH<sub>4</sub> emitters.

### METHODOLOGY

This study was conducted during January to March 2010, in a similar manner to the comparison of ryegrass and pelleted feed diets and their effect on CH<sub>4</sub> emissions from groups of 10 known high emitting and 10 low emitting sheep described in the preceding section.

The animals were housed individually in metabolism crates for feed digestibility and digesta kinetics measurements over a seven-day period. Daily dry matter (DM) intake was calculated from the amounts of feed offered and refused, faecal output was measured, and digestibility of the feed eaten was calculated. Retention time of feed particles and of feed dissolved in the rumen liquid (solutes) were estimated by staining soluble fibre (NDF) with chromium so it could be used as a marker for feed particles. Co-EDTA was used in a similar fashion as a marker for solutes. Sheep were dosed once with the

markers at a known time, and samples of faeces were then taken at pre-determined times to see how long it took for them to move through the digestive tract. Following the digesta kinetics measurement phase, animals were moved to the respiration chambers for a two-day CH<sub>4</sub> measurement period.

### RESULTS AND DISCUSSION

Methane yield was higher for the group of high emitting sheep than for the low emitting sheep (17.7 vs. 14.0 g/kg DM eaten) and was higher for ryegrass than for the pelleted diet (23.2 vs. 8.6 g/kg DM eaten), as found in the earlier trial. Also, while not statistically significant, the difference between high and low emitting groups appeared higher for pellet-fed (69%) than ryegrass-fed (13%) sheep.

Rumen retention time of particles was greater in the high than in the low emitting groups (35.1 vs. 31.7 hours respectively) however this difference was not statistically significant. Solute retention time in the rumen was significantly longer for the high emitting sheep compared with the low emitting sheep (15.5 vs. 13.6 hours respectively). Particle retention time was significantly greater for the pelleted than for the ryegrass diet (36.9 vs. 29.9 hours respectively) but solute retention time did not differ, averaging 14.6 hours in each case.

Results from this study support findings from other research that suggests CH<sub>4</sub> emission may be associated with the rumen solute retention time, but further work is required to confirm if this. The solute fraction of the digesta is responsible for flow of small feed particles out of the rumen and shorter solute retention times are associated with shorter residence time of protozoa in the rumen before they are washed out. As well as promoting fibre breakdown and therefore H<sub>2</sub> production in the rumen, there is a symbiotic relationship between protozoa and methanogens that produce CH<sub>4</sub> using H<sub>2</sub>, in the rumen.

The lower CH<sub>4</sub> yield from the pelleted feed was expected, as digestion of concentrate diets is known to produce less CH<sub>4</sub> than forage diets. However the longer retention time in the rumen was unexpected, and this may have been associated with the processing and pelleting process used, as this effect has been previously noted.

## Future work

The above study suggested that animal-to-animal differences in CH<sub>4</sub> emission for animals known to be high or low CH<sub>4</sub> emitters were associated with rumen retention time of digesta. This in turn suggests underlying differences exist in extent of digestion of fibre in the rumen and that CH<sub>4</sub> emission may be associated with net feed efficiency (or residual feed intake, i.e. the difference between an animal's actual feed intake and its expected feed intake based on its size and growth over a specified test period). The number of animals used in the study was small however, and similar work needs to be repeated with larger numbers of animals to make further progress in this important area.

Priority areas for further research include:

- Maintain and measure high and low CH<sub>4</sub> emission sheep selection lines and estimate the degree to which the low CH<sub>4</sub> emission trait can be captured by selection (realised heritability) and how this correlates with other production traits
- Estimate the consistency of emissions from high and low selection lines by testing their emissions with fresh forage; and measuring feed flow rates, digestibility, and rumen volume on more animals
- Investigating consistency of CH<sub>4</sub> emissions across physiological states by testing lines of lactating and pregnant animals
- Make the selection line animals and samples available to microbiological genomics and microbial ecology teams to ascertain reasons for differences in CH<sub>4</sub> emissions.



# FEEDS AND GHG EMISSIONS

## BACKGROUND

The primary feed source for New Zealand's grazing animals is pasture, plus a small amount of grazed crops such as brassicas and annual ryegrasses. Dairy cows also may receive a proportion of their annual feed requirements from supplements such as pasture silage and hay, palm kernel extract, maize silage and a small amount of concentrate feeds such as maize and barley grain.



Forages are composed of cells that contain highly digestible cell contents covered by cellulose-rich cell walls or 'fibre'. Fibre is indigestible to monogastrics such as chickens, pigs, and humans. Ruminants can digest cell walls, a major component of forages, because of the microbes that live in the rumen, and convert them into useful products such as meat and milk. Unfortunately, during the digestion of feed in the rumen, hydrogen ( $H_2$ ) is formed which is the main energy source for methanogens which evolve hydrogen ( $H_2$ ), the energy source for methanogens which produce methane ( $CH_4$ ) in the rumen.

Concentrate feeds generally have a lot more grain in their make-up and are known to produce less  $CH_4$  per unit of feed eaten than forage. However, New Zealand grazing systems would lose their current cost advantage if a much larger component of concentrates was used as feed rather than forage. Therefore identification of forages that produce less  $CH_4$  when digested could lead to a useful GHG mitigation option for our grazing systems.

Two methodologies are commonly used for measuring direct  $CH_4$  emissions from animals in PGgRc trials; the sulphur hexafluoride ( $SF_6$ ) tracer technique and respiration chambers.

The  $SF_6$  technique allows the measurement of emissions from free-ranging animals, sampling exhaled breath in containers placed in halters around the neck of the animal. Levels of  $SF_6$ , a non-toxic, physiologically inert tracer gas released from permeation tubes implanted in the rumen are measured and methane emission calculated from the release rate of  $SF_6$  and concentration of  $SF_6$  and  $CH_4$  in the containers in excess of background level.

In respiration chambers all of the exhaled breath from the animal is collected and gas concentrations determined directly. This is considered the standard method for estimating methane emissions from animals, as the environment can be controlled and measurements made in a stable and reliable manner. However, there is a risk that the chamber creates an artificial environment which can affect animal behaviour and therefore emissions.

PGgRc funded research has measured  $CH_4$  emissions from a range of forages over the last five years, and has identified some forages that when eaten by sheep appear to result in less  $CH_4$  emission per unit of feed eaten. This opens up some exciting avenues for future research.



## Effects of feeding white clover or perennial ryegrass on methane emissions from sheep

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### INTRODUCTION

Measurements using the SF<sub>6</sub> tracer technique have suggested sheep fed white clover have lower CH<sub>4</sub> yields (g CH<sub>4</sub> per kg dry matter (DM) intake) than sheep fed perennial ryegrass. Thus, white clover may offer an opportunity to reduce CH<sub>4</sub> emissions from pastoral-based livestock systems if its lower CH<sub>4</sub> yield can be confirmed using respiration chambers. Comparisons between SF<sub>6</sub> tracer and respiration chamber techniques to measure CH<sub>4</sub> emissions from ruminants have shown good agreement, but there is evidence that CH<sub>4</sub> emissions can be affected by SF<sub>6</sub> gas permeation rates, which could exacerbate variation in CH<sub>4</sub> estimates.

The purpose of this study was to measure CH<sub>4</sub> emissions from sheep fed fresh white clover or perennial ryegrass forages in respiration chambers, at several intake levels, to validate previous comparisons of these forages using the SF<sub>6</sub> tracer technique.

### METHODOLOGY

Two experiments were carried out between May and November 2009. Freshly harvested white clover and ryegrass were fed to 16 sheep in experiment one; with eight sheep per diet, and 32 sheep in experiment two; with 16 sheep per diet. Animals were fed at 1.6 times metabolisable energy (ME) requirements for maintenance ( $\times$  ME<sub>m</sub>) in experiment one, and either 0.8 or 2.0  $\times$  ME<sub>m</sub> in experiment two (i.e. eight animals per diet per feeding level). In both experiments, sheep were acclimatised to diets and feeding level treatments for 10 days before individual CH<sub>4</sub> emissions and feed intakes were measured for two consecutive days in respiration chambers.

### RESULTS AND DISCUSSION

In experiment one, CH<sub>4</sub> yield (g/kg DM intake) was 12% lower (P=0.04) for sheep fed white clover (19.8) compared with ryegrass (22.5).

In experiment two, forage treatment did not affect CH<sub>4</sub> yields (g/kg DM intake) from sheep fed at the lower feed intake level of 0.8  $\times$  ME<sub>m</sub> (27.1 and 25.5 for white clover and ryegrass forages, respectively), but at the higher feed intake (2.0  $\times$  ME<sub>m</sub>), CH<sub>4</sub> yields were 7% higher (P=0.05) for sheep fed white clover compared to ryegrass (23.4 and 21.7, respectively).

Combined analysis across both experiments showed there was no significant effect of diet on CH<sub>4</sub> yield from sheep, however, with increasing feed intake level (0.8 to 2.0  $\times$  ME<sub>m</sub>) CH<sub>4</sub> yield was reduced by 14% (P=0.01).

Previous research using the SF<sub>6</sub> technique has reported lower CH<sub>4</sub> emissions from sheep grazing white clover, red clover, sulla and chicory, compared to ryegrass. Thus, the lack of a difference in CH<sub>4</sub> yields from sheep fed white clover and ryegrass diets across these experiments using respiration chambers was unexpected, particularly as there were marked differences in chemical composition between the two forages (i.e. white clover contained 50% less neutral detergent fibre (NDF), 30% more crude protein (CP) in the DM, and nearly a three-fold greater readily fermentable to structural carbohydrate ratio (RFC:SC), compared to ryegrass).



The reason for investigating white clover as a potential option for reducing CH<sub>4</sub> emissions is the high growth rates of lambs fed white clover due to its lower NDF content and higher feed intake compared to ryegrass. The low NDF content and high RFC:SC in white clover compared to ryegrass is shown to have rapid digestion in the rumen, a high rate of passage, and a decrease in acetate to propionate ratio; all which are expected to lower CH<sub>4</sub> yield. However, the similar CH<sub>4</sub> yields from sheep consuming either white clover or ryegrass in the experiments reported here suggests there are no simple relationships between chemical components of fresh forages and CH<sub>4</sub> yields from sheep, emphasising the contribution of alternative factors to methanogenesis.

These trials agree with the consensus that increasing feed intake reduces CH<sub>4</sub> yield from ruminants. The relationship requires more investigation because it offers an opportunity to lower CH<sub>4</sub> emissions whilst increasing productivity. Despite similar CH<sub>4</sub> yields from sheep fed white clover and ryegrass in this study, high growth rates of lambs fed white clover reduce emissions per unit of product relative to ryegrass. Moreover, intake of clover diets are directed towards more growth rather than maintenance thus reaching slaughter live weight more quickly compared to ryegrass diets.



Sheep feeding on a clover diet in a respiratory chamber

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## Reference

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## Sheep fed chicory or perennial ryegrass have similar methane emissions

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### INTRODUCTION

Chicory is a high quality forage with a relatively high rate of passage through the rumen which might be expected to lower CH<sub>4</sub> emissions per unit feed eaten. It has been previously reported from trials using the SF<sub>6</sub> technique that sheep grazing chicory had lower CH<sub>4</sub> emissions than those grazing perennial ryegrass. These trials were conducted to investigate whether the earlier reports of lower CH<sub>4</sub> emissions could be repeated.

### METHODOLOGY

Fresh chicory and perennial ryegrass in a vegetative state were fed to 24 wether sheep housed in metabolism crates, at two feeding levels: 1.3x and 2.2x maintenance. Eight of the sheep were rumen fistulated to allow sampling of the rumen digesta, and two fistulated and four non-fistulated animals were allocated to each feed and feeding level treatment combination. Animals were initially acclimatised to the feeds, then acclimatised for an additional 13 days in the crates. Dry matter intake, feed digestibility, rumen fermentation characteristics (pH, volatile fatty acids [VFAs], and ammonia) and rumen liquid passage rate (using Co-EDTA as a marker) were measured, followed by two days of CH<sub>4</sub> emissions measurement in respiration chambers. This work was carried out during April/May 2009.

In addition, *in sacco* DM degradation studies were carried out using two rumen fistulated cattle fed ryegrass hay. In *in sacco* trials feed samples in fine mesh bags were placed in the rumen of fistulated cattle and then sequentially withdrawn for analysis after predetermined times.

### RESULTS AND DISCUSSION

The chemical composition of the feeds differed markedly for chicory and ryegrass (Table 1). Digestibility of chicory was higher than ryegrass but the rumen liquid outflow rate was similar. There were no significant effects of treatments on rumen fermentation characteristics, apart from rumen ammonia concentrations being lower for chicory fed sheep.

**Table 1:** Chemical composition and digestion of Chicory and Ryegrass

	Chicory	Ryegrass
Crude Protein	11.4	19.7%
Readily fermentable carbohydrate concentration	22.8	12.4%
Neutral detergent fibre (NDF)	23.9	42.3%
Digestibility	76.3	74.7%
Rumen liquid outflow	0.16	0.155/h

Despite the different chemical compositions of the forages, CH<sub>4</sub> yield did not differ between chicory and ryegrass (22.0 and 23.6g/kg DM intake respectively). This suggests chemical composition as measured by conventional laboratory analytical techniques does not influence CH<sub>4</sub> yield. Methane yield on the ryegrass diet was lower at the high intake (21.5 g/kg DM) than the low intake (25.6) level but the difference was not statistically significant for chicory (22.6 vs. 21.4g/kg DM). Methane yield was lower for the high (21.5g/kg DM) than for the low (24.1) feeding level, as found in other trials. As rumen pH and VFA concentrations were similar for the two feeding levels the combined result suggests rumen fermentation is not a driver of the differences in CH<sub>4</sub> yields.

It has been suggested that rumen outflow rate is a major driver of CH<sub>4</sub> yield from different feeds and this work would tend to support that view, as outflow rates and CH<sub>4</sub> yield were similar for the two feeds, while outflow rates and CH<sub>4</sub> yield differed across feeding levels.

This work differed from earlier research using the SF<sub>6</sub> technique where CH<sub>4</sub> yield was lower for chicory than for ryegrass.

### Reference

Sun XZ, Hoskin SO, Zhang GG, Molano G, Muetzel S, Pinares-Patiño CS, Clark H, Pacheco D (2012) Sheep fed forage chicory (*Cichorium intybus*) or perennial ryegrass (*Lolium perenne*) have similar methane emissions. *Animal Feed Science and Technology* 172, 217-225.



## Effects of chicory and perennial ryegrass on methane emissions *in vitro* and from sheep

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### INTRODUCTION

Chicory has been reported to reduce CH<sub>4</sub> emissions compared to some other forages, when measured using the SF<sub>6</sub> technique. Chicory is a very productive forage herb that is used in New Zealand predominantly for provision of high quality feed in the warm season. This research compared chicory and ryegrass harvested and fed fresh at the pre-flowering and flowering stages, and differs from the previous study which used vegetative chicory and ryegrass.

### METHODOLOGY

Chicory was harvested just prior to flowering, and ryegrass was harvested in the early flowering stage in November 2009. Groups of eight wether sheep were fed the two forages at 1.3x maintenance for seven days, and then for five days in metabolism crates. Methane and H<sub>2</sub> yields were measured for two days in respiration chambers. Rumen samples were taken using stomach tubes and volatile fatty acid (VFA) composition measured.

In addition, samples of the forages used in the feeding trial, plus samples from vegetative chicory and ryegrass collected in April, were used for an *in vitro* experiment. Half of each sample was freeze-dried and ground, while the other half was minced while frozen to compare the effects of sample preparation for these assays. This is important because drying forages, as commonly done for *in vitro* tests, do not represent the physical characteristics of a fresh forage after ingestion and mastication. Each of these "substrates" were incubated in two separate runs using rumen inoculums from different cows on each occasion. Duplicate samples were run, with one sample used for measuring CH<sub>4</sub> evolution and one for products of fermentation.

### RESULTS AND DISCUSSION

The chemical composition of the feeds differed markedly. Crude protein concentration was 11.7 vs. 8.5%, readily fermentable carbohydrate concentration was 17.2 vs. 12.1% and neutral detergent fibre (NDF) was 28.1 vs. 49.9% for chicory and ryegrass respectively. The molar proportion of acetate measured in the animal trial tended to be higher for chicory than for ryegrass (69.7 and 67.7mol/100mol of total VFA respectively) and the proportion of propionate lower for chicory (16.7 and 18.8mol/100 mol respectively). Therefore, the acetate to propionate ratio was highest for chicory, indicating that chicory did not give a more favourable (lower methane) fermentation than ryegrass. In the *in vitro* trial acetate was the predominant VFA produced and the substrates ranked vegetative chicory > pre-flowering chicory > vegetative ryegrass > flowering ryegrass. Propionate production was highest for vegetative chicory.

Sheep emitted similar amounts of CH<sub>4</sub> from both feeds (22.8 and 23.8g/kg DM for chicory and ryegrass respectively) in the feeding trial. The yield of H<sub>2</sub> was very small (<0.1g/day) and less for sheep fed ryegrass than chicory. Methane evolution was similar for both forages at both stages of maturity in the *in vitro* trial. Minced forage yielded more CH<sub>4</sub> than ground forage.

As in the earlier trial (see previous) although the chemical composition of the forages differed markedly there were no differences in CH<sub>4</sub> yield. The results reinforce the findings of other studies which show that the stage of forage maturity does not affect CH<sub>4</sub> yield.

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### Reference

Sun XZ, Hoskin SO, Muetzel S, Molano G, Clark H (2011) Effects of forage chicory (*Cichorium intybus*) and perennial ryegrass (*Lolium perenne*) on methane emissions *in vitro* and from sheep. *Animal Feed Science and Technology* 166-167, 391-397.



## Methane emissions from sheep fed fresh brassicas compared to perennial ryegrass

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### INTRODUCTION

Forage brassicas are frequently used as forage crops in New Zealand because of their rapid growth and high production of high quality forage. They are considered high quality as they contain high concentrations of non-structural carbohydrate and low concentrations of neutral detergent fibre (NDF). The most commonly grown brassicas are kale, rape, turnips and swedes. Brassicas also contain secondary plant metabolites including polyphenol oxidase, S-methyl cysteine sulfoxide (SMCO) and glucosinolates. These compounds may inhibit the performance of grazing animals. Human gut bacterial communities have been shown to be altered by adding glucosinolate-containing vegetables to the diet, but there are no reports of any of these secondary compounds influencing rumen methanogens. Methane is formed in the rumen by methanogens which use  $H_2$  formed during fermentation of feed. Nitrate and sulphate in the diet can be effective as alternative  $H_2$  sinks and can reduce  $CH_4$  production in sheep. Brassicas are known nitrate accumulators and this might affect methanogenesis.

In this work  $CH_4$  emissions from sheep fed brassicas were compared with emissions from sheep fed perennial ryegrass, the major sown grass species in New Zealand. Concentrations of secondary compounds in the brassicas were also measured and correlated with  $CH_4$  production.

### METHODOLOGY

The  $CH_4$  yields of four forage brassicas (kale, turnips, rape and swedes) were compared with perennial ryegrass when fed to sheep. Sixty cryptorchid sheep (scrotum removed, testicles retained) were allocated to five groups of 12, one group for each of the forages. The sheep were acclimatised to their allocated feed outdoors for two weeks, then indoors for one week at 1.5x maintenance requirements. Turnip and swede bulbs were pulled, washed and diced and combined with leaves before feeding. In the last two days indoors, rumen samples were obtained by stomach tube. Ten sheep from each group were then transferred to metabolism crates and fed individually for three days, at which point intakes and excreta were measured in five sheep per treatment to estimate diet digestibility. Following this,  $CH_4$  emissions from nine sheep per treatment were measured in respiration chambers over two days. Forage samples were analysed for chemical composition and secondary plant compound concentrations. The indoor phase of the trial was conducted during June/July 2010.

### RESULTS AND DISCUSSION

Sheep ate similar amounts of all brassicas other than swedes which they consumed at a slightly lower rate (about 6% less) than the others. Sheep consumed ryegrass at a higher rate (14% more) than the average for the brassicas excluding swedes, probably because ryegrass was offered in greater quantities to ensure similar amounts of metabolisable energy was allocated to the animals daily. Dry matter digestibility was highest for swedes (89%), followed by the other brassicas (average 81.2%), and ryegrass was lowest (66.5%). Metabolisable energy (ME) concentrations followed the same pattern (14.1, 12.7 and 9.4 MJ ME/kg DM intake respectively).

Methane yield was highest for ryegrass (22.0g/kg DM intake), intermediate for turnips and kale (20.6 and 19.8 respectively) and lowest for swedes and rape (16.9 and 16.4 respectively). The difference between ryegrass and swedes and rape was statistically significant. Hydrogen emissions from sheep fed brassicas was more than twice those of sheep fed ryegrass (0.045 to 0.052 vs. 0.015g/kg DM intake respectively). Methane yields were not significantly related to the chemical composition of the diet, although  $H_2$  yields did increase with increasing pectin, sugar and lignin concentrations and increasing ratios of readily fermentable carbohydrates to structural carbohydrates; and decreased with increasing neutral and acid detergent fibre concentrations. Methane yields decreased with increasing digestibility of organic matter and neutral and acid detergent fibre, and with metabolisable energy (ME) content. These variables individually explained 22-49% of the  $CH_4$  yield variation measured for the five forage treatments.

Feeding brassicas reduced the ratio of acetate to propionate in the rumen from 2.6 pre-feeding to an average of 1.5 post feeding; in contrast, acetate to propionate ratios in the rumen of sheep fed the ryegrass diet were 3.6 and 2.9 respectively. Low acetate to propionate ratio is associated to rumen conditions that lead to less methane being produced.

Methane production decreased with increased nitrate concentration ( $r=-0.96$ ), but none of the secondary compounds were significantly correlated. Nitrate is considered to be an  $H_2$  sink in the rumen, and hence the negative relationship with  $CH_4$  yield is logical. However, estimates of the effect on methane production attributable to nitrate content in the feed suggest that nitrate accounted for only 5-11% of the

difference in methane yield between brassicas and ryegrass.

Feeding brassica forages to sheep reduced CH<sub>4</sub> yield per unit feed intake, when compared with feeding perennial ryegrass. The effect was greatest with rape and swedes (25% reduction). Reductions in CH<sub>4</sub> yield appeared to not be directly related to the chemical composition of the forage, but was inversely related to digestibility.

In addition to the trials described in the published work above, additional feeding trials were carried out to validate these initial results. Methane emissions from lambs were measured in respiration chambers after seven or 15 weeks of continuous feeding in the field with either forage rape or perennial ryegrass. Lambs fed forage rape had 30% lower emissions per unit of organic matter or digestible dry matter intake after seven weeks feeding compared with lambs fed ryegrass. After 15 weeks CH<sub>4</sub> emissions from rape were 20% lower. This confirmed the earlier findings and suggested the effect of feeding rape on CH<sub>4</sub> emissions persists for at least three months.

## Future work

The potential for widespread use of brassicas to feed New Zealand's farmed animals will be limited because of their agronomic characteristics. Mechanisms underlying the effects of brassicas on CH<sub>4</sub> emissions need to be worked out, as a better understanding of these would assist in development of a range of forages well-adapted to different environments and management systems that could be used as practical mitigation options for farmers.

Understanding the mode of action of brassicas could also assist understanding of between animal differences in CH<sub>4</sub> yields, provide useful leads for the Inhibitor programme, and also provide insights into possible interactions amongst various mitigation technology options.

Further information is required on the effect of varying the proportion of supplementary feed in an animal's diet on CH<sub>4</sub> emissions.

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## Reference

Sun XZ, Waghorn GC, Hoskin SO, Harrison SJ, Muetzel S, Pacheco D (2012) Methane emissions from sheep fed fresh brassicas (*Brassica* spp.) compared to perennial ryegrass (*Lolium perenne*). *Animal Feed Science and Technology* 176, 107-116.



Methane measurement using the SF<sub>6</sub> technique on brassica crop



# MITIGAS TESTING PROCEDURE

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## BACKGROUND

An increasing number of studies are being conducted to assess the ability of feeds or inhibitors to reduce methane (CH<sub>4</sub>) emissions from the rumen. The most favoured screening method involves *in vitro* laboratory based batch culture systems as they are low cost and straight forward to operate. Screening of multiple substances is most effectively done using automated systems. However, most of these systems, while being capable of monitoring gas production over time, require tedious manual sampling for determining composition of those gases.

With support from the PGgRc and MPI, AgResearch has developed a testing protocol or 'testing pipeline' for systematic and cost-effective analysis of the ability of candidate feeds and compounds to reduce CH<sub>4</sub> emissions from the rumen.

## THE MITIGAS PROTOCOL

There are three sequential components to the protocol:



The preliminary batch culture step identifies whether the substance being tested specifically reduces CH<sub>4</sub> production, as opposed to a more generic effect on total fermentation gas emissions. Total gas production is compared against a high quality ryegrass 'negative' control that is routinely incubated in every test run alongside the test substances. If the test substances do not themselves support normal fermentation the ryegrass control is included as a basal substrate with the test substances. A 'positive' ryegrass control containing a potent methanogen inhibitor (bromoethane sulfonate) is also incubated in each run.

The test substances and the ryegrass controls are incubated in duplicate bottles containing buffered rumen fluid from a rumen fistulated sheep or cow, and results from the duplicates are averaged. Total gas, CH<sub>4</sub> and hydrogen (H<sub>2</sub>) evolution are automatically calculated at frequent intervals throughout the 48 hour incubation run. At the end of the run a sample from each bottle is taken and analysed for short chain fatty acid (SCFA) concentration and proportions. Methane production data are used to determine inhibitory effects of the test substances and along with results for SFCAs and H<sub>2</sub> production can be used to assist in identifying the actual inhibitory principles in the test substances.

If the substance reduces CH<sub>4</sub> production and increases H<sub>2</sub> production it is considered a 'true' inhibitor of methanogens. Usually this is accompanied by a small increase in the SFCAs propionate and butyrate. A decrease in H<sub>2</sub> production over the incubation period suggests the microbial community has adapted to the effects of the substance and raises doubts as to its potential as a practical inhibitor.

If CH<sub>4</sub> is emitted, H<sub>2</sub> production is not increased and propionate production is increased, meaning the test substance either has a fermentation modifying ability or contains sugars that are fermented into propionate thereby decreasing the H<sub>2</sub> available for methanogens.

If CH<sub>4</sub> production is reduced but H<sub>2</sub> and propionate production are not increased, the test substance may contain an H<sub>2</sub> sink like nitrate or sulphate. If it is NO<sub>3</sub> the production of the SCFA acetate is increased, but the effect may be masked where the test substance is plant material.

In some cases response run is useful, particularly if a substance reduces CH<sub>4</sub> production in combination with a reduction in total gas and SCFA production.

An experiment was conducted using ryegrass as a base substrate to demonstrate/validate this approach. The methanogen inhibitor bromoethane sulfonate (BES) and the sugar rhamnose (Rh) only slightly decreased gas production compared to the control ryegrass, while nitrate (NO<sub>3</sub>) addition reduced total gas production. All the compounds reduced CH<sub>4</sub> by 25-30% compared to the control ryegrass. The inhibitor BES led to an H<sub>2</sub> accumulation, but this effect was minor for rhamnose or NO<sub>3</sub>. There was no change in total SCFA or the proportion of the individual fatty acids with NO<sub>3</sub>, whereas rhamnose also did not increase SCFA total concentration but doubled propionate production. Inhibition of CH<sub>4</sub> production by BES led to a decrease in total SCFA production and a minor increase in propionate and butyrate production.

The above approach has been used to screen 100 plant species grown in New Zealand to date. Both rape and swedes have been shown to reduce CH<sub>4</sub> production using this testing method.

### CONTINUOUS CULTURE INCUBATION

Substances that are considered worthy of further evaluation following short term batch culture screening can then be tested using a recently developed automated continuous culture fermenter. This allows the effects of the candidate substances to be studied over a longer period in a stable *in vitro* test environment. The short term approach is rapid and can handle large numbers of samples with minimum cost, however it has the disadvantage that it does not enable longer term effects to be assessed and it also allows the accumulation of fermentation end-products in the incubation bottles. The continuous culture procedure enables any likely changes in the rumen microbial community and/or fermentation pathways to be identified over a longer period of time. Such changes could mean the inhibitory effects of a test substance will not persist or are reduced over time when utilised in a commercial rather than a short-term 'test-tube based' research environment. This approach also allows effects on fibre digestion (which has a critical effect on value of feeds to animal production) to be identified. Another advantage is that substrates can be tested in a controlled environment and effects of palatability or toxicity to the host organism are no issue.

Batteries of incubation vessels containing rumen fluid in buffer are seeded with test substrates, and fresh substrate is infused into each vessel either continuously or in pulses. Overflow liquid is collected, measured and analysed daily for amount of undegraded feed, and SCFA and ammonia concentrations. Total gas, CH<sub>4</sub> and H<sub>2</sub> production in the fermentation vessels are automatically monitored. Results are compared with those from an appropriate control substance.

A complete run takes less than 20 days, including an initial settling down period to allow the continuous fermentation process to equilibrate, and uses about 600gm (dry matter) of test substance per vessel, with two "duplicate" vessels run for each substance.



Continuous culture fermenter for *in vitro* studies.

## ANIMAL TRIALS IN RESPIRATION CHAMBERS

The final proof of efficacy of a mitigation option needs to occur using live animals i.e. *in vivo* evaluation. The most accurate means of making the required CH<sub>4</sub> emission measurements involves the use of respiration chambers where animals are individually housed. This approach is the most expensive of the three-stage protocol and is used once batch culture and continuous culture analysis has suggested merit in proceeding to animal trials. The ability to conduct these trials is strongly limited by the availability of respiration chambers and approval by the animal ethics committee. Normally this work is carried out using sheep as the facilities are more extensive and sheep have lower feed requirements. However, cattle can be used if required.

Animals are adapted to their experimental diet for at least seven days in pens, then for three days in metabolism crates where monitoring of individual intake commences. Animals that do not adapt to this regime are excluded at this stage.

Two general experimental designs are used. Firstly, an experimental diet can be compared with a control. Experimental groups involving eight sheep for conserved feed and 10 sheep for fresh feeds allow a 10% difference in CH<sub>4</sub> emissions to be detected. Alternatively, a 'shift-up' design may be used where CH<sub>4</sub> emissions without an experimental treatment are measured, then the experimental treatment is imposed and the effects on emissions are monitored. This latter approach is useful where the effect of an inhibitor may be short-lived and changes in the rumen microbial community are to be monitored e.g. for testing the effect of bro-moethane sulfonate or acetylene.

Once the sheep are in the air tight respiration chambers air flow through the chambers is controlled and gas concentrations of air in and out are measured. The chambers are opened twice daily to remove faeces and urine, replace the water supply and feed the animals. The actual measurement period in the chambers lasts for two days. Methane, H<sub>2</sub> and carbon dioxide production, along with feed dry matter intake are measured. Methane production (g CH<sub>4</sub>/day) and CH<sub>4</sub> yield (g CH<sub>4</sub>/kg feed dry matter intake) are calculated.

In shift-up experiments CH<sub>4</sub> on the control diet is measured for three to five days, and then the animals are released into pens for at least two days to recover from confinement. The animals then go back into the chambers and treatments are either started immediately, or control measurements are made for another couple of days before the treatment starts.

The process can include an additional six-day period in metabolism crates but outside the respiration chambers when feed intake and total faecal output are measured and feed digestibility is calculated. This step can also be carried out in the respiration chambers if required.

## Future work

It is possible to consume large amounts of resources and funding in attempts to identify which of a vast array of compounds and feed options might have future value in mitigating rumen CH<sub>4</sub> emissions.

There is little doubt such work should be a critical part of the search for solutions that can reduce New Zealand's large CH<sub>4</sub> emission profile. However resources and funding for this work are strongly constrained and so it is critical they are utilised to best effect. The Mitigas protocol is a cost-effective, methodical step-wise process for screening feeds and other substances that might reduce rumen-derived CH<sub>4</sub> emissions. The test pipeline approach moves through relatively simple, quick and cheap mass screening in batch culture in the laboratory, through longer term screening using continuous culture in the laboratory, to relatively expensive whole animal screening in respiration chambers.

This test pipeline is being utilised within the PGgRc research programme, and is also available to and being used by other parties.

Now the process has been conceptualised, documented and validated, future work will revolve around using and where necessary refining the procedures to increase the accuracy of identifying and developing potent and practical GHG mitigation options for farmers.

# NITROUS OXIDE

## BACKGROUND

In pastures grazed by farmed animals, the conversion of consumed nitrogen (N) into products is low and a substantial amount of the N consumed (more than 70%) is deposited back onto pastoral soils in the form of urine and dung. This reflects the fact that ruminant animals are not efficient utilisers of the N in their diet and a basic dilemma that pasture plants require a significantly higher concentration of N to grow at optimal rates than is needed by the grazing animal. For example, the maximum dietary N concentration needed by cattle and sheep is approximately 2.5% but New Zealand pastures often have an N concentration of 3-4%.

Nitrous oxide ( $N_2O$ ) loss from agricultural and industrial activities is significant for the environment, as it is thought to be a cause of both global warming and stratospheric ozone depletion. Nitrous oxide is an intermediate product of the soil processes of nitrification and denitrification, where biological processes break down nitrogen sources in the soil to nitrate and nitrite. These processes are affected by a number of soil factors, such as soil oxygen and moisture contents, temperature, mineral N content, available soil C and pH. Weather conditions, such as rainfall, can affect soil moisture and oxygen contents, and are therefore also important factors in the production of  $N_2O$ . In grazed pasture soils,  $N_2O$  gas is mostly generated from N present in the dung and urine of grazing animals and fertiliser N.

## WHY ITS IMPORTANT

Nitrous oxide as well as being a significant GHG gas is also a loss of nutrient to the farm system and as such it is an inefficiency that could be reduced. Within the PGgRc nitrous oxide mitigation research has focused on the application of Dicyandiamide (DCD) to soils to inhibit the transformation of N into nitrate and nitrous oxide. This product although very effective at reducing N losses (>50%) under urine spots was removed from the market in January 2013. Our research has also demonstrated that management changes can have modest effects on emissions and that a continual focus on efficient nutrient use will be a part of the package of mitigations into the future.



Drawing samples of  $N_2O$



## A review of management options to reduce nitrous oxide emissions from intensively grazed pastures

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### BACKGROUND

In New Zealand, Australia and parts of North and South America and Europe, most pastoral land is managed with high per-hectare animal productivity as an important goal for the pastoral farmers. The input of resources, including nitrogen (N) fertiliser, to the managed pastures can be substantial, resulting in a large surplus of N. The ability of soils to hold this surplus N is limited and so the majority is lost through leaching as nitrate ( $\text{NO}_3^-$ ) or emitted as gaseous N (ammonia ( $\text{NH}_3$ ), nitric oxide ( $\text{NO}$ ), nitrous oxide, ( $\text{N}_2\text{O}$ ) and dinitrogen ( $\text{N}_2$ )), causing economic and environmental impacts.

High  $\text{N}_2\text{O}$  emission rates observed in grazed pastures primarily come from animal urine and dung, which then degrades in anaerobic (“lacking in air”) conditions caused by soil compaction due to animal treading. Wet soil conditions soon after N fertilisation or grazing can also cause high  $\text{N}_2\text{O}$  emissions from pastures. The application of farm effluents and slurries to pasture soils also promotes emissions. Reviews on the sources of N, processes regulating  $\text{N}_2\text{O}$  emissions, emissions measurements in grazed pastures and their mitigation all provide a greater understanding of the mechanisms involved in  $\text{N}_2\text{O}$  emissions.

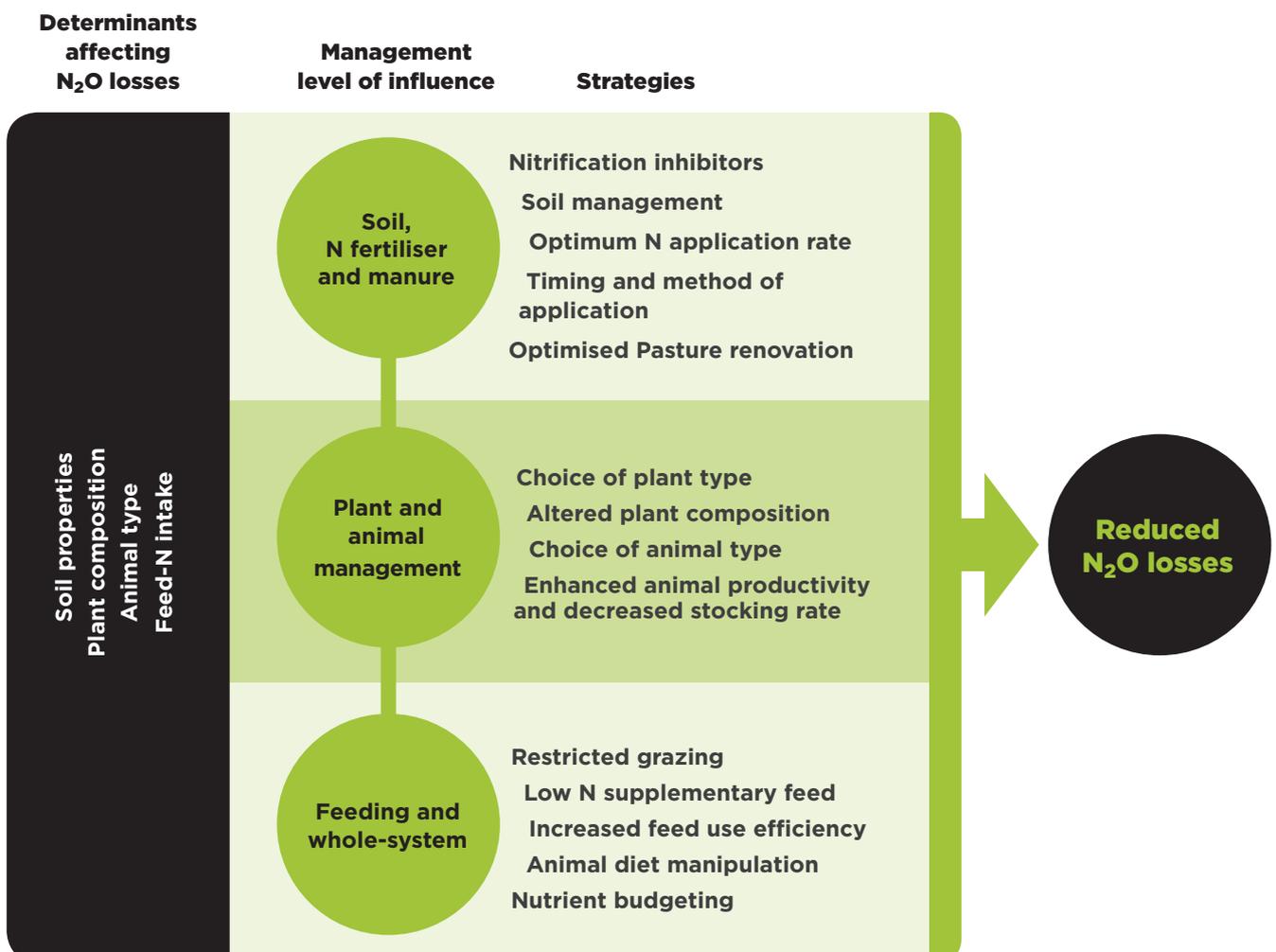


Figure 1. Management strategies for reducing  $\text{N}_2\text{O}$  losses (adapted from Ledgard and Luo, 2008).

A range of on-farm management options exist that have the potential to reduce  $N_2O$  emissions from grazed pastures (see Figure 1). Some are in use by farmers, while others require further research and development. The effectiveness and cost of practices differ and the preferred option, or options, varies between farms depending on economics and practicality.

### SOIL MANAGEMENT

Different soil types have different N loss and  $N_2O$  emission risk properties. For example poorer-draining clay-textured soils generally have higher denitrification and  $N_2O$  losses and lower N leaching than well-draining soils. It is therefore possible that small reductions in  $N_2O$  losses could be achieved by altering soil conditions (e.g. liming, improving drainage and avoiding soil compaction). In practice however the general applicability of these techniques is limited and the results vary.

Soil compaction and moisture are significant risk factors for  $N_2O$  emissions. Forage crops grazed in winter are frequently high in moisture content and especially vulnerable to compaction and high  $N_2O$  emissions. Intensive tillage practices promote soil compaction and therefore are thought to have a significant effect on  $N_2O$  emissions. The use of direct drilling methods to establish forage crops and restricting grazing when soils are wet should reduce the risk of increasing  $N_2O$  emissions. Soil aggregate size is also an important factor as larger soil aggregates are capable of withstanding significantly higher soil compaction.

Renovation of grazed pastures is also a high risk area for  $N_2O$  emissions. Farmers can reduce emissions by minimising the amount of N on pasture before and during the renovation process. Techniques that have been used include removing grazing animals, avoiding N fertiliser application, sowing in spring rather than autumn (when soils are drier) and not returning pasture swards back into the soil. Ploughing during renovation can also lower  $N_2O$  emissions by increasing the aeration of soils.

### APPLICATION OF N FERTILISERS AND FARM EFFLUENT/MANURE

In order to improve the efficiency of fertiliser N use by plants, farmers should consider climate and soil factors (e.g. rainfall and soil drainage) at the time of application and seasonal patterns of plant growth. As  $N_2O$  emissions are highest in wet soils, limiting the amount of N fertiliser applied during late-autumn/winter or early spring, when pasture growth is slow and soil is wet, can decrease  $N_2O$  emissions from grazed pastures. This also reduces the potential for indirect losses of  $N_2O$  from direct leaching of fertiliser N during this high risk period. There are also advantages to delaying effluent application after grazing events to reduce the level of surplus mineral N in soil. While these techniques can reduce  $N_2O$  emissions, they have the potential to lead to N pollution swapping by increasing direct  $N_2O$  emissions and leaching from the soil.  $CO_2$  emissions can also increase because more fuel is required for effluent/manure application.

### NITROGEN PROCESS INHIBITORS

Technologies employing urease and nitrification inhibitors (UI and NI) can be used as effective mitigation alternatives to control N losses. UI's generally act by slowing urea hydrolysis, reducing ammonia volatilisation while NI's delay nitrification and retain soil N in the more immobile  $NH_4^+$  form in soil. Studies have shown that both  $NO_3^-$  leaching and  $N_2O$  emissions from urine patches can be potentially reduced by up to 70% with land application of NI onto pastures.

### PLANT AND ANIMAL SELECTION

Plant characteristics that help reduce  $N_2O$  emissions include increased rooting depths, tannin content, water soluble C content and improved residue quality. Plants with increased rooting depth are able to remove N from a greater depth of soil, potentially reducing the risk of N losses. Changing the composition of plants (e.g. tannin, C and residue contents) can reduce the amount of N in animal feed and therefore reduce N in urine, which is a highly concentrated N source for  $N_2O$  emissions.

The simplest approach to mitigate N<sub>2</sub>O in grazed pasture systems is to reduce animal numbers and therefore the amount of N deposition on pasture through urine and dung. However, as the human population increases so too will animal protein consumption and therefore reducing animal numbers to lower N<sub>2</sub>O emissions is unlikely to be acceptable, unless the animal productivity per animal can be substantially increased. Breeding and selection of grazing animals for increasing productivity is therefore an attractive option to reduce N losses.

### LOW NITROGEN FEED SUPPLEMENT

Pasture typically contains an excess of protein compared to what an animal actually requires. Feed supplements with a low protein concentration (e.g. maize silage) can increase the efficiency of N utilisation. Reducing the protein content of supplement feed has been shown to give up to 20% reductions in estimated N excreted in urine and faeces of dairy cattle. The practice can be effective but must be managed to ensure diets meet animal requirements and do not effect production.

### DIET ADDITIVES OR MANIPULATION

Animal supplementation studies have shown that addition of salt to feed can increase urine volume, decrease urine-N concentrations and increase spread of urine, thereby possibly increasing N efficiency and decreasing N losses. Increasing the hippuric acid concentration in urine has also been shown to reduce N<sub>2</sub>O emissions. Hippuric acid, found naturally in the urine of herbivores, inhibits denitrification in soil.

### RESTRICTING GRAZING

In temperate environments with winter grazing, practices involving the use of stand-off/feed pads or housing systems that are adopted to reduce soil physical damage due to grazing on wet soils, can reduce N<sub>2</sub>O emissions and NO<sub>3</sub><sup>-</sup> leaching. Animals are kept off grazing paddocks so that excreta deposition is reduced at a time when it leads to greatest N losses (e.g. late-autumn/winter). This provides the opportunity to control N losses, as the dung and urine can be collected and applied evenly to the pasture at targeted rates and optimum times when the risk for N losses is minimal. Manure management systems must be closely managed to avoid N pollution swapping (e.g. reducing N leaching from paddocks but increasing NH<sub>3</sub> loss from animal houses). Anaerobic digestion of the animal manure during storage also has the potential advantage of producing CH<sub>4</sub> as biofuel.

### GREENHOUSE GAS EMISSIONS FROM WHOLE FARM SYSTEM AND LIFE-CYCLE-BASED ASSESSMENT

Management of all factors involved in the N cycle to reduce N losses in animal grazing systems is complex, and requires a whole farm systems approach. If management practices are used to reduce N loss in one part of the system, the preserved N can be lost elsewhere in the system if all parts are not equally well managed. Increasing N efficiency within the soil, plants and animals also has the potential to increase pasture and animal productivity, which can adversely affect other GHG emissions on farm. A complete assessment of the impact of a mitigation strategy across the entire farm system is therefore necessary.

A system approach also needs to consider the whole food chain and account for multiple environmental emissions and the efficiency of resource use (e.g. energy). Life Cycle Assessment (LCA) is one tool for such an analysis. Its purpose is to ensure that a full environmental footprint for each system is considered and that potential (and un-intended) issues such as pollution swapping or likely additional energy costs that come from a particular management system and mitigation option are accounted for. There are limitations to LCA assessments as they can be based upon a range of assumptions, for which there is little supporting field data.

### ADOPTION OF MITIGATION OPTIONS

Actual farmer practices and the likelihood of adoption of mitigation technologies is dependent on many factors, including economic, production, environmental and social factors. In order to adopt mitigation technologies farmers need to understand the benefits that come from controlling on-farm emissions. Farmers must also have choice to ensure that there are mitigation practices that fit with their farm business. Adopting N<sub>2</sub>O mitigation strategies has the potential to improve productivity and soil quality and also reduce N and C losses from farm systems (which may be linked to financial reward or penalty).

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## The affect of nitrification inhibitor's and restricted grazing on nitrous oxide and greenhouse gas emissions from grazed pastures

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### BACKGROUND

In many regions of the world animal housing, particularly over winter, is a common practice and has been shown to reduce N<sub>2</sub>O emissions from grazed pasture land. Animals are kept off grazing paddocks for most of the day and as a result less dung and urine is deposited on fields at a time when it could lead to greatest N loss. In temperate regions of the world with milder winter climates, such as New Zealand, animals generally graze pastures all year round and animal housing is less common. However, the practice has been adopted by some farmers in New Zealand to reduce the physical damage to wet soils from grazing during the winter season. Restricted grazing provides the opportunity to control N losses, as animal dung and urine can be collected and applied evenly to the pasture at targeted rates and at times when the risk for N losses is reduced.

<sup>1</sup>DCD was voluntarily removed from the market in January 2013 until there is an international codex residue standard in place for rumen livestock products.

Nitrification inhibitors (NI), such as dicyandiamide (DCD<sup>1</sup>), have also been used to reduce N<sub>2</sub>O emissions from urine patches on pasture. Inhibitors delay nitrification and help to retain nitrogen in the soil (in the form of ammonium ions (NH<sub>4</sub><sup>+</sup>)). A dairy farmlet study in southern New Zealand found that N<sub>2</sub>O emissions in the late autumn/winter seasons were 30-90% lower in the DCD treated farmlets than those that were not treated.

NI's are also being promoted in New Zealand as having potential to decrease nitrate (NO<sub>3</sub><sup>-</sup>) leaching from soils as well as N<sub>2</sub>O emissions, as they may indirectly reduce NO<sub>3</sub><sup>-</sup> production, but there is little information available on this effect for grazed pastures.

Combining the use of both restricted grazing in winter and nitrification inhibitors therefore has potential to further reduce N<sub>2</sub>O emissions from grazed pasture systems. In this study work was undertaken to see if greater reductions in N<sub>2</sub>O emissions from grazed pasture could be achieved by combining the strategies, than the use of either option alone. Along with N<sub>2</sub>O emissions, the farm system as a whole was monitored to determine what effect reducing N<sub>2</sub>O had on total GHG emissions including CH<sub>4</sub> and CO<sub>2</sub>.



## METHODOLOGY

A three year field study was conducted to compare annual N<sub>2</sub>O emission rates from a “tight nitrogen” grazed farmlet with those from a control. The farmlets were established in the Waikato region of New Zealand in 2006 and were 10-20% white clover (*Trifolium repens L.*) and 80-90% perennial ryegrass (*Lolium perenne L.*) pasture on silt loam soil, which was poorly drained with compact subsoil and slow permeability. Winter and spring were relatively wet with cool temperatures, while summer and early autumn were generally moist and warm.

The control farmlet was managed under a conventional rotational all-year grazing regime with 10-20 0.5ha paddocks with a stocking rate of three cows/ha. The “tight nitrogen” farmlet was under similar stocking and grazing regimes, except during winter and early spring when cows grazed for about 6 hours per day with the other 18 hours spent in animal shelters. A nitrification inhibitor (dicyandiamide, DCD) was applied onto the “tight nitrogen” farmlet at 10kg/ha immediately after grazing through winter and early spring. A chamber technique was used to measure N<sub>2</sub>O emissions in several paddocks from each farmlet during three contrasting seasons each year.

The IPCC (Intergovernmental Panel on Climate Change) inventory methodology was used to estimate CH<sub>4</sub> from animal rumen fermentation and indirect N<sub>2</sub>O emissions from sources that were not included in the field measurements, including leached N, land-applied effluent N and volatilised ammonia N. The N flows for the farm systems were calculated using the OVERSEER<sup>®</sup> nutrient budget model which has been validated for New Zealand dairy farm systems.

Life Cycle Assessment (LCA) methodology was used to calculate CO<sub>2</sub> emissions from the farm systems; including the use of fuel, electricity, fertiliser, lime, feeds, effluent and DCD. The method accounted for all components of these systems including extraction of raw materials, manufacturing, transportation and application.

The individual and combined effects of restricted grazing and DCD use on N<sub>2</sub>O emissions were also determined.

## RESULTS AND DISCUSSION

This study is the first publication that has presented measurements of N<sub>2</sub>O emissions from grazed dairy pasture farmlets covering a three year period.

It was found that during late spring/summer and autumn, N<sub>2</sub>O emission rates were generally similar between the control and farmlet. When restricted grazing and DCD were used in combination during the winter/early spring seasons, N<sub>2</sub>O emissions from “tight nitrogen” farmlet were significantly reduced when compared to the control where the strategies were not used. N<sub>2</sub>O emissions were 43-55% less in year one, 64-79% in year two and 45-60% in year three over the winter/early spring. On average the annual N<sub>2</sub>O emission rate from the “tight nitrogen” farmlet was 20% lower than the control, demonstrating the effectiveness of these mitigation technologies. However the use of either restricted grazing or DCD alone gave similar results, showing that there was no significant benefit seen in this study by combining the two technologies. As both strategies target the same source of nitrogen at the same time, this result is not unexpected. Further benefits might be seen if the timing of reduced grazing and the application of nitrification inhibitors were staggered.

When measuring total GHG emissions for the farm system it was found that there was a 5% overall reduction in emissions over the study period. Total annual emissions for the sum of the three major GHGs, N<sub>2</sub>O, CH<sub>4</sub> and CO<sub>2</sub>, were estimated at 13,267 kg CO<sub>2</sub>-equivalent per hectare from the control and 12,669 from the “tight nitrogen” dairy farm. There were slight increases in methane and CO<sub>2</sub> production in the trial and this in combination with the greater proportion of GHG emissions being due to methane (CH<sub>4</sub>;57-60%, N<sub>2</sub>O;24-27%), meant that the full effect of the nitrous oxide mitigation was offset. The result, while still positive, highlights the importance of looking at the impact of mitigation strategies on a whole farm system, rather than in isolation, when evaluating the value of GHG mitigation practices.

Reducing emissions could have potential economic benefits if carbon costs are imposed. Based on a 5% reduction in GHG per year and assuming a carbon cost of \$25 per tonne of CO<sub>2</sub> equivalent, the benefit of using restricted grazing and nitrification inhibitors has been estimated at \$15 ha<sup>-1</sup> yr<sup>-1</sup>. While in practice this saving is small compared to the saving from reduced nitrogen fertiliser use (c. \$50 ha<sup>-1</sup> yr<sup>-1</sup>) or the costs of DCD (c. \$200 ha<sup>-1</sup> yr<sup>-1</sup>) or animal housing facilities (c. \$150 ha<sup>-1</sup> yr<sup>-1</sup>), other benefits that result from using these mitigations, such as reduced nitrogen losses to waterways and reduced soil damage, may be significant if whole-system economics are taken into account.

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Luo J, Ledgard SF, Lindsey SB 2013. Nitrous oxide and greenhouse gas emissions from a grazed pasture as affected by use of a nitrification inhibitor and a restricted grazing regime. *Science of the Total Environment* 465: 107-114.



## Nitrous oxide emissions from animal urine application on a New Zealand pasture

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### BACKGROUND

The nitrogen (N) deposited onto grazed pastures in urine by livestock provides high localised concentrations of N and C in soils and is the main source of N<sub>2</sub>O emissions from pasture. Typically, 70-90% of the N in urine is present as urea; the rest is present in amino acids and peptides. Urea is converted to ammonium ions (NH<sub>4</sub><sup>+</sup>) and releases N<sub>2</sub>O as the NH<sub>4</sub><sup>+</sup> undergo nitrification and denitrification processes. Animal urine has been identified as the single largest source of N<sub>2</sub>O on pasture.

Studies have shown that variation in N<sub>2</sub>O emissions and denitrification rates can be due to weather patterns, particularly patterns of rainfall. In general N<sub>2</sub>O emissions are increased when the water content of the soil increases, which usually occurs in winter. Understanding the extent and seasonal variation of N<sub>2</sub>O emissions from animal urine is important for the development of best management practices for reducing N<sub>2</sub>O losses.

### METHODOLOGY

In a two year study the application of urine to pasture was monitored and compared to pasture where no urine was applied. The experimental site was a white clover based pasture (perennial ryegrass, *Lolium perenne*; white clover, *Trifolium repens*) on Te Kowhai silt loam soil. The soil was poorly drained with compact subsoil and slow permeability. In the two months before the trial no fertiliser was applied or grazing allowed on the site.

Urine was applied to the pasture at a rate of 1000kg N/ha on the experimental site. Nothing was applied to the control. Nitrous oxide emissions were measured using a closed soil chamber technique. Soil properties including soil water content (SWC), N content and soil porosity were recorded over the course of the study and values for water filled pore space (WFPS) calculated from the data.

### RESULTS AND DISCUSSION

Over the course of the study there was a marked difference in seasonal temperature and rainfall; winter and spring were relatively wet with cool temperatures while summer and early autumn were dry and warm.

Application of cow urine to the experimental site significantly increased N<sub>2</sub>O emissions above those of the control for up to six weeks following application, the duration of the increase depending on the season. Nitrous oxide emissions were higher during the winter and spring when the moisture levels were high and the soil was saturated with water (i.e. the water filled pore space (WFPS) was high; >65%). Emissions were lower during the summer and autumn periods, when the soil was drier. Applying urine also increased soil nitrate (NO<sub>3</sub><sup>-</sup>) N. Increased N<sub>2</sub>O emissions generally coincided with increased soil NO<sub>3</sub><sup>-</sup> concentrations, particularly when soil moisture levels were high enough for denitrification.

There was a definite seasonal effect seen in the trial suggesting that a reduction in urine on the soil (e.g. through use of stand-off pads or animal housing) under wet conditions in New Zealand could potentially reduce N<sub>2</sub>O emissions from pastoral soils.



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Luo J, Lindsey S B, Ledgard S F 2008. Nitrous oxide emissions from animal urine application on a New Zealand pasture. *Biology and Fertility of Soils* 44: 463-470.



## Effects of dairy farming intensification on nitrous oxide emissions

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### BACKGROUND

Estimates indicate that N<sub>2</sub>O emissions in 2004 from New Zealand agriculture produced 39,980 tonnes of N<sub>2</sub>O annually, which represents 16.5% of national GHG (CO<sub>2</sub>-equivalent) emissions. New Zealand's target for 2008-2012 under the Kyoto Protocol is to reduce GHG emissions to 1990 levels. However, the estimated emissions of N<sub>2</sub>O in 2004 were about 25% higher than in 1990. The increase is has been linked to livestock farming intensification, particularly on dairy farms where New Zealand has steadily increasing stocking rates, increased feed supply and supplementary forage feeds.

There are a number of possible management options that can reduce N<sub>2</sub>O emissions from dairy farms. These options include using restricted grazing regimes, nitrification inhibitors, applying fertiliser at optimum times and using low-N feed supplements as an alternative to using N-rich pasture. This study was undertaken to determine the effect of combining fertiliser N-boosted pasture with low-protein maize silage in-order to reduce environmental N emissions.

### METHODOLOGY

The experimental site was a white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) pasture on Te Kowhai silt loam soil in the Waikato region of New Zealand. The soil was poorly drained with compact subsoil and slow permeability. Winter and spring at the study site were relatively wet with cool temperatures, whereas summer and early autumn were dry and warm.

Prior to this study a Resource Efficient Dairy (RED) farmlet system trial had been established at the experimental site to evaluate intensive dairy farm systems involving treatments with integration of N fertiliser, maize silage and winter management strategies. The RED trial was made up of six farmlets stocked with Holstein-Friesian cows. Milk yield, crude protein and N concentration were measured by weekly herd tests and detailed information on the animal management regime, pasture composition and external inputs was available for this system. Nitrous oxide emissions from two of the farm systems in the RED farmlet system trial were evaluated in this study. The first was the



control, which operated a normal rotational pasture grazing regime with no external feed brought in and the second a trial which used maize supplementation to cows on pasture. The maize supplement farmlet used the same rotational grazing regime, but the pasture received urea application through the year (175kg urea-N ha<sup>-1</sup> y<sup>-1</sup>) and maize silage (5.6 t DM ha<sup>-1</sup>) was brought in annually, from a maize growing field located nearby. Maize silage was fed in autumn and winter when pasture growth was slow.

A closed chamber technique was used to measure N<sub>2</sub>O emissions both on the grazed pasture and on the maize-growing land during the trial. Measurements on pasture were staggered around grazing events and always occurred at least one month after urea fertiliser application so direct emissions from the urea would not affect the trial results.

The New Zealand IPCC inventory methodology was used to calculate N<sub>2</sub>O emissions from sources that were not included in the field measurements including leached N, land-applied effluent N and volatilised ammonia N.

Soil properties including soil water content (SWC), N content and soil porosity were recorded over the course of the study and values for water filled pore space (WFPS) calculated from the data.

## RESULTS AND DISCUSSION

Nitrous oxide emission rates from the grazed pastures showed markedly different seasonal variation. Rates were highest in spring/early summer, followed by late autumn/winter and then summer/early autumn. This is largely explained by seasonal variations in soil water content and also higher grazing intensity on the pastures during late autumn and winter where more N is released onto compacted water rich soils. Annual average N<sub>2</sub>O emissions were slightly lower on the maize supplementation pasture than on the control pasture.

Nitrous oxide emission rates from the maize growing land significantly increased when compared to grazed pasture. The higher emission rates were probably due to higher nitrate-N levels resulting from the application of N fertiliser to the land and short term physical changes in soil properties associated with land use changes between pasture and arable cropping.

Overall the use of maize supplementation increased the total N<sub>2</sub>O emissions per hectare of grazed pasture by about 4% compared to the control farm. Reductions in the measured emission on the maize supplementation pasture were offset by increased N<sub>2</sub>O emissions from the maize growing land, N fertiliser use and land application of farm effluent when the whole farm system was considered. Total annual N<sub>2</sub>O emissions were 7.71 and 8.00kg N<sub>2</sub>O-N per hectare of dairy farm on the control and maize supplement farm systems, respectively.

Average annual milk production was measured as 13,437kg ha<sup>-1</sup> on the control farm and 17,925kg ha<sup>-1</sup> on the maize supplement farm, while the total amount of N in dung and urine excreted was similar. Calculations show that N<sub>2</sub>O emission per kg of milk production was actually 22% lower when using maize supplementation compared to the control. Much higher milk production is due to the high conversion of N in low-protein maize into milk. The results therefore suggest that using low-protein forage can be an effective management practice to reduce the adverse environmental effects N<sub>2</sub>O emissions from increasing stocking rates in the New Zealand dairy farm systems, if N<sub>2</sub>O emissions per unit of milk production are considered.

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# PGgRc RESEARCH OUTPUTS

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Luo J, Lindsey SB, Ledgard SF, Sun W, Kear M (2007) Nitrous oxide emissions from "prototype farms for dairy farming's future". A poster presentation at the International Greenhouse gases and animal agriculture conference, Christchurch, 26-29 November 2007.

Luo J, Lindsey S, Ledgard S, Sun W, Kear M (2008) Nitrous oxide emissions from dairy farmlets, as affected by use of a winter restricted grazing strategy and a nitrification inhibitor: year 1. A poster presentation at the Massey FLRC workshop.

Luo J, Saggart S (2007) Nitrous oxide and methane emissions from a dairy farm stand-off pad. A poster presentation at the International Greenhouse gases and animal agriculture conference, Christchurch, 26-29 November 2007.

Pinares-Patino,C,S;Rochette,Y;Koolaard,J,P;Jouany,J.-P.;Clark,H;Martin,C 2007. Effect of SF<sub>6</sub> tracer permeation rate upon calculated ruminal methane production rates using rumen head space gas composition. Proceedings of the 3rd International Greenhouse Gases and Animal Agriculture Conference (GGAA2007), held at The Hotel Grand Chancellor, Christchurch, 26-29 November.

Patino, C, S; Lovejoy, P, J; Hunt, C; Martin, R; Molano, G; Waghorn, G; Clark, H 2008. A versatile sheep respiration chamber system for measurement of methane emission. New Zealand Society of Animal Production Annual Conference.

Gina Pedersen, D. Neil Wedlock, Peter Janssen, Michel Denis and Bryce M. Buddle Development of a vaccine to reduce methane emissions. Queenstown Molecular Biology Meeting from 2-4th September 2008.

Luo J, Lindsey S, Ledgard S, Sun W and Kear M (2008) Nitrous oxide emissions from dairy pasture systems in New Zealand. In: Multifunctional grasslands in a changing world, Volume 1 (ed. Organizing Committee of 2008 IGC/IRC Conference), pp. 327-327. Guangdong People's Publishing House.

Luo, J; Sun, W; Lindsey SB; Ledgard, SF (2008) Denitrification N loss from a grazed pasture as affected by the use of a nitrification inhibitor in wet winter. Paper presented to Soils 2008 at Massey University, Palmerston North, New Zealand.

Luo J, Aspin M, Ledgard SF, de Klein CAM, Lindsey S (2009) Management options to reduce nitrous oxide emissions from dairy farms. In: Nutrient management in a rapidly changing world. (Eds L.D. Currie and C. L. Lindsay). Occasional Report No. 22. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. Pp 47-55.

Sun W, Luo J, Lindsey S, Ledgard S (2008) Nitrogen losses through denitrification from a grazed pasture as affected by the use of a nitrification inhibitor. In: Multifunctional grasslands in a changing world, Volume 1 (ed. Organizing Committee of 2008 IGC/IRC Conference), pp. 385-385. Guangdong People's Publishing House.

Ledgard SF, Luo J (2008) Nitrogen cycling in intensively grazed pastures and practices to reduce whole-farm nitrogen losses. In: Multifunctional grasslands in a changing world, Volume 1 (ed. Organizing Committee of 2008 IGC/IRC Conference), pp. 292-297. Guangdong People's Publishing House.

Kenters N, Henderson G, Kittelmann S, Janssen PH (2009) Rapid isolation of novel anaerobic bacteria. Poster presentation. 68th Annual Assembly of the Schweizerische Gesellschaft für Mikrobiologie, 4 - 5 June 2009. University of Lausanne, Lausanne, Switzerland.

Jeyanathan J, Ronimus R, Hoskin SO, Janssen PH (2009) Diversity of rumen methanogens in New Zealand ruminants fed different diets. Oral presentation. 11 August 2009. Institute of Veterinary, Animal, and Biomedical Sciences, Massey University, Palmerston North, New Zealand.

Wedlock N, Pedersen G, Dey D, Janssen PH, Denis M, Buddle B (2009) Development of a sub-unit vaccine to reduce methane emissions in ruminants. Poster presentation. 39th Annual Meeting of the Australasian Society for Immunology, 6 - 10 December 2009. Broadbeach, Queensland, Australia.

Kelly WJ, Leahy SC, Altermann E, Ronimus RS, Pacheco DM, Li D, Yeoman CJ, Kong Z, McTavish S, Sang C, Lambie S, Janssen PH, Dey D, Attwood GT (2009) The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium*: identifying targets for controlling ruminant methane emissions. Oral presentation. COMBIO 2009, 6 - 10 December 2009. Christchurch, New Zealand.

Henderson G, Naylor GE, Leahy SC, Janssen PH (2009) Identification of new, potentially homoacetogenic bacteria in the rumen after analysis of formyltetrahydrofolate synthetase gene sequences. Oral presentation. COMBIO 2009, 6 - 10 December 2009. Christchurch, New Zealand.

Pedersen G, Wedlock N, Dey D, Janssen PH, Denis M, Buddle B (2009) Development of a sub-unit vaccine to reduce methane emissions in ruminants. Poster presentation. COMBIO 2009, 6 - 10 December 2009. Christchurch, New Zealand.

Attwood GT, Altermann E, Kelly B, Leahy SM, Zhang L, Morrison M (2010) Exploring rumen methanogen genomes to identify targets for methane mitigation strategies. Invited lecture. GGAA2010 conference, 3 - 10 Oct 2010, Banff, Canada.

Dynes RA, Burggraaf VT, Dalley DE, Goulter CG. (2010). Farming Systems of Canterbury. Keynote address joint meeting of Australian Agronomy Society and New Zealand Grasslands Association 26 November 2010.

Dynes RA, Smeaton DC, Rhodes AP, Fraser TJ, Brown MA. (2011) Modelling farm management scenarios that illustrate opportunities farmers have to reduce greenhouse gas emissions while maintaining profitability. Invited presentation in a contract: Continuing to increase productivity while maintaining our clean green image: Proceedings of the NZ Society of Animal Production 71 149-178.

Hegarty RS, McEwan LC (2010) Genetic opportunities to reduce enteric methane emissions from ruminant livestock. Invited presentation. 9th World Congress on Genetics Applied to Animal Production, 1-6 August 2010, Leipzig, Germany.

Hoogendoorn CJ, Brown MA, Dynes RA, Fraser TJ, Smeaton DC, Rhodes AP, Walcroft JL (2011) Raising farmer awareness and understanding of climate change and green house gases - impacts, adaptations and mitigations. Invited lecture. 2011 International Forum on Grassland Ecology, 13-14 July 2011, Hoh Hot, Inner Mongolia, China.

Janssen PH (2011) Rational discovery of methane mitigation strategies for NZ farming systems. Oral presentation. New Zealand Agricultural Greenhouse Gas Research Centre Conference, Public Day, 22 February 2011. Massey University, Palmerston North, New Zealand.

Janssen PH, Jeyanathan J, Kittelmann S, Henderson G (2010) Ruminant methane mitigation - applied microbial ecology. Invited lecture. New Zealand Microbiological Society and New Zealand Society for Biochemistry and Molecular Biology Joint Meeting, 30 Nov - 3 Dec 2010, Auckland, New Zealand.

Jeyanathan J, Dey D, Molano G, Pinares-Patiño C, Knight TW, Clark H, Ronimus RS, Janssen PH (2010) Rumen Cluster C (RCC) - an uncultured archaeal group living in the rumen. Oral presentation. New Zealand Microbiological Society and New Zealand Society for Biochemistry and Molecular Biology Joint Meeting, 30 November - 3 December 2010. Auckland, New Zealand.

Kittelmann S, Janssen PH (2010) Characterisation of ciliate communities in domestic ruminants - an opportunity for methane mitigation. Oral presentation. 13th International Symposium on Microbial Ecology; 22 - 27 Aug 2010, Seattle, USA.

Muetzel S, Hunt CL, Tavendale MH (2011). A new automated *in vitro* rumen batch culture system for continuous measurements of total gas, methane and hydrogen production. Oral presentation. Proceedings of the New Zealand Society of Animal Production Society 71th Annual Conference, 29 June - 1 July 2011, Invercargill, New Zealand.

Pinares-Patiño CS, Gere JI, Williams KE, Gratton R, Juliarena MP, Molano G, Fernandez M, MacLean S, Sandoval E, Taylor G, Koolaard JP (2010) Extending the collection length of breath samples for methane emission estimation using the SF6 tracer technique. Oral presentation. GGAA2010 conference; 3 - 10 Oct 2010, Banff, Canada.

Janssen PH (2012) Manipulating rumen microbial processes to mitigate methane emissions in grazing systems. Oral presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

McEwan J (2012) Irish beef genetics research or there is no such thing as a free lunch. Oral presentation at Teagasc Workshop, Portlaoise, Ireland, 28 March 2012.

McEwan J (2012) Animal variation in methane emission. Oral presentation at Teagasc Workshop, Portlaoise, Ireland, 28 March 2012.

McEwan J (2012) Animal variation in methane emission. Oral presentation at UCD, Dublin, Ireland, 29 March 2012.

McEwan J, Pinares-Patiño C (2012) Heritability estimates for methane emissions in sheep and correlations with production traits. Oral presentation at Australia-NZ Workshop: Reducing Emissions from Livestock through Genetics and Breeding, Sydney, New South Wales, Australia, 15 May 2012.

Morgavi DP, Kelly WJ, Janssen PH, Attwood GT (2011). Rumen microbial (meta) genomics and its application to ruminant production. Oral presentation at 8th International Symposium on the Nutrition of Herbivores (ISNH8). Aberystwyth, UK, 6 - 9 September 2011.

Pinares-Patiño CS, MacLean S, McEwan J, Hickey S, Dodds K (2012) Measurements of methane in NZ sheep - experimental design and repeatability of various methods. Oral presentation at Australia-NZ Workshop: Reducing Emissions from Livestock through Genetics and Breeding, Sydney, New South Wales, Australia, 15 May 2012.

Pinares-Patiño CS, MacLean S, McEwan J, Hickey S, Dodds K (2012) Associations between rumen characteristics and methane emissions from sheep. Oral presentation at Australia-NZ Workshop: Reducing Emissions from Livestock through Genetics and Breeding, Sydney, New South Wales, Australia, 15 May 2012.

Ronimus RS (2012) Enhanced discovery of anti-methanogen inhibitors. Oral presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

Sun XZ, Pacheco D, Hoskin SO, Luo DW (2012) Rumen fermentation characteristics are influenced by feeding frequency in sheep fed forage chicory and perennial ryegrass at two feeding levels. Oral presentation at 2012 Joint Meeting of the Australian Society of Animal Production and the New Zealand Society of Animal Production, Lincoln, New Zealand, 2 -5 July 2012.

Wedlock DN, Shu D, Zheng T, Dey D, Janssen PH, Buddle BM (2011) Development of a vaccine to reduce methane emissions in ruminants. Oral presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

Wedlock N (2012) Development of a vaccine to reduce methane emissions in ruminants. Oral presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

## Posters

Altermann E, Kelly W, Leahy S, Lambie S, Janssen PH, Zhang L, Klieve A, Ouwerkerk D, Morrison M, Attwood G. (2010) A comparative genomics analysis of rumen methanogens. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Carbone V (2010) Crystallisation and *in silico* modelling of enzymes from the methanogen *Methanobrevibacter ruminantium* for the discovery of novel inhibitors. Poster presentation. Queenstown Molecular Biology Meeting - QMB2010, 02 - 03 September 2010, Queenstown, New Zealand.

Hammond K, Hoskin SO, Muetzel S, Burke JL, Waghorn GC (2010) Effect of feeding fresh white clover or perennial ryegrass on methane emissions from sheep. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Kittelmann S, Janssen PH (2010) Characterisation of ciliate communities in domestic ruminants - an opportunity for methane mitigation. Poster presentation. GGAA2010 conference; 3 - 10 Oct 2010, Banff, Canada.

Knight T, Ronimus RS, Dey D, Tootill C, Naylor G, Evans P, Molano G, Smith A, Tavendale M, Pinares-Patino C, Clark H (2010). Medium-term effects on methanogens and rumen function in cattle due to a methanogenesis inhibitor. Poster presentation. Greenhouse Gases and Animal Agricultural Conference (GGAA) 2010, 3-8 October 2010, Banff, Canada.

Pinares-Patiño CS, Knight T, Ronimus RS, Dey D, Tootill C, Naylor G, Evans P, Molano G, Smith A, Tavendale M, Clark H (2010) Medium-term effects of chloroform on methanogens and rumen function in cattle. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Pinares-Patiño CS, McEwan JC, Dodds KG, Muetzel S, Cárdenas EA, Hegarty RS, Koolaard JP, Clark H (2010) Repeatability of methane emission from sheep. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Schofield L (2010) Expression and purification of enzymes from the methanogen *Methanobrevibacter ruminantium* for discovery of novel inhibitors. Poster presentation. 13th International Conference on the Crystallisation of Biological Macromolecules, 12-16 September 2010, Dublin, Ireland.

Sun XZ, Hoskin SO, Muetzel S, Molano G, Clark H (2010). Chicory does not reduce methane emissions from sheep. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Swainson N, Hoskin SO, Brookes I, Clark H (2010) The SF6 technique for measuring CH4 emissions: Effect of residence time of permeation tubes. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Wedlock DN, Pedersen G, Wilson T, Baker J, Dey D, Janssen PH, Buddle BM (2010) Immunological tools to reduce greenhouse gas emissions. Poster presentation. GGAA2010, 03 - 10 October 2010, Banff, Canada.

Altermann E, Schofield LR, Lambie S, Boschian A, Kelly B, Leahy SC, Attwood GT (2011) Initial characterisation of the novel *Methanobrevibacter ruminantium* virus phi mru. Poster presentation at the Gordon Archaea Conference, Waterville Valley, New Hampshire, USA, 31 July - 5 August 2011.

Altermann E, Tootill C, Zhang Y, Al-Attar S, Engelhart R, Wolf M, Beattie AK, Tootill CM, Dey D, Muetzel S, Cook GM, Sutherland-Smith A, Attwood GT, Carbone V, Ronimus RS (2011) Crystallisation, X-ray structure determination and *in silico* modelling of *Methanobrevibacter ruminantium* enzymes for the discovery of novel inhibitors. Poster presentation at the Gordon Archaea Conference, Waterville Valley, New Hampshire, USA, 31 July - 5 August 2011.

Carbone V, Schofield LR, Beattie AK, Attwood GT, Sutherland-Smith AJ, Ronimus RS (2011) The crystal structure of methenyltetrahydromethanopterin cyclohydrolase from *Methanobrevibacter ruminantium*: modelling substrate binding of N5-formyl-5,6,7,8 tetrahydromethanopterin. Poster presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

Carbone V, Schofield LR, Beattie AK, Attwood GT, Sutherland-Smith AJ, Ronimus RS (2012) The crystal structure of Apo- and GTP-bound GTP cyclohydrolase from *Methanobrevibacter ruminantium*. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

Cookson AL, Lambie S, Kelly WJ, Leahy SC, Henderson G, Attwood GT, Janssen PH, Hatton V, Clark H (2012) Global co-ordination of efforts towards the development of worldwide methane mitigation and rumen adaptation technologies using a Rumen Microbial Genomics Network approach. Poster presentation at 8th INRA-RRI Symposium on Gut Microbiology, Clermont-Ferrand, France, 17 -20 June 2012.

Cox F, Zethof M, Heidarian Miri V, Kittelmann S, Janssen PH, Henderson G (2011) Comparison of different DNA extraction methods for cow and sheep rumen samples. Poster presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

Cox F, Zethof M, Heidarian Miri V, Kittelmann S, Janssen PH, Henderson G (2012) Comparison of different DNA extraction methods for cow and sheep rumen samples. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

Henderson G, Tavendale M, Raju P, Janssen PH (2012) Identifying alternative hydrogen utilisers in ruminants. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

Kelly WJ, Attwood GT, Cookson AL, Goodwin L, Woyke T, Henderson G, Leahy SC, (2012) The Hungate 1000. A catalogue of reference genomes from the rumen microbiome. Poaster at 8th INRA-RR1 Symposium on Gut Microbiology, Clermont-Ferrand, France, 17 -20 June 2012.

Kittelmann S, Naylor GE, Koolaard JP, Janssen PH (2011) Molecular detection of novel groups of anaerobic fungi (class *Neocallimastigomycetes*) in New Zealand ruminants and development of a revised taxonomy suitable for sequence-based high-throughput community structure analysis. Poster presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

McEwan JC, Hickey S, Dodds KG, Young E, McLean, S, Kjestrup H, Sandoval E, Molano G, Pinares-Patiño C. Rapid measurement of methane emissions for estimation of animal variation. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Conference, Palmerston North, New Zealand, 1- 2 February 2012.

Ronimus RS, Dey D (2011) Effect on the growth of the rumen methanogen *Methanobrevibacter ruminantium* by antibiotics, mutagens and known antimicrobial compounds. Poster presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

Ronimus RS, Zhang Y, Al-Attar S, Engelhart R, Wolf M, Beattie AK, Tootill C, Dey D, Muetzel S, Cook GM, Sutherland-Smith A, Attwood GT, Carbone V, Schofield LR (2011) Using enzymes from *Methanobrevibacter ruminantium* to help discover novel methanogen-specific inhibitors for mitigation of rumen methane emissions. Poster presentation at ComBio 2011, Cairns, Queensland, Australia, 25 - 29 September 2011.

Schofield LR, Zhang Y, Al-Attar S, Engelhart R, Wolf M, Beattie AK, Tootill CM, Dey D, Muetzel S, Cook GM, Sutherland-Smith A, Attwood GT, Carbone V, Ronimus RS (2011) Expression, purification, crystallisation, X-ray structure determination and *in silico* modelling of *Methanobrevibacter ruminantium*

enzymes for the discovery of novel inhibitors. Poster presentation, Queenstown Molecular Biology Conference (QMB2011), Queenstown, New Zealand, 29 - 31 August 2011.

Schofield LR, Zhang Y, Sang C, Beattie AK, Dey D, Sutherland-Smith AJ, Carbone V, Ronimus RS (2012) Utilising chemogenomics techniques to rapidly find novel rumen methanogen control agents. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Conference, Palmerston North, New Zealand, 1- 2 February 2012.

Wedlock DN, Shu D, Zheng T, Attwood GT, Janssen PH, Buddle BM (2012) Development of a vaccine to reduce methane emissions in ruminants. Poster presentation at New Zealand Agricultural Greenhouse Gas Research Centre Annual Conference, Palmerston North, New Zealand, 31 January 2012.

Zhang Y, Schofield LR, Beattie AK, Carbone V, Ronimus RS (2011) Expression, purification and characterisation of L-sulfolactate dehydrogenase (ComC) from *Methanobrevibacter* species SM9. Poster presentation at New Zealand Microbiological Society Annual Meeting, Palmerston North, New Zealand, 23 - 25 November 2011.

## Other outputs

Hoskin SO, Nicolson MJ, Swainson NM, Joblin KN (2007) Lowering ruminant methane: deer, diet and protozoa. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 22-23. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Janssen PH (2007) Techniques for monitoring changes in the rumen. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 16-17. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Kleive A, Joblin K (2007) Comparison in hydrogen utilisation of ruminal and marsupial reductive acetogens. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 34-35. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Nicholson MJ, Janssen PH, Evans PN, Joblin KN (2007) Analysis of methanogen diversity in the rumen using temporal temperature gradient gel electrophoresis. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 18-19. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Ronimus R, Tootill C, Dey D, Kirs M (2007) Methanogen-protozoal interactions. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 20-21. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Olson K, Ronimus R (2007) Applications of phage therapy to reduce ruminant methane emission. In: Kennedy R (editor) Pastoral Greenhouse Gas Research Consortium (PGgRc) 5 Year Science Progress Report, pp. 24-25. Pastoral Greenhouse Gas Research Ltd, Wellington, New Zealand [ISBN 978-0-473-13021-3]

Luo J, Lindsey S, Ledgard S (2008) Nitrous oxide emissions from DairyNZ's Prototype dairy farms (2006-2007) – Progress report. Report prepared for PGGRC.

Luo J, Ledgard S.F, Lindsey S.B. (2007). Nitrous oxide emissions from dairy farms: sources and management options to reduce emissions. In: PGGRC (Pastoral Greenhouse Gas Research Consortium) 5 year Progress Report 2002-2007. Pp. 72-73.

Luo J, Lindsey S, Ledgard S (2008) Nitrous oxide emissions as affected by the use of dairy effluent . Report prepared for PGGRC.

Luo J, Saggart S, Ledgard S.F. (2007) Nitrous oxide and methane emissions from a livestock stand-off pad. In: PGGRC (Pastoral Greenhouse Gas Research Consortium) 5 year Progress Report 2002-2007. Pp. 76-77.

Buddle, B.M. Vaccine to reduce methane emissions. National Radio, 29 November 2007.

Lee A. 2008. Greenhouse Gas Conference: simple jab may beat methane. Dairy Exporter, February 2008 (based on an interview with Bryce Buddle).

Buddle, B.M., Denis, M., Janssen, P., Pedersen, G., Wedlock, D.N. Immunological tools to reduce greenhouse gas emissions. NZ branch Australasian Society for Immunology meeting, Wellington, 5-6 June 2008.

Wedlock, D.N., Denis, M., Pedersen, G., Janssen, P., Buddle, B.M. 2008. A vaccine to reduce greenhouse gas emissions. Presentation for the World Environment Day, Palmerston North, 5 June 2008.

Buddle, B.M., Denis, M., Pedersen, G., Wedlock, D.N. 2008. Towards a vaccine against methanogens. Presentation to the Pastoral Greenhouse Gas Research Consortium Board of Directors, 6 June 2008.

Buddle, B.M., Wedlock, D.N., Denis, M. Vaccine against methanogens. Climate Change Summit. Palmerston North, 13-14 February 2008.

Luo J, Lindsey S, Ledgard S (2008) Nitrous oxide emissions from DairyNZ's Prototype dairy farms (2006-2008) – progress report. Prepared for PGGRC.

Luo J, Ledgard SF, Lindsey S (2009) An update of the study on nitrous oxide emissions from DairyNZ's Prototype dairy farms and effects of DCD on denitrification. Prepared for PGGRC.

Luo J, Ledgard SF, de Klein CAM (2009) Nitrous oxide emissions from intensively grazed pastures and potential mitigation measures. Prepared for PGGRC.

Janssen PH (2009) Rumen microbial ecology. Oral presentation to Pastoral Greenhouse Gas Research Consortium Commercial Advisory Group, 27 July 2009. Bio Commerce Centre, Fitzherbert Science Centre, Palmerston North, New Zealand

Janssen PH (2009) Lytic enzymes and chemogenomics. Oral presentation to Pastoral Greenhouse Gas Research Consortium Commercial Advisory Group, 27 July 2009. Bio Commerce Centre, Palmerston North, New Zealand

Janssen PH, Altermann E, Ronimus RS, Buddle BM (2009) Microbiology and immunology research to mitigate ruminant methane. Oral presentation to Hon. David Carter, Minister of Agriculture, 29 September 2009. Grasslands Research Centre, AgResearch, Palmerston North, New Zealand

Janssen PH (2010) Sustainable Land Management and Climate Change research at AgResearch. Oral presentation to Combined Meeting of the Peak Group and Technical Working Groups for Sustainable Land Management and Climate Change, 18 March 2010. Pastoral House, Wellington, New Zealand

Janssen PH (2010) Methane mitigation: targeting rumen methanogens. Oral presentation to Global Research Alliance on Agricultural Greenhouse Gases Senior Officials, 10 April 2010. Bio Commerce Centre, Palmerston North, New Zealand

Janssen PH (2010) Methane mitigation: targeting rumen methanogens. Oral presentation to Meat and Wool New Zealand Board, 4 May 2010. New Zealand Agricultural Greenhouse Gas Research Centre, Palmerston North, New Zealand

Janssen PH (2010) New Zealand Agricultural Greenhouse Gas Research Centre. Oral presentation to GreenDrinks Meeting, 18 May 2010. Steeple Conference Centre, Kingsgate Hotel, Palmerston North, New Zealand

Janssen PH, Buddle BM (2010) Methane mitigation: targeting rumen methanogens. Oral presentation to Understanding Climate Change and Greenhouse Gas Emissions, Impacts, Adaptations, and Mitigation Options on Farms – Farmers Project Summit, 25 May 2010. AgResearch, Palmerston North, New Zealand

Brown M (2011). Presentation at B+L Regional Managers Workshop, Napier.

Carbone V, Schofield L, Ronimus R (2011) Synchrotron Report from AgResearch to the Royal Society of New Zealand: Foundation investor access on the MX2 beam line at the Australian Synchrotron (AS111/MX2/3182). Report to the Royal Society of New Zealand.

Dynes RA. (2010). Farmers understanding climate change and GHG: what have scientists learnt? Presentation to Lincoln Plant Sciences Seminar Series, 15 August 2010.

McEwan JC (May 2011) Presentation 'DNA samples types and storage'. GRA International Workshop 'Enteric CH<sub>4</sub> mitigation using animal selection, genetics and genomics', Auckland, NZ.

McEwan JC and Pinares C. (2011) Breeding low methane sheep [www.agresearch.co.nz/our-science/animal-performance/sustainability/Pages/default.aspx](http://www.agresearch.co.nz/our-science/animal-performance/sustainability/Pages/default.aspx)

Morris CA (2011). Travel Report to New Zealand Society of Animal Production on visit to Research Stations in the UK to consider laser measurements for methane estimation in individuals from large groups of cattle or sheep. Report to New Zealand Society of Animal Production.

Pinares-Patiño CS (2011). The SF<sub>6</sub> tracer technique for methane emission measurement. Oral presentation. GRA International Workshop, Palmerston North, New Zealand.

Pinares-Patiño CS, McEwan JC, Morris C, Dodds KG (2011). Methane measurement techniques and animal variation in CH<sub>4</sub> emission. Oral presentation. GRA International Workshop on Enteric CH<sub>4</sub> mitigation using animal selection, genetics and genomics, Auckland, New Zealand.

Pinares-Patiño CS (2010). Recent research advances on mitigation of enteric methane emissions from ruminants in NZ Department of Animal Science. Seminar to staff and postgraduate students, September 2010. University of Padova, Padova, Italy.

Pinares-Patiño CS (2010). Distance education program on Greenhouse gas emissions from livestock farming. July –August 2010, REDCAPA, Brazil.

Pinares-Patiño CS (2010). Presentations, International course 'Livestock and environment'. Universidad Nacional de Colombia, 6-30 July 2010, Bogota, Colombia.

Rhodes AP, Dynes R (2010) Small Footprints and thin wallets – implications for sheep and beef farmers. Oral presentation at Fertiliser and Lime Research Centre Workshop, Massey University, 15 February, Palmerston North.

Rhodes T (2011). Presentations at PGG Wrightson-Leading Thinking in Agriculture.

Rhodes T (2011). Presentations at MAF CC and GHG Technology Transfer – Train the Trainers Programme 2010 and 2011(Napier, Palmerston North, New Plymouth, Nelson, Hokitika, Christchurch, Timaru, Dunedin, Balclutha, Invercargill).

Smeaton D (2011). Presentation at MOTU Mitigation Workshop, May 2011, Hamilton.

Smeaton D (2011). 4 presentations to RPs at Massey University (13 July 10), Rotorua (20 July 10), Newstead, Hamilton (June, 10), Whangarei (15 July 10) to audiences each of approximately 50 people.

Sun XZ (2010) New Zealand studies on methane emissions from ruminants. China Agricultural University, Shandong Academy of Agricultural Sciences, Shandong Agricultural University, Nanjing Agricultural University, Zhejiang University (several dates in September 2010).

Carbone V, Schofield LR, Ronimus RS (2011) Enhanced discovery of methanogen-specific inhibitors of *Methanobrevibacter ruminantium*. Report to the Royal Society of New Zealand resulting from recent Synchrotron visit. 11 November 2011.

Carbone V, Schofield LR, Ronimus RS (2012) Enhanced discovery of methanogen-specific inhibitors of *Methanobrevibacter ruminantium*. Report to the Royal Society of New Zealand on the recent visit to the Australian Synchrotron, 29 March 2012.

Carbone V, Schofield LR, Ronimus RS (2011) Australian Synchrotron Beamline Proposal: Enhanced discovery of methanogen-specific inhibitors of *Methanobrevibacter ruminantium*. 15 September 2011.

Carbone V, Schofield LR, Ronimus RS (2012) Australian Synchrotron Beamline Proposal: Structural characterisation of methanogen and archaeal-specific enzymes. 15 May 15, 2012.

Dynes RA, Smeaton DC, Rhodes AP, Fraser TJ, Brown MA (2011) Modelling farm management scenarios that illustrate opportunities farmers have to reduce greenhouse gas emissions while maintaining profitability. Proceedings of the NZ Society of Animal Production 71: 149-178.

Engelhart R, (2011) Characterizing methanogen marker proteins by structure determination and operon analysis. MSc Thesis Internship Report, University of Wageningen, The Netherlands.

Henderson G, Tavendale M, Janssen PH (2011) The methane-free rumen – the solution to all problems? Release (NZAGRC Quarterly Newsletter), April 2011, p. 4.

Janssen PH (2011) New Zealand Agricultural Greenhouse Gas Research Centre. Oral presentation at Massey Agriculture Student Professional Development Workshop, 12 August 2011, Massey University, New Zealand.

Janssen PH (2011) Methanogen microbiology for methane mitigation. Oral presentation at Massey Agriculture Student Professional Development Workshop, 12 August 2011, Massey University, New Zealand.

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