Ministry for Primary Industries
Manatū Ahu Matua

Identification of methanogen-specific inhibitors and cross-reactive vaccine antigens

SLMACC C10X1104

Accelerated methane mitigation for ruminants

MPI /TechnicalPaperNo: 2018/32

Prepared for MPI by Neil Wedlock and Ron Ronimus

ISBN No: 978-1-77665-897-8

25th June 2012

New Zealand Government

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Executive summary

This project aimed to produce tools that will assist in the reduction of methane emissions from ruminants. The two strategies that were investigated were (1) the identification of specific compounds which could be used to inhibit the activity of methanogens in the rumen and (2) identification of proteins from methanogens which could be incorporated into a vaccine to reduce methane emissions.

In strategy one, the goal was to discover novel compounds that are able to specifically inhibit the growth of rumen methanogens. Our hypothesis was that by employing powerful *in silico* screening and molecular modelling techniques, potent and specific inhibitors of the dominant rumen methanogens species could be discovered and employed to mitigate methane production in ruminants. The large majority of this work has centered on using already-published crystal structures for modelling using the respective *Methanobrevibacter ruminantium* sequences. Three enzyme structure models of a dominant rumen methanogen were constructed based on already-published and publicly available crystal structures and used to screen chemical libraries containing tens of thousands of compounds. The enzymes targeted catalyse key steps in archaeal lipid synthesis (hydroxymethyl glutaryl CoA reductase), methanogen cofactor synthesis (F420 CofD, 2-phospho-L-lactate transferase) and Mer of the methanogenesis pathway (N(5),N(10)-methylenetetrahydromethanopterin reductase). Initial crystallisation attempt were carried out on HMG CoA with little or no success. A large number of compounds were identified as plausible inhibitor candidates. The active sites of the enzymes were studied and subsets of these compounds obtained for testing *in vitro* assays. A number of compounds were found to be inhibitory to rumen methanogen growth in pure culture experiments and *in vitro*. Contracted outputs (see Appendices) have been met with three enzymes structure models constructed and used to screen libraries. Our hypothesis has been validated with its successful implementation and identification of inhibitors of rumen methanogen enzyme activity (HMG-CoA – *see appendices*) and growth in pure culture.

The development of a vaccine to reduce methane emissions from ruminants such as sheep, deer and cattle will require key vaccine antigens to be identified from methanogens. Our hypothesis is that ideal vaccine candidates are proteins which are membrane associated and are exposed on the surface of the methanogen cells. Such proteins would be accessible to binding of anti-methanogen antibodies that enter the rumen via the saliva following vaccination of animals. Existing methods to produce sub-cellular fractions from methanogens to identify vaccine targets have tended to identify predominately intracellular proteins and this has been an obstacle to identifying suitable vaccine candidates. In this project, a novel method was developed to identify surface-associated proteins in methanogens. Cell surface extracts which were enriched for membrane associated proteins were produced from dominant rumen methanogens. From these extracts, 13 potential vaccine antigens were identified by mass spectrometry methods. Four of the proteins were produced as recombinant proteins in *Escherichia coli*, using the protein sequences from a dominant rumen methanogen, *Methanobrevibacter* sp. SM9. These proteins were evaluated as antigens for a prototype anti-methanogen vaccine. This new strategy to identify antigens for a vaccine is complementary to the already established procedure of predicting vaccine candidates from an analysis of methanogen genome sequences. Contracted outputs (*see appendices*) have been met with >10 potential vaccine candidates being identified and three or more recombinant proteins produced and tested. Our work has developed a novel approach to identifying vaccine targets which will assist in our search for vaccine antigens, in particular membrane-associated proteins. Our hypothesis that ideal vaccine candidates are surface exposed proteins will be

further tested in our on-going programme of identifying and testing membrane associated proteins as vaccine antigens. While *E. coli* is commonly used to express recombinant proteins, yeast may be a better host for expressing membrane associated proteins and proteins that are glycosylated. To explore this possibility, an alternative expression system for producing recombinant methanogen protein was developed. Two methanogen proteins were expressed in the yeast *Pichia pastoris*. Although the proteins were expressed at low levels, it should be possible to optimise the levels of expression in this host. This part of the project was additional to the contracted work and is relevant to our goal of testing candidate proteins for a vaccine.

1. Identification of methanogen-specific inhibitors

Introduction

Small molecule inhibitors have routinely demonstrated the potential in achieving the nearly complete knockdown of methane emissions in ruminants by targeting key methanogenesis and co-enzyme synthesis pathways (Van Nevel and Demeyer 1995, Dumitru 2003). In the past a number of patents have been developed or issued (for example Miner et al US 2003/0219467) describing extensive numbers of chemical compounds and their derivatives designed solely to inhibit the growth of methanogenic archaea and therefore decrease methane emissions. It is also hoped that in turn this would lead to an increase in feed efficiency and reduce caloric loss in livestock.

Here we present a new paradigm in the investigation, selection and analysis of new inhibitors of methanogenesis in archaea by avoiding the pitfalls of previous studies including toxicity and nonspecific activity. By utilising *in silico* methods including molecular modelling and compound database docking we are able to purposely screen, select and test specific and mostly non-toxic compounds of a particular target. These include (and are reported here), CofD an enzyme involved in the biosynthesis of F_{420} coenzyme and HMG-CoA, essential in the production of organic molecules involved in lipid synthesis.

Structure based discovery of novel inhibitors for the *Methanobrevibacter ruminantium* enzyme 2-phospho-L-lactate transferase (CofD) from database screening and molecular docking

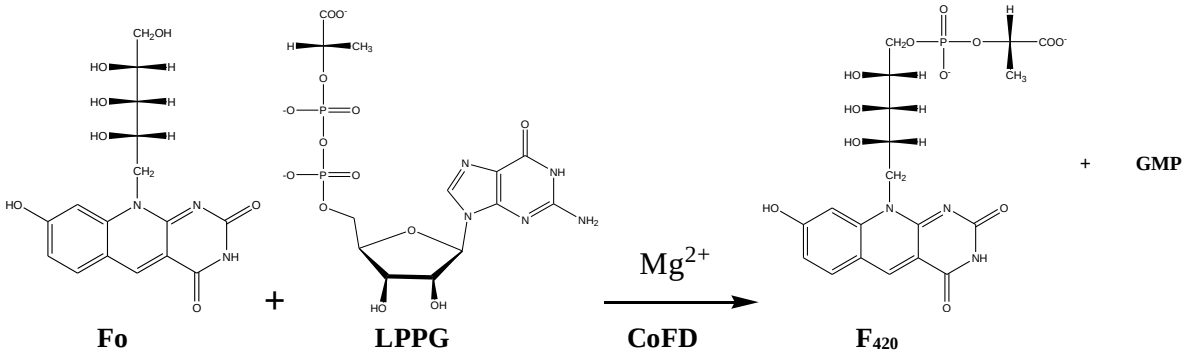
Vincenzo Carbone, Debjit Dey and Ron S. Ronimus

AgResearch Limited, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand.

Summary

Coenzyme F_{420} (CofD), a hydride carrier, is uniquely found in Archaea and some Gram positive bacteria and has crucial roles in methanogenesis, antibiotic biosynthesis and DNA repair (Forouhar 2008, Graupner 2001, Graupner 2003). It catalyses the terminal step in the

biosynthesis of F_{420} coenzyme by transferring the lactyl phosphate moiety of lactyl(2)diphospho-(5')guanosine (**LPPG**) to 7,8-didemethyl-8-hydroxy-5-deazariboflavin ribitol (**Fo**; **Scheme 1**). For these reasons, and the availability of crystal structures with well delineated active site interactions and binding modes showcasing a number of natural substrates, it was targeted for *in silico* modelling. Additionally, the sequence similarity with the *M. ruminantium* protein (mru_1844; **Figure 1**) suggested that a reasonable overall *in silico* model could be derived, one that included an exceptionally homologous active site domain(s).



Scheme 1: Substrate reaction of catalysed by CofD

Materials and methods

Materials

Inhibitors of the highest purity were purchased from the chemical synthesis company ChemBridge™, USA.

Modelling of *Methanobrevibacter ruminantium* CofD

The *M. ruminantium* models of CofD were constructed using the structure modelling software *Coot* (Emsley 2004) and a combination of the substrate bound structures of 2-phospho-(*S*)-lactate transferase from *Methanosarcina mazei* (PDB codes 3CGW, 3C3E and 3C3D; Forouhar 2008) as templates. With the ClustalW sequence alignment of CofD (Figure 1), individual residues were mutated taking care to correctly fit rotamers to produce the most accurate homology model (Figure 2). The active sites were identified via close contacts (<4 Å) residues made with the substrate(s) and inhibitor (Figure 1). The two distinct guanosine diphosphate (**GDP)**molecules bound and **Fo** bound domains of the enzyme suggested two individual scenarios for focused targeting of inhibitor screening and selection. This report centres on the Fo domain of CofD (Figure 3).

In silico database screening

Molecular docking experiments were carried out using the programme GOLD (Genetic Optimization for Ligand Docking), Version 5.1 (Verdonk 2003). Docking protocols were established utilising scoring functions and modes that could best mimic the binding poses of the crystal structure ternary complexes with bound substrate(s). Docking functions that consistently scored highest or prioritised such a poses were chosen. Each GOLD run was centred on all residues that fell within 5.5–6.0 Å of the **Fo** binding site (Figure 1 and 3). The

Chemscore function and CHEMPLP rescore facility utilising default parameters were used to sort and score all potential inhibitor poses from commercially available databases. Side-chain residues were rigid and no water molecules were defined as part of the active site. The diverse solutions option of GOLD was turned on (Cluster size 2, RMSD 2) with automatic GA settings of 6 or 10 and 100% search efficiency. Structural databases from commercial distributors chosen for the docking process included the Chembridge building block library (12,975 compounds; http://www.hit2lead.com/search.asp), MP biomedicals database (18,066 compounds) and Sigma database (15,186 compounds). Compounds that ranked highest were prioritised and then purchased and evaluated as inhibitors *in vitro*.

***M. ruminantium* whole cell assays**

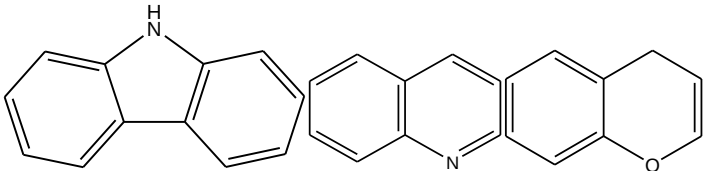
Inhibition assays were conducted in a similar fashion to that described by Wedlock 2010 in experiments designed to test the effects of antisera on growth of cultures of *M. ruminantium*. Inhibitors were prepared as stock solutions in DMSO, and gassed with oxygen-free CO_2 to remove oxygen for at least 30 min prior to addition to the cultures in Balch tubes. Growth was monitored by absorbance at 600 nm until the growth reached stationary phase and were performed in duplicate. Inhibitors were added when the cultures reached a density of approximately 0.1. Growth usually ceased in the control tubes with DMSO alone when the density reached approximately 0.4. Inhibition was estimated relative to controls with DMSO (as part of a final reaction volume of 5%).

Results

Identification of novel inhibitors of CofD / post-database docking analysis

Our post-docking analysis and compound selection heavily favoured candidates comprised of heterocyclics or polycylic aromatic compounds. These included pharmacophores in the form of carbazoles (as seen in the derivatives of harmane),Quinoline and Benzopyrane/Chromene(see Scheme 2, Table I and Figure 4). The selection of these polycyclics was to be expected when the structure of natural product **Fo** (Scheme 1 and Figure 3) fits a binding pocket lined by a number of aliphatic amino acids such as Ala45, Thr49, Tyr52, Tyr64, Leu86 and Ile152. Our analysis and careful screening found that five out of our initial nine compounds tested thus far produced close to 100% inhibition of cell growth at 1 mM concentrations, with a sixth compound (4-methyl-3-(2-oxopropoxy)-7,8,9,10-tetrahydro-6H-benzo[c]chromen-6-one) producing total inhibition at 250 μM.

These new structural paradigms for ruminant methanogen inhibitors are a promising and new avenue of investigation and have correctly validated our enzyme target and *in silico* approach. We are also further heartened to the fact that the carbazoles, quinolines and chromenes have a diverse number of naturally occurring derivatives and pharmacological activities (Ahmed 2012, Thatcher 2012, Areias 2012, Curini 2006). They have also demonstrated properties such as antibiotic resistance (Ahmed 2012, Nagappan 2011), enzyme inhibition (Wincent 2012, Dixon 2011) and can act as possible chemopreventative agents (Bandgar 2012, Kok 2012).



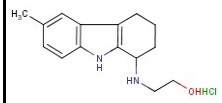
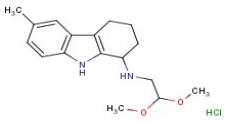
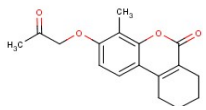
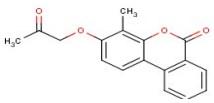
Carbazole **Quinoline** **Benzopyrane/Chromene**

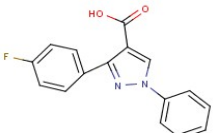
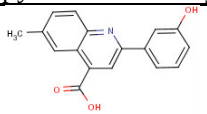
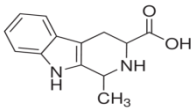
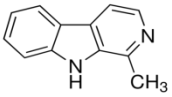
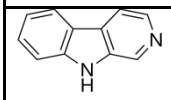
Scheme 2: Comparison of the active pharmacophores/polycyclic organic compounds discovered during CofD *in silico* docking

Conclusion/future work

In order to produce an effective means of mitigation of archaeal methanogenesis in the rumen, producing targeted small molecule inhibitors is a valid methodology. Producing a single small molecule inhibitor of CofD represents a promising target due to its nearly unique presence amongst methanogens and its very limited distribution in bacteria and its role in the biosynthesis of the coenzyme F_{420} involved in methanogenesis. Our inhibitor candidates closely mimicked large moieties within the substrates of a number of methanogenesis enzymes (in particular CofD our target), a result of our careful screening and selection. A majority of our candidates were inhibitory and will require further optimisation in producing more potent derivatives.

Table I: Candidate molecules selected following *in silico* screening and their inhibitory values at **1 mM** in subsequent *in vitro* whole cell assays

Structure	**ID**	**% Inhibition (O.D. – *M.rum* - whole cell assay) / concentration**
 2-[(6-methyl-2,3,4,9-tetrahydro-1H-carbazol-1-yl)amino]ethanol	**6046217**	~ 60% @ 1mM
 N-(2,2-dimethoxyethyl)-6-methyl-2,3,4,9-tetrahydro-1H-carbazol-1-amine	**5259158**	inactive
 4-methyl-3-(2-oxopropoxy)-7,8,9,10-tetrahydro-6H-benzo[c]chromen-6-one	**6148717**	100 % @ 250 uM
 4-methyl-3-(2-oxopropoxy)-6H-benzo[c]chromen-6-one	**6146055**	inactive

 3-(4-fluorophenyl)-1-phenyl-1H-pyrazole-4-carboxylic acid	**5867817**	100 % @ 1mM
 2-(3-hydroxyphenyl)-6-methylquinoline-4-carboxylic acid	**3013482**	100 % @ 1mM
 1,2,3,4-Tetrahydroharmane-3-carboxylic acid	**H0500**	100 % @ 1mM
 Harmane	**103276**	100 % @ 1mM
 Noharmane	**N6252**	100 % @ 1mM

Figures

```
CofD (mma_3C3D)    -MIIFSGGTGTPKLLDGLKEILPEEELTVVVNTAEDLWVSGNLISPDLDT 49
CofD (mru_1844)     ITILSGGTGTPKLIQGIKEIYPEEEISVIVNTVENEYFSGGYVAADCDT 50

3C3D (mma_3C3D) VLYLFSDQIDRKRWWGIENDTFGTYERMKELGIEEGLKLGDRDRATHIIR 99
CofD (mru_1844) VMYTFADMIDDQFFYGIIGDTFKIRNILIEMGTTELLKIGDMDRAIKIQK 100

3C3D (mma_3C3D)    SNIIRDGASLTDSTVKLSSLFGIKANILPMSD-DPVSTYIETAEGIMHFQ 148
CofD (mru_1844)    TILLEEGKTLSEIVEHQKNKLGVKAKIIPMSDEDSEIKIITKEHGELDFH 150

3C3D (mma_3C3D) DFWIGKRGEPDVRGVDIRGVSEASISPKVLEAFEKEENILIGPSNPITSI198
CofD (mru_1844) DFLIKHQCEGEVLEVKY---SDVKPSPNVIGTIKDADKVIIGPSNPITSI197

3C3D (mma_3C3D)    GPIISLPGMRELLKKKKVVAVSPIIGNAPVSGPAGKLMPACGIEVSSMGV248
CofD (mru_1844)    RPIIAIEGVEEALKEKKVIAVSPFLGEAAFSGPAAKFMNAFGYEASSKGV247

3C3D (mma_3C3D) AEYYQDFLDVFVFDERDRADEFAFERLGCHASRADTLMTSTEKSKELAEI 298
CofD (mru_1844) AELYKPFLDTFIIDNEDKDKKEELEKIIPNIVVANTFMKTIEDKINLAKV 297

3C3D (mma_3C3D) VVQAFLEHHHHHH 311
CofD (mru_1844) VLEL--------- 301
```

Figure 1: ClustalW (Thompson 1994) sequence alignment of *M. ruminantium* CofD (mru_1844) and 2-phospho-(*S*)-lactate transferase from *Methanosarcina mazei* (mma; pdb code 3C3D). Identical residues are highlighted in yellow. Residues identified as interacting with **GDP** are in red and residues that interact with **Fo** are in green. Both enzymes contain the **PSNP*XX*SI** motif that is conserved amongst F_{420}-producing CofD homologs

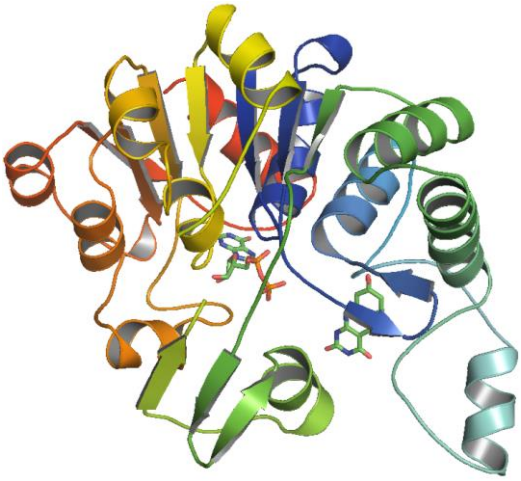


Figure 2: A ribbon representation of the *M. ruminantium* model of CofD. In stick format are the **Fo** (right) and **GDP** (left) binding sites, the centres of our *in silico* screening attempts

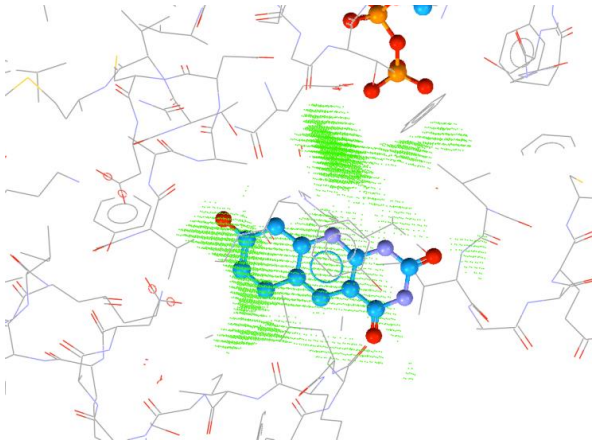


Figure 3: Centre of the modelled *M. ruminantium* CofD active site (**Fo** binding domain) designated for database docking. The green shading indicates the size of the *in silico* search domain probed

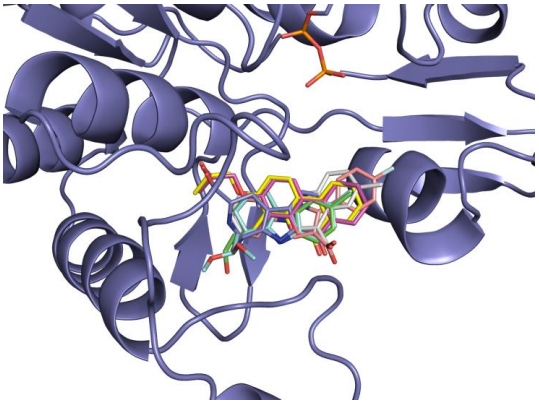
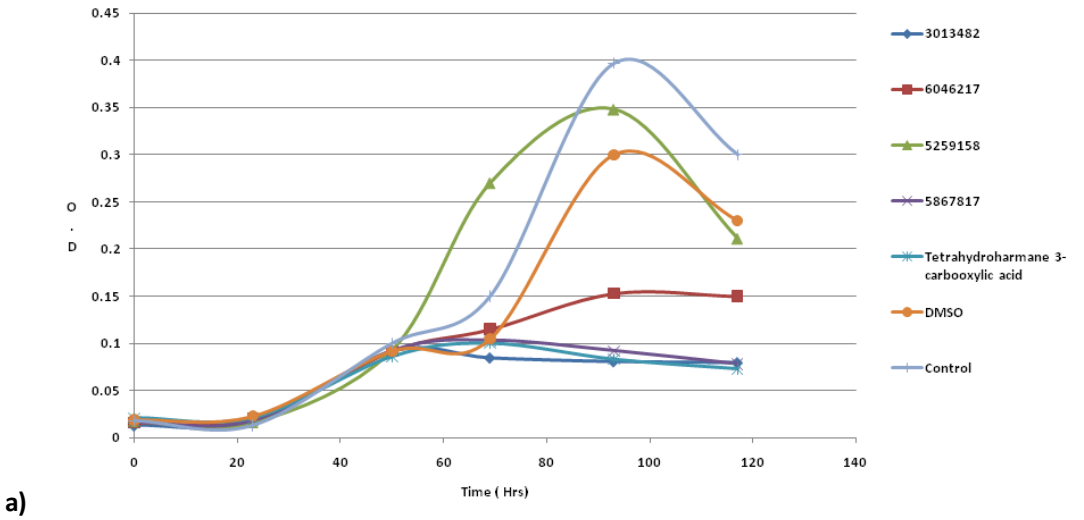
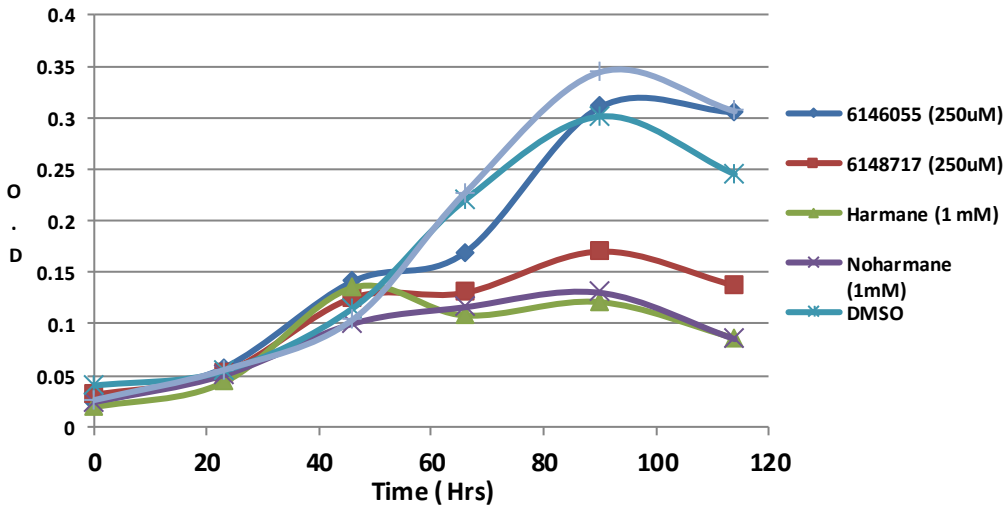


Figure 4: Cartoon representation of the *M. ruminantium* model of CofD and the docked (superimposed) compounds selected for inhibitor trials. All figures were prepared using PyMOL (DeLano 2002)



a)



b)

Figure 5: Small molecule inhibition experiments using whole cells of *M. ruminantium*. Methanogen growth (OD) was measured in the presence or absence of DMSO (control) and compounds discovered during the *in silico* screening process at a concentration of **a)** 1 mM and **b)** 1 mM and 250 µM

Structure based discovery of novel inhibitors for the *Methanobrevibacter ruminantium* enzyme hydroxymethylglutaryl-coenzyme A reductase from database screening and molecular docking

Vincenzo Carbone, Debjit Dey, Yanli Zhang, Sinan Al-Attar, Linley R. Schofield and Ron S. Ronimus

AgResearch Limited, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand.

Summary

Hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA) is essential for the production of isoprenoid subunits, a large class of organic molecules which help derive the building blocks of lipid synthesis in fungi, mammals and selected archaea (Takahashi 1999). While not strictly a unique or specifically archaeal enzyme, HMG-CoA has been widely studied in mammals with respect to structural characterisation (Bochar 1999, Istvan 2000, Istvan 2001, Tabernero 1999), and enzyme inhibition (Istvan 2001b, Sarver, 2008, Pfefferkorn 2007, Pfefferkorn 2008). The homologous nature of the *M. ruminantium* sequence with that of the truncated form of the human enzymes crystal structure (Figure 1), its high resolution and the multiple forms available co-crystallised with substrate and inhibitors made it priority as a target for *in silico* modelling and compound library docking. Our endeavours involved using multiple compound libraries, specifically tailored docking protocols and the targeting of multiple active site domains and morphologies. It is hoped that we will fast track the discovery of several compounds able to mitigate rumen methanogen growth and therefore the methanogenesis process *in vivo*.

Materials and methods

Materials

Materials of the highest purity including NADPH, HMG (3-Hydroxy-3-methylglutaryl coenzyme A), MOPS (3-(N-morpholino)propanesulfonic acid), dithiothreitol (DTT) and Bis-Tris Propane (BTP) were purchased from Sigma-Aldrich. Inhibitors of the highest purity were purchased from the chemical synthesis company ChemBridge™, USA.

Cloning, protein expression and purification of HMG-CoA

HMG-CoA protein was cloned, expressed and purified using previously established in-house protocols. In brief, the gene of HMG-CoA was amplified using the forward PCR primer 5′-CACCATGAAAGTAAAAAACAATATTATAC and reverse primer 5′-TTACTATCTGCCTAGTTCTTGATG. The PCR reaction utilised the high fidelity AccuPrime *Taq* DNA polymerase (Invitrogen, USA) in a 50 µL reaction with 0.2 µM of each primer, 1.5 U *Taq*, 0.3 µM dNTP, approximately 20 ng of *M. ruminantium* strain DSMZ 1093 DNA, and 1 × buffer. The plasmid used for expression was sequenced to verify that the gene was in frame and that the sequence was correct (Leahy 2010) and then transformed into Rosetta 2 cells (Novagen, USA). *E. coli* BL21-Rosetta2 cells were then grown for approximately 16 h with vigorous shaking at 30 °C before harvesting (10,400 *g*, 15 min, and 4 °C). Cell pellets were lysed and cell debris removed. HMG-CoA was then purified to homogeneity from the cell-free extract using nickel-affinity chromatography, then pooled and

concentrated using a stirred ultrafiltration cell (Amicon, UK) with a 10 kDa pore size. The buffer was exchanged using dialysis to 25 mM MOPS, 50 mM NaCl, 100 µM EDTA and stored at 4°C.

HMG-CoA *in vitro* inhibitor assays

Enzyme stock solutions (0.6 mg mL^{-1}) were pre-incubated in 50 mM BTP pH 6.5 with 400 mM KCl (or NaCl) and 10 mM DTT at 4 °C for 2 hours and at 37 °C for 20 minutes. Assay solutions (with final reaction volumes of 200 µl) were comprised of 100 µl of 0.1 M BTP, pH 6.5 (at 37°C), 10 µl of 5mM HMG, 15 µl of 5mM NADPH, with variable inhibitor stock solution (final concentrations between 1 mM – 10 uM) and MilliQ H_2O. Solutions were mixed well and allowed to equilibrate for 90 seconds. The assay was initiated by the addition 10 µl of pre-incubated HMG-CoA enzyme (final concentration 30 µg ml^{-1}) and recorded for 3 min at 366 nm using a Heλios γ UV Vis spectrophotometer (Thermo Scientific, USA). The temperature was maintained at 37 °C while measuring the decay of NADPH (366 nm, ε 3,300 M^{-1} cm^{-1}).

***M. ruminantium* whole cell assays**

Inhibition assays were conducted in a similar fashion to that described by Wedlock *et al* (2010) in experiments designed to test the effects of antisera on growth of cultures of *M. ruminantium*. Inhibitors were prepared as stock solutions in DMSO, and gassed with oxygen-free CO_2 to remove oxygen for at least 30 min prior to addition to the cultures in Balch tubes. Growth was monitored by absorbance at 600 nm until the growth reached stationary phase and were performed in duplicate. Inhibitors were added when the cultures reached a density of approximately 0.1. Growth usually ceased in the control tubes with DMSO alone when the density reached approximately 0.4. Inhibition was estimated relative to controls with DMSO (as part of a final reaction volume of 5%).

Modelling of the *M. ruminantium* HMG-CoA

The *M. ruminantium* models of HMG-CoA were constructed using the structure modelling software *Coot* (Emsley 2004) and the substrate and inhibitor bound structures of the catalytic portion of human HMG-CoA (PDB codes 1DQA and 2Q6B respecively; Istvan 2000b and Pfefferkorn 2007) as a template. With the ClustalW sequence alignment of Figure 1, individual residues were mutated taking care to correctly fit rotamers to produce the most accurate homology model (Figure 2). A substrate bound model bound with CoA and 3-hydroxy-3-methyl-glutaric acid (MAH) and a pyrrole inhibitor-based model (which induces a large conformational change in the active site of HMG-CoA and is of beneficial consequence when attempting to design and/or retrieve plausable or alternatively fashioned inhibitors that will "fit" the active site) were created prior to *in silico* screening. The active sites were identified via close contacts (<4 Å) residues made with the substrate(s) and inhibitor (Figure 1).

***In silico* database screening**

Molecular docking experiments were carried out using the programme GOLD (Genetic Optimization for Ligand Docking), Version 5.1 (Verdonk 2003). Docking protocols were established utilising scoring functions and modes that could best mimic the binding poses of crystal structure ternary complexes with bound substrate or inhibitor (Figure 3a). Docking functions that consistently scored highest or prioritised such a poses were chosen (Figure 3b). Structural databases from commercial distributors chosen for the docking process included the Chembridge database (1,224,316 compounds; http://www.hit2lead.com/search.asp) used

exclusively for virtual screening, the Chembridge building block library (12,975 compounds), MP biomedicals database (18,066 compounds), Sigma database (15,186 compounds) and LOPAC library of pharmacollogically active compounds (1,743 compounds). **Table I** lists the compound libraries, techniques and portions of the active site targeted for individual *in silico* screenings. The focus of this research centres exclusively on the virtual screening event that utilised the entire Chembridge database and the pyrrole-enhanced active site of HMG-CoA.

Virtual screening

Each GOLD virtual screening run was centred on all residues that fell within 11 Å of the approximate centre of the pyrrole based inhibitor binding site (Figure 3a and 3c). The CHEMPLP scoring function was utilised with default parameters and automatic GA settings of 4 and a 10% search efficiency. The top 10% of ligand hits were then resubmitted for more intensive screening. This time each GOLD *in silico* screen was centred on all residues that fell within 12.5 Å of the approximate centre of the pyrrole based inhibitor binding site, the CHEMPLP scoring function was utilised with default parameters and automatic GA settings of 6 and 100% search efficiency. The diverse solutions option of GOLD was turned on (Cluster size 2, RMSD 2) and the top 1000 "hits" output and investigated. Side-chain residues were rigid throughout the screening process and no water molecules were defined as part of the active site. Compounds that ranked highest were prioritised and then purchased and evaluated as inhibitors *in vitro*.

Results

Post docking analysis of the *M. ruminantium* HMG-CoA active site modulated by the pyrrole-based inhibitor preselected a number of polyphenolic compounds. These were remarkably reminiscent of the statin compounds that routinely inhibit the enzyme (Istvan 2003), suggesting that our ligands prior to testing had some accurate steric complementarity with the *M. ruminantium* model and that our docking protocol was sound. Additionally a number of compounds possessed a hexanoic or propanoic moiety, which consistently occupied the 3-hydroxy-3-methyl-glutaric acid-binding site, observed in the substrate bound crystal structures of HMG-CoA (Istvan 2000b). Another predominant pharmacaphore that was consistently selected was theisoindol-5-sulfonyl benzoic acid and 1,3-dioxo-isoindol moieties of nine of the seventeen compounds preselected for testing (Table II). Unfortunately due to either solubility problems or inordinate levels of background signal (during the *in vitro* assays at 366 nm) while using modest concentrations of inhibitor, obtaining a reasonable inhibitor profiles for thirteen of seventeen compounds was not possible. We did however produce significant inhibition with three compounds in particular 3-{[1-(4-methoxy-phenyl)-4,5 diphenyl-1H-imidazol2yl]thio}propanoic acid, the most statin like of all our inhibitors at 1mM. Additional testing will need to occur in the future to establish IC_{50} values for these compounds.

The rumen whole cell *in vitro* assays found that six compounds out of eight tested were inhibitory of *M. ruminantium* growth at concentrations of 1 mM (see Table II and Figure 5). These results helped in some cases overcome the problems associated with the enzyme *in vitro* assays and continued to validate our target and compound selection. In others such as 3-(5-{[4-(acetyloxy) benzoyl]amino}-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoic acid, where there was no inhibition in the enzyme assay (at reasonable concentration regardless of background noise) and inhibition of the whole cell growth, we would have to assume a nonspecific or non HMG-CoA interaction taking place. We would not regard these as specific

"hits", but are still valuable leads in the effort of mitigating *M. ruminantium* growth. At lower concentrations (500 uM), when tested, our candidates failed to completely mitigate growth.

Conclusions and future work

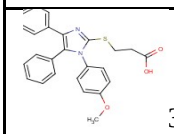
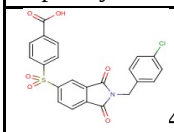
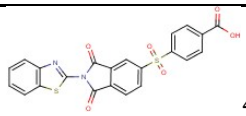
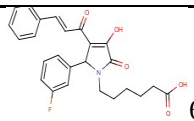
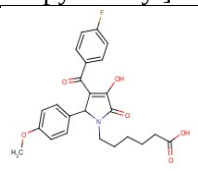
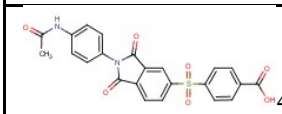
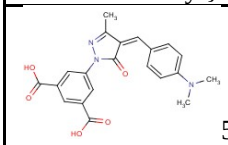
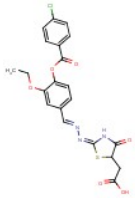
These inhibitor discovery methods accurately represent our unique approach within the multiple methane mitigation programmes at AgResearch to find and optimize small molecule inhibitors of methanogenesis in ruminant animals. HMG-CoA represents a feasible target in mitigating methane in ruminants. We have developed an *in vitro* and an *in silico* means to modulate its activity thereby producing the first experimental reports of potent, potentially micro-molar inhibitors for the *M. ruminantium* version of the enzyme. A paper on identifying inhibitors will be presented at an upcoming New Zealand conference subject to meeting any confidentially requirements. It is hoped that a number of these compounds will become a template for which a number of more potent and specific compounds will be based on in the future and further tested *in vitro*. A number of compounds remain to be tested and will be prioritised immediately.

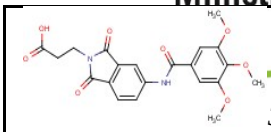
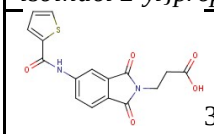
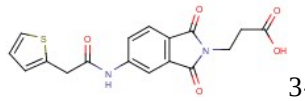
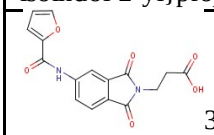
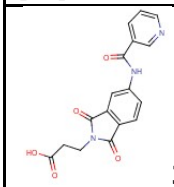
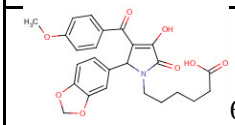
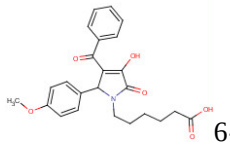
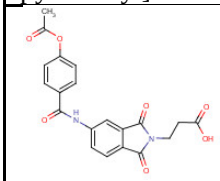
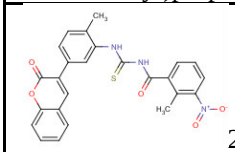
Tables

Table I: Screened chemical libraries against HMG-CoA including binding sites investigated and scoring functions utilised using the docking program GOLD (Verdonk 2003)

	Chembridge database	MP Biomedicals	Sigma	LOPAC	Chembridge Building Blocks
Compounds Screened	1,224,316 (virtual screen)	18,066	15,186	1,743	12,975
MAH binding site	-	-	-	+	+
Active site (MAH-Coenzyme A binding)	-	+	+	+	-
Active site (modulated by pyrrole based inhibitor)	+	-	-	+	-
Scoring function Gold/CHEMPLP	-/+	+/-	+/-	+/+	+/-
Compounds ordered	17	*N/A*	*N/A*	*N/A*	*N/A*
Active compounds *(in vitro)*	4	*N/A*	*N/A*	*N/A*	*N/A*
Active compounds *(whole cell assays)*	6	*N/A*	*N/A*	*N/A*	*N/A*

Table II: Candidate molecules selected following *in silico* screening and their inhibitory values in subsequent *in vitro* assays

Structure	ID	% Inhibition (enzyme assay)	% Inhibiton (O.D. – *M.rum* - whole cell assay)
 3-{[1-(4-methoxy-phenyl)-4,5 diphenyl-1H-imidazol2yl]thio}propanoic acid	**6874583**	90.9 % inhibition @ 1 mM	€
 4-{[2-(4-chloro-benzyl)-1,3-dioxo-2,3-dihydro-1H-isoindol-5-yl]sulfonyl} benzoic acid	**7354475**	53.0 % inhibition @ 1 mM	€
 4-{[2-(1,3-benzothiazol-2-yl)-1,3-dioxo-2,3-dihydro-1H-isoindol-5-yl]sulfonyl}benzoic acid	**5745674**	*€	*€
 6-[3-cinnamoyl-2-(3-fluorophenyl)-4-hydroxy-5-oxo-2,5-dihydro-1H-pyrrol-1-yl]hexanoic acid	**6040868**	No inhibition @ 50 µM#	40-50 % @ 1mM
 6-[3-(4-fluoroben-zoyl)-4-hydroxy-2-(4-methoxyphenyl)-5-oxo-2,5-dihydro-1H-pyrrol-1-yl]hexanoic acid	**6042079**	#	€
 4-({2-[4-(acetylamino)phenyl]-1,3-dioxo-2,3-dihydro-1H-isoindol-5-yl}sulfonyl)benzoic acid	**5766221**	84.8 % inhibition @ 1 mM	€
 5-{4-[4-(dimethylamino)benzylidene]-3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl}isophthalic acid	**6977990**	*€	*€
 [2-({4-[(4-chlorobenzoyl)oxy]-3-ethoxybenzylidene}hydrazono)-4-oxo-1,3-thiazolidin-5-yl]acetic acid	**6638731**	No inhibition @ 50 µM#	€

 *3-{1,3-dioxo-5-[(3,4,5-trimethoxybenzoyl) amino]-1,3-dihydro-2H-isoindol-2-yl}propanoic acid*	**7926036**		€
 3-{1,3-dioxo-5-[(2-thienylcarbonyl)amino]-1,3-dihydro-2H-isoindol-2-yl}propanoic acid	**7572210**	No inhibition @ 50 µM[#]	~15 % @ 100 uM
 3-{1,3-dioxo-5-[(2-thienylacetyl)amino]-1,3-dihydro-2H-isoindol-2-yl}propanoic acid	**7951889**	No inhibition @ 50 µM[#]	100 % @ 1mM
 3-[5-(2-furoylamino)-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl] propanoic acid	**7492979**	No inhibition @ 50 µM[#]	100 % @ 1mM
 3-{1,3-dioxo-5-[(3-pyridinyl-carbonyl)amino]-1,3-dihydro-2H-isoindol-2-yl}propanoic acid	**9199228**	No inhibition @ 50 µM[#]	100 % @ 1mM
 6-[2-(1,3-benzodioxol-5-yl)-4-hydroxy-3-(4-methoxybenzoyl)-5-oxo-2,5-dihydro-1H-pyrrol-1-yl]hexanoic acid	**5947846**	No inhibition @ 10 µM[#]	100 % @ 1mM ~50 % @ 500uM
 6-[3-benzoyl-4-hydroxy-2-(4-methoxyphenyl)-5-oxo-2,5-dihydro-1H-pyrrol-1-yl]hexanoic acid	**6040603**	No inhibition @ 50 µM[#]	100 % @ 1mM ~50 % @ 500uM
 3-(5-{[4-(acetyloxy) benzoyl]amino}-1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoic acid	**7612748**	No inhibition @ 500 µM[#]	100 % @ 1mM ~50 % @ 500uM
 2-methyl-N-({[2-methyl-5-(2-oxo-2H-chromen-3-yl)phenyl] amino} carbonothioyl)-3-nitrobenzamide	**7967187**	27.4 % inhibition @ 100 µM[#]	€

***Solubility problems occurred and were either completely insoluble or only partly soluble in DMSO, DMF, NaOH, Etoh, Acetone, acetonitrile and water and could not be adequately assayed *in vitro*. #Too much background noise to be adequately assayed at 366 nm using higher concentrations. €Currently untested**

Figures

```
HMGCoA (hsap_1DQA) GAMASSVLVTQEPEIELPREPRPNEECLQILGNAEKGAKFLSDAEIIQLV 50
HMGCoA (mru_1092)  ------------------------------------MDKEEIINKL 10

HMGCoA (hsap_1DQA) NAKHIPAYKLETLIETHERGVSIRRQLLSKKLSEPSSLQYLPYRDYNYSL 100
HMGCoA (mru_1092)  MAGEMKLYQIDKFTENATEALDIRREFIERHSN--CELNQIANYTLDMEK 58

HMGCoA (hsap_1DQA) VMGACCENVIGYMPIPVGVAGPLCLD----EKEFQVPMATTEGCLVASTN146
HMGCoA (mru_1092)  AFSKNIENPIGTVQVPIGVVGPLKINGEHADDEFFVPLATSEGALLASVN108

HMGCoA (hsap_1DQA) RGCRAIGLGGGASSRVLADGMTRGPVVRLPRACDSAEVKAWLETSEGFAV 196
HMGCoA (mru_1092)  RGCSAITASGGVNARVIGDKMTRAPVIKTDSVVEAVKVKSWFET--KFDE 156

HMGCoA (hsap_1DQA) IKEAFDSTSRFARLQKLH-TSIAGRNLYIRFQSRSGDAMGMNMISKGTEK245
HMGCoA (mru_1092)  LKSIAESTTSHGKLVKIDPIIIVGNYVYPRFVYSTGDSMGMNMVTIATEK206

HMGCoA (hsap_1DQA) ALSKLHEYFPEMQILAVSGNYCTDKKPAAINWIEGRGKSVVCEAVIPAKV 295
HMGCoA (mru_1092)  VLSKLYDDL-GVHAIALSGNLCVDKKPAAINLVEGRGKTVVADILIPEEI 255

HMGCoA (hsap_1DQA) VREVLKTTTEAMIEVNINKNLVGSAMAGSIGGYNAHAANIVTAIYIACGQ345
HMGCoA (mru_1092)  VKSKLKTTAKAMEEVNIAKNLIGSAAAGSMA-FNAHFANMIGAIFLATGQ304

HMGCoA (hsap_1DQA) DAAQNVGSSNCITLMEASGPTNEDLYISCTMPSIEIGTVGGGTNLLPQQA 395
HMGCoA (mru_1092)  DEAHVVEGSLGITTAEDR---DGDLYFAVSMPDLPIATIGGGTRLETASE 351

HMGCoA (hsap_1DQA) CLQMLGVQGACKDNPGENARQLARIVCGTVMAGELSLMAALAAGHLVKSH445
HMGCoA (mru_1092)  GLSIIDCLGSGKVN------KFAEIVISTVLAGELSLVGALAAGHLAKAH395

HMGCoA (hsap_1DQA) MIHNRSKINLQDLQGACTKKTA 467
HMGCoA (mru_1092)  QELGR---------------- 400
```

Figure 1: ClustalW (Thompson 1994) sequence alignment of the truncated form of the human HMGCoA (hsap) enzyme and *M. ruminantium* sequence (mru_1092). Identical residues are highlighted, while active site residues in red are typically within 4 Å of the substrate CoA, residues in green are within 4 Å of 3-hydroxy-3-methyl-glutaric acid (MAH), residues in purple are within 4 Å of CoA and MAH and residues that are underlined are within 4 Å of coenzyme (NADP)

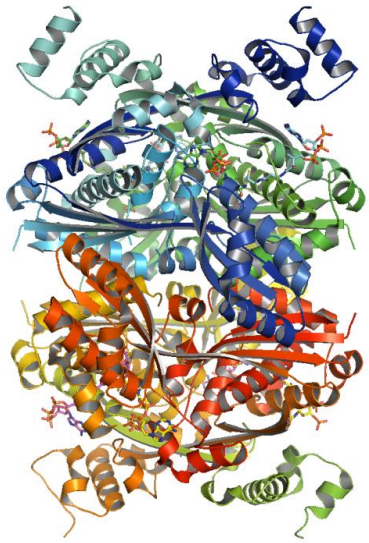


Figure 2: A ribbon representation of the modelled quaternary structure of HMG-CoA from *M. ruminantium*

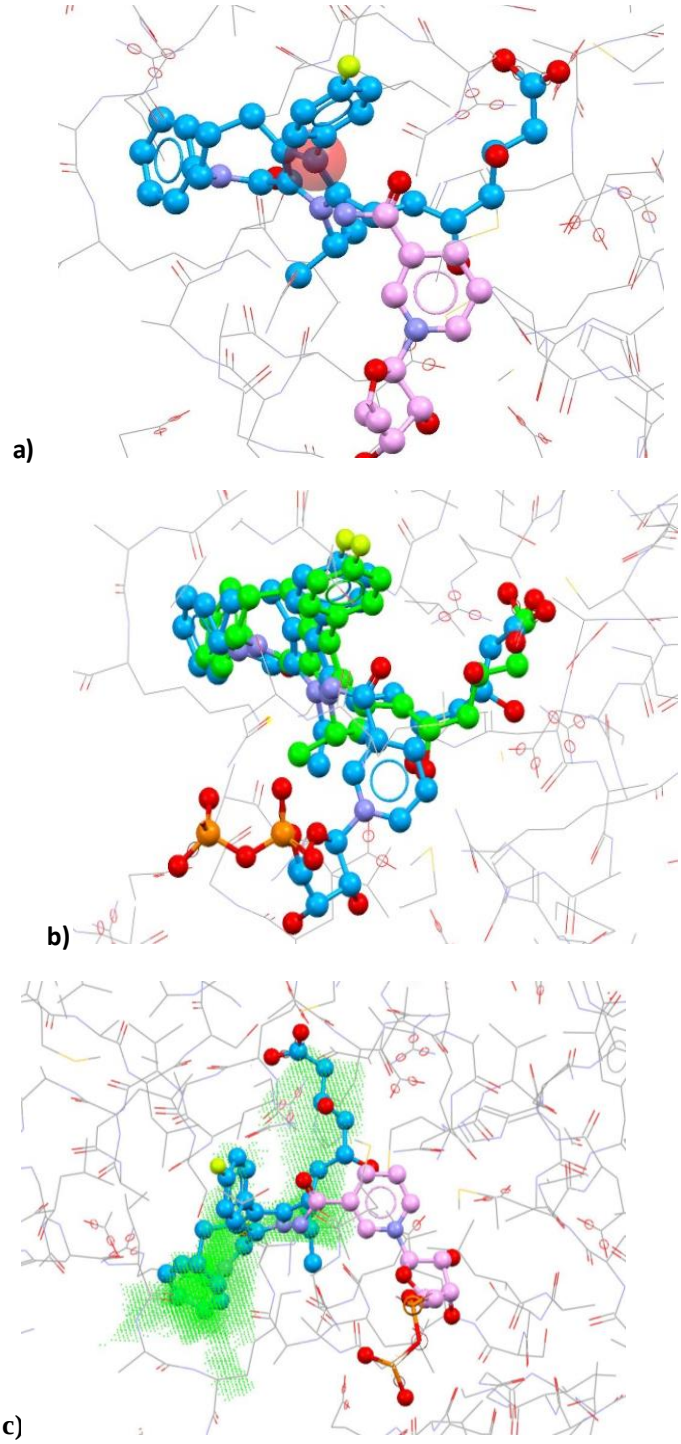
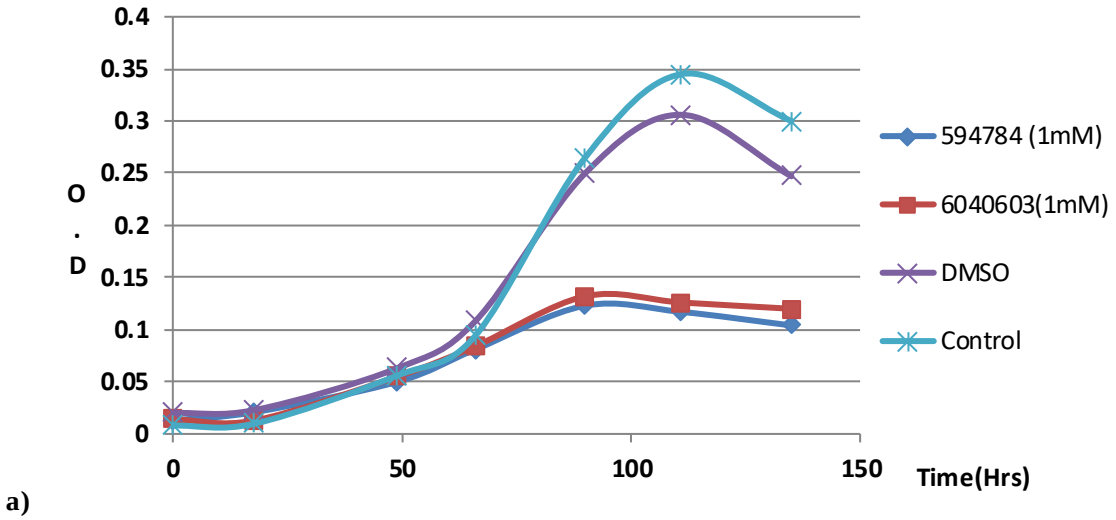
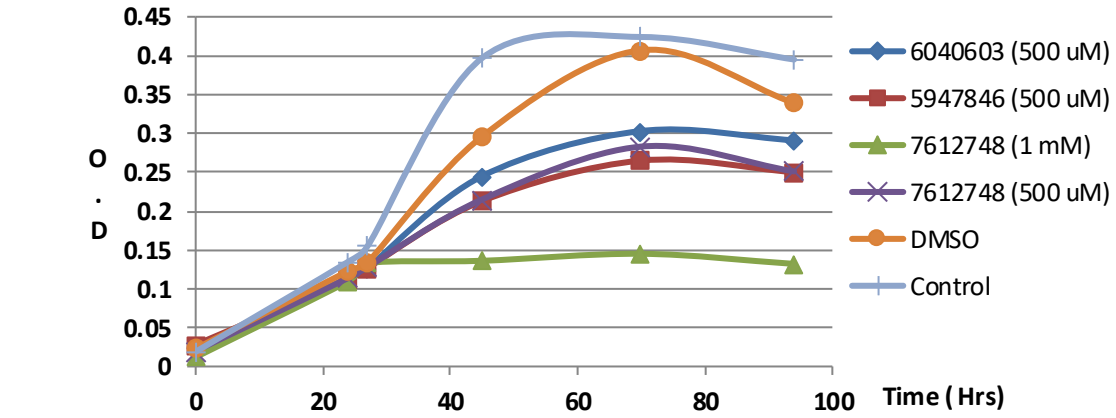


Figure 3: a) Centre (red sphere) of the inhibitor modelled *M. ruminantium* HMG-CoA active site designated for database docking. **b)** The pyrrole based HMG-CoA inhibitor docked back into the *M. ruminantium* model using our formulated docking protocol. The docked inhibitor in green closely mimics the orientation found in the crystal structure of human HMG-CoA (2Q6B) in blue. **c)** Centre of the modelled HMG-CoA active site (pyrrole inhibitor binding mode) designated for database docking. The green shading indicates the size of the *in silico* search domain probed



a)



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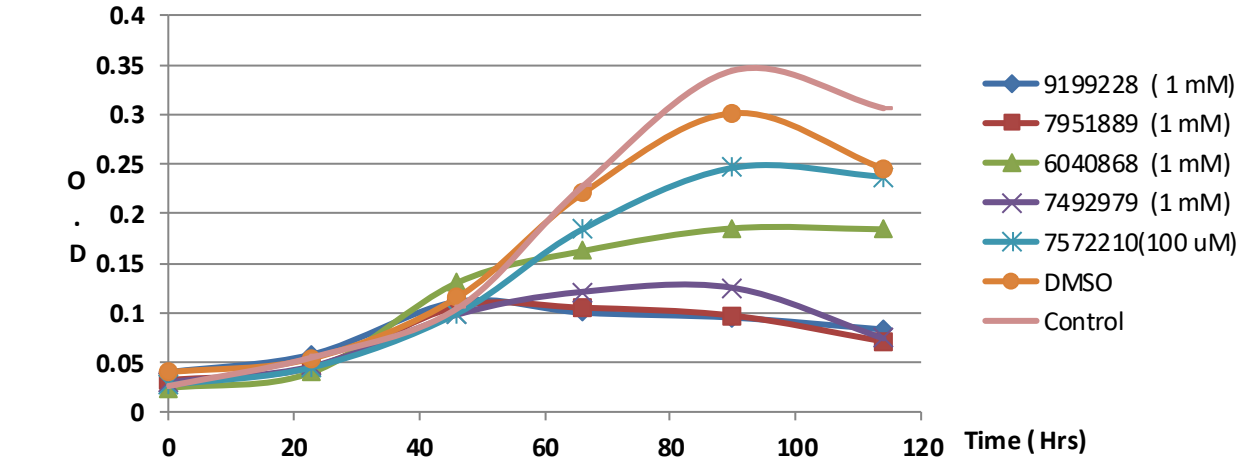

c)

Figure 5: Small molecule inhibition experiments using whole cells of *M. ruminantium*. Methanogen growth (OD) was measured in the presence or absence of DMSO (control) and compounds discovered during the *in silico* screening process at a concentration of **a)** 1 mM, **b)** combinations of 1mM and 500 uM, **c)** 1mM and 100 µM

N^5-N^{10}-methylenetetrahydromethanopterin reductase modelling and screening

Vincenzo Carbone and Ron S. Ronimus

AgResearch Limited, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand.

Summary

A three dimensional model has been produced for the enzyme Mer (N^5-N^{10}-methylenetetrahydromethanopterin reductase) of the methanogenesis pathway using sequence data from the rumen methanogen *M. ruminantium* (mru 0569) and available crystal structures (Aufhammer 2004, Aufhammer 2005, Shima 2000). The native enzyme is tetrameric and consists of a single subunit and catalyses an essential step in methanogenesis and thus represents a strong target, although developing an assay system for this enzyme may prove to be difficult. A number of compound libraries including the Chembridge building block library which contains small compounds that are readily modified into more efficacious drugs have been tested and screened, however results suggest that the docking protocol currently developed is insufficient and will require re-development.

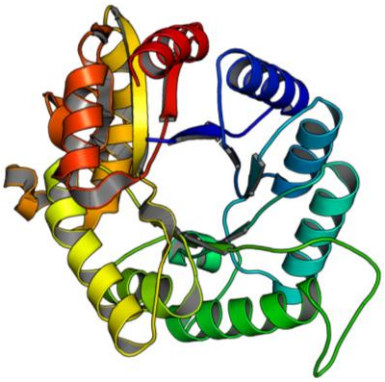

Figure 1: Model for the *M. ruminantium* Mer enzyme

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2. Identification of methanogen targets for a vaccine

Introduction

An attractive option being investigated to reduce methane emission from ruminants is to harness their immune system. This would be achieved by vaccinating animals to generate a substantial salivary antibody response that delivers a high yield of antibodies to the rumen and reduces the ability of methanogens to produce methane. Development of an anti-methanogen vaccine will require identifying key vaccine antigens. Due to the diversity of the methanogen population within the rumen, a successful methanogen vaccine will need to contain a number of antigens present on the major groups of methanogens found in the rumen.

Here we report the development of a novel method to identify vaccine candidates, conserved proteins that are exposed on the methanogen cell surface. This strategy to identify antigens for a vaccine is complementary to the already established procedure of predicting vaccine candidates from an analysis of methanogen genomic sequences. A number of potential vaccine candidates were identified and vaccine candidates from the dominant rumen methanogen *Methanobrevibacter* sp. SM9 were expressed in *E. coli* for further evaluation (reported here). In addition, the use of yeast as an alternative host for expressing methanogen antigenic proteins was explored.

Novel cell surface extracts from methanogens identify potential antigenic targets for an anti-methanogen vaccine

Dairu Shu, Bryce M. Buddle and D. Neil Wedlock

AgResearch Limited, Hopkirk Research Centre, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand.

Summary

A method was developed to produce sub-cellular extracts enriched for surface exposed proteins with potential as vaccine candidates, including targets not predicted by bioinformatics analysis of methanogen genomes. Fractions were produced from the dominant rumen methanogens, *M. ruminantium* M1 and *M.* sp. SM9 and shown to contain a higher proportion of membrane-associated proteins compared to fractions produced by other methods. A number of potential vaccine candidates were identified from analysis of the new cell surface extracts prepared from *M. ruminantium* M1 and four of these proteins were produced as recombinant proteins. The use of these new cell surface extracts to identify potential vaccine targets is complementary to predicting targets by bioinformatics analysis of methanogen genomes and has the potential to identify cross-reactive antigens for a vaccine.

Introduction

Development of an anti-methanogen vaccine will require identifying key vaccine antigens and there is evidence that antibodies against critical components of methanogens can impair their

ability to grow and produce methane (Buddle et al., 2010; Wedlock et al., 2010). Ideal vaccine candidates are proteins which are membrane-associated and are exposed on the surface of the methanogen cell. Bioinformatics has been used to identify such proteins in *M. ruminantium* M1 (Leahy et al., 2010). We hypothesised that some proteins which have a cell surface location or possess surface exposed domains could be missed for example, due to absence of canonical signal sequences or predicted transmembrane domains. Some of these proteins may have an unknown function. We have recently developed a new technique which has potential for identifying proteins that are both immunogenic and surface exposed. In this study, we produced new sub-cellular extracts from *M. ruminantium* M1 and *M.* sp. SM9. This method could be applied to other methanogens and since vaccine candidates are likely to be proteins that are conserved across a number of methanogen species, the extracts, and antisera produced against them could be used to determine cross-reactivity between different species of rumen methanogens. The proteins in the new fractions were identified and potential vaccine candidates identified using bioinformatics on *M. ruminantium* M1 and the more recently available genome sequences from *M.* sp. SM9 genomic and data from other methanogens. Selected targets were produced as recombinant proteins for evaluation as suitable antigens for inclusion in a prototype anti-methanogen vaccine.

Materials and Methods

Preparation of cell extracts

Extracts enriched for cell surface-exposed proteins were prepared from *M. ruminantium* M1 and *M.* sp. SM9 cells by *in vivo* biotin labelling of exposed proteins followed by streptavidin affinity purification. The procedure was adapted from methods described by Francoleon et al, 2009. Briefly, freshly grown methanogen cells were resuspended in phosphate-buffered saline (PBS) and incubated with an equal volume of 10 mM NHS-LC-biotin for 30 min at room temperature. The cells were washed three times with 100 mM glycine in PBS to quench and remove excess biotin reagent. The cells were subjected to gradient centrifugation over PercolPlus (density 1.038 g/ml) at room temperature and following washing with PBS resuspended in PBS with complete protease inhibitors (Roche). The cells were disrupted by repeated cycles of freeze thawing in liquid nitrogen and grinding with glass beads. Following centrifugation at 20,000 g for 10 min at 4^{o}C, the supernatant (whole cell lysate proteins, designated WCLP) was treated with 2% SDS in10 mM Tris-HCl and heated to 70^{o}C for 30 min to produce a derivative fraction designated as WCLS. Following centrifugation, the two different preparations, WCLP and WCLS containing biotin-labelled proteins were desalted and the labelled proteins isolated by streptavidin affinity purification using Dynabeads MyOne Streptavidin T1 (Invitrogen). The beads were washed with PBS containing 0.1% Tween 20 (PBS-T) prior to loading on the proteins in the desalted WCLP and WCLS preparations. Unbound proteins were removed by washing the beads 3 times with PBS containing 0.1% bovine serum albumin (BSA) and 2 times with PBS-T. Deionised water was added to the beads and the mixture incubated at 70^{o}C for 2 min and the biotinylated proteins were separated from the beads using the MagnaRack (Invitrogen) and concentrated by ultracentifugation with a 10 kDa Mol. wt cut off membrane. The isolated proteins were analysed by SDS-PAGE (NuPAGE 4-12% Bis-Tris gels, Invitrogen) and Western blotting using a streptavidin-HRP conjugate (DAKO).

Identification of proteins in extracts

The proteins in the extracts were separated by SDS-PAGE and the identity of the proteins was determined by LC-MS/MS (conducted by Lincoln, AgResearch) with access to AgResearch's *M. ruminantium* M1 and *M.* sp.SM9 genomic databases.

Bioinformatic analysis of selected proteins in the surface extracts

Selected proteins in the various extracts were subjected to bioinformatic analysis at AgResearch.

Expression of recombinant proteins

The proteins selected for expression in *E. coli* were analysed by TOPCONS consensus prediction of membrane protein topology to predict transmembrane domains and extracellular domains. For two of the proteins, MtrF and MtrG, the entire protein coding region was selected for expression (68 and 83 amino acids respectively for MtrF and MtrG), while for the other two proteins, NikA2 (amino acids 34-543) and AAABC (amino acids 27-278) corresponding to the predicted extracellular domains were selected. The genes coding these proteins, using the *M.* sp. SM9 amino acid sequences were synthesised using *E. coli* codon preference (GeneArt/Invitrogen). Constructs were made in the vector pET-32a (Novagen) to express the four proteins/domains and these plasmids were transformed into *E. coli* strain BL21 pLysS. Methods to culture the transformants and induce expression of recombinant protein and purify proteins using immobilised metal chelating chromatography (IMAC) have been described previously (Wedlock et al., 1999). Recombinant proteins were analysed by SDS-PAGE and Western blotting using mouse anti-His tag monoclonal antibody (Novagen) and anti-mouse IgG HRP (DAKO).

Production of antibodies and analysis

Production of antisera against recombinant proteins was undertaken in sheep according to methods described for producing antisera to methanogen fractions (Wedlock et al. 2010). Four 5 month old female lambs, Romney cross (Romney dam – Texel X East Friesian Sire) were used to produce antibodies against recombinant proteins. All animals were grazed on pasture, and were provided water *ad libitum*. Animal ethics approval was granted by the AgResearch Grassland's Animal Ethics Committee, Palmerston North, New Zealand, for all procedures involving animals. Saponin was used as the adjuvant rather than Freund's incomplete adjuvant as used previously. Saponin (from Quillaja bark, Sigma S4521) was prepared at 10 mg ml^{-1} in PBS and sterilised by filtration through a 0.22 μm membrane filter and mixed with the antigenic fractions to give a 5 mg of saponin per vaccine dose. Each vaccine contained 0.1 mg of recombinant protein and was administered subcutaneously in a 2 ml volume. Sheep were vaccinated 3 x at 3 week intervals. At 2 weeks after the third vaccination, serum and saliva were obtained and the antibody responses determined by ELISA and Western blotting according to methods previously described (Wedlock et al., 2010). Pre-immune sera was obtained from each of the sheep prior to the first vaccination.

Determination of inhibition of methanogen activity

The ability of antibodies in the sera to inhibit growth of methanogens and reduce production of methane in *in vitro* pure cultures of methanogens was determined according to previously described methods (Wedlock et al., 2010).

Results

Preparation of cell-surface extracts and identification of proteins

Three different cell surface extracts were prepared by *in vivo* biotin labelling of exposed proteins followed by affinity purification with streptavidin. Two extracts containing cell surface-exposed proteins were isolated from *M. ruminantium* M1 and one extract was prepared from *M.* sp. SM9 cells. The proteins in the various preparations were analysed by SDS-PAGE and Western blotting. Figure 1 shows a SDS-PAGE gel of the biotin-labelled proteins in one of the extracts (the second extract) prepared from *M. ruminantium* M1cells. The proteins eluted from the Dynabeads (lanes 2, 3) strongly reacted with streptavidin labelled antibody confirming that the proteins were biotin labelled. The dominant Coomassie blue stained protein in the eluted fractions was BSA as the purification buffer contained this protein.

In order to identify the proteins in the cell surface extracts, the proteins in the extracts were separated by SDS-PAGE, stained with Coomassie blue (see Figure 1 for example) and the visible protein bands that showed reactivity with streptavidin HRP conjugate antibody were excised from the gels. From the first extract that was prepared from *M. ruminantium* M1, 8 protein bands were excised. The proteins in the excised gel fragments were identified by LC-MS/MS with access to the *M. ruminantium* M1 and *M.* sp. SM9 databases. Subsequently, 13 bands from a second extract prepared from *M. ruminantium* M1 were also submitted for analysis by LC-MS/MS.

The data from analysis of the protein bands excised from the first extract prepared from M1 become available first and this information was used to select vaccine targets for the next stage of investigation. In the first extract from *M. ruminantium* M1, 48 different proteins were identified within the 8 excised protein bands. Of these proteins, 46 had a known or predicted functional classification, while 2 proteins had no known function. Table I shows the 13 proteins (representing 28% of the total number of proteins with known function) considered likely to be membrane-associated or have extracellular domains, based on several criteria including known function, protein topology, presence of canonical signal sequences. Several targets were selected from this sub-set of proteins for expression in *E. coli* as described below. With the exception of BtcC, these proteins had not been identified in methanogen fractions prepared previously (work conducted in the PGgRc programme) using different techniques. BtcC had been identified in different fractions prepared from *M. ruminantium* M1 and has been expressed in *E. coli* and now has also expressed in yeast (additional work conducted in this programme and reported below).

Three of the membrane-associated proteins identified in the new cell-surface fractions of *M. ruminantium* M1 (Table I) were selected to express in *E. coli*. These were the MtrG and two other membrane-associated proteins, nickel ABC transporter substrate-binding protein NikA2 (mru_1710), and amino acid ABC transporter substrate-binding protein (AAABC) (mru_1777). Bioinformatic analysis was performed on the proteins using TOPCONS to identify topology and identify which parts of the protein were predicted to span membrane or lie outside the cell membrane and also determine the degree of conservation of amino acid sequence across a range of methanogens, including *M. ruminantium* M1, *M.* sp. SM9, and other methanogens including YLM1, Abm4, CM1, BRM9 and 3F5. Since two of the Mtr

subunits were identified in the new extract, an analysis of all the known Mtr subunits was also done to determine which Mtr subunits had extracellular domains (ectodomains). With the exception of MtrH, all sub-units were predicted to be membrane associated and have ectodomains of varying lengths. The Mtr subunit F was selected for expression, rather than MtrA1, the protein identified in the extract. MtrA1 has only a very short predicted ectodomain in M1 and this may be even shorter in the SM9 'version' of the protein, whereas MtrF has a longer predicted extracellular domain compared to the MtrA1 subunit. NikA1 was not selected as this protein was less conserved compared to NikA2 and was not present in SM9. Each of the four selected proteins was conserved in *M. ruminantium* M1 and *M.* sp. SM9 and at least two other methanogens. Thus antibody which reacts to the *M. sp.* SM9 'version' of the targets has the potential to cross-react with the homologous protein found in a range of methanogens.

The proteins in the second extract prepared from M1 were identified by LC-MS/MS. Some additional potential vaccine targets were identified and bioinformatic analysis was performed on five of these proteins. These were molybdate ABC transporter substrate-binding protein, ModA (mru_0201), ATPase RIL (mru_0507), transporter Na+/H+ antiporter family protein (mru_0405) AtwA1 methyl-coenzyme M reductase component A2 (mru_1262) and Atp V-type ATP synthase subunit I (AhaI) (mru_0696). The results from this analysis indicated that ATPase RIL, AtwA1 and Aha1 were conserved across seven methanogens while the other proteins were conserved in three-four of the methanogens. Mod A and AhaI may be the best candidates for further investigation. The second extract also contained proteins identified in the first extract including adhesin-like proteins, NikA1 and NikA2 and BtcC.

An extract was prepared from *M.* sp. SM9 (shown above in Figure 1) using the same methods as employed for *M. ruminantium* M1. The proteins in this extract were analysed by LC-MS/MS and a number of surface proteins were identified. These proteins have not been investigated further at this stage.

Expression of recombinant proteins

Four potential vaccine candidates, MtrF, MtrG, NikA2 and AAABC were expressed in *E. coli*. For all four proteins, the amino acid sequences from *M.* sp. SM9 were used for expression rather than the *M. ruminantum* M1 sequences. This was done to enable cross-reactivity between the two species of methanogens to be determined as antibody produced against the *M.* sp. SM9 proteins could be tested for anti-methanogen activity in *M. ruminantium* M1 cultures. Constructs were made in the vector pET-32a (Novagen) by Invitrogen using *E. coli* codon preference and placing the gene coding for the methanogen protein in frame with thioredoxin. Each of the plasmids was transformed into an expression strain of *E. coli* strain. The *E. coli* transformants were cultured in expression medium and protein expression induced by addition of IPTG to the cultures. All four *M.* sp. SM9 proteins were expressed successfully in *E. coli*, although there was some variability in yield and also solubility of the proteins. The proteins were purified using immobilised metal chelating chromatography via the poly-histidine tag incorporated into protein. Sufficient quantities of each protein were produced for immunising sheep to produce antisera.

The purified proteins were confirmed by SDS-PAGE and Western blotting with mouse anti-His•Tag monoclonal antibody (IgG1) (Novagen) and rabbit anti-mouse Immunoglobulins/HRP (DAKO). Figure 2 shows reactivity of anti-his antibody with proteins of the expected molecular size.

Production of antisera to the recombinant proteins

Sheep were immunised with each of the recombinant proteins mixed with the adjuvant saponin to produce antisera (antibodies) against the proteins. Serum antibody titres in the vaccinated sheep were measured by ELISA. Vaccination with the proteins mixed with saponin induced strong target-specific antibody responses. Antibody titres post-vaccination were 500-1000 fold higher compared to pre-vaccination titres indicating the proteins are highly immunogenic, an important requirement for a vaccine antigen. Figure 3 shows the antibody titres in the sheep vaccinated with the four different recombinant proteins. Each of the purified recombinant proteins were blotted with the antisera produced against the proteins and the results are shown in Figure 4. Antibody reacted with proteins of the expected mol. wt.

Western blotting of *M.* sp. SM9 proteins with antisera produced against M1 fractions to determine cross-reactivity

We have previously produced antisera against a range of fractions prepared from *M. ruminantium* M1. It was of interest to determine whether these antisera would contain cross-reactive antibodies that recognise the *M.* sp. SM9 recombinant proteins. In an initial experiment, the recombinant proteins were run on SDS-PAGE and blotted with antisera produced against crude antigenic fractions prepared from *M. ruminantium* M1. There was strong reactivity of antisera produced against a cytoplasmic fraction prepared from *M. ruminantium* M1 with the recombinant AAABC protein and weak reactivity with NikA2 and MtrG (Figure 5). A similar pattern of reactivity was seen with antisera against a cell-wall derived fraction of M1 (data not shown). Western blotting was also performed using pre-immune sera. Interestingly, pre-immune sera also showed reactivity against AAABC, NikA2 and MtrG, although in the case of NikA2 this reactivity was weaker compared to reactivity with the antisera. No reactivity was seen when the proteins were blotted with secondary antibody alone as a control. Crude antigenic fractions previously prepared from *M.* sp. SM9 were blotted with the antisera prepared against the various recombinant proteins (data not shown). The antisera showed little reactivity to proteins in the fractions, but Western blotting would need to be repeated with newly prepared fractions as the fractions had been prepared some time ago and the proteins may have degraded.

Effect of antisera on growth of methanogens and production of methane in *in vitro* pure cultures of methanogens

The antisera produced against each of the recombinant *M.* sp. SM9 proteins was tested for the ability of target specific antibodies to inhibit methanogen growth and production of methane in *in vitro* pure cultures of methanogens. A preliminary trial with *M. ruminantium* M1 showed that addition of antisera produced against the different targets to cultures of *M. ruminantium* M1 did not reduce methanogen growth or inhibit production of methane. More recently, the ability to test antibodies for inhibition of methanogens has been developed for *M.* sp. SM9. In future studies, testing of the antisera with *M.* sp. SM9 will be done to determine if the antisera against the proteins will inhibit this species of methanogen and the testing with *M. ruminantium* M1 will be repeated.

Discussion

Vaccine targets are likely to be membrane-associated proteins with surface exposed domains. Previous sub-cellular fractions prepared from both *M. ruminantium* M1 and *M.* sp. SM9 had a very high proportion of intracellular proteins and there were few proteins known or predicted

to be surface associated. This has hindered the identification of vaccine targets using techniques such as Western blotting with antisera produced against the various fractions. This limitation has prompted us to investigate better ways of producing sub-cellular fractions for mining vaccine antigens. The method developed in this study used a novel approach of firstly biotinylating exposed surface proteins before cell lysis. The biotinylated proteins were then captured via binding to streptavidin. This procedure was developed from a published procedure to locate surface exposed proteins and was adapted for both *M. ruminantium* M1 and *M.* sp. SM9 cells. This approach to produce sub-cellular fractions from methanogens which are more amenable to identifying potential vaccine targets was successful. While the extracts contained a number of intracellular proteins, they had a greater abundance of membrane associated proteins than extracts prepared by other methods. With the exception of BtcC, which had been identified previously in a cytoplasmic fraction prepared from *M. ruminantium* M1, the membrane-associated proteins identified using the new cell surface extracts prepared in this project had not been identified in previous methanogen fractions. Some of these proteins would not necessarily have been predicted as potential vaccine targets from bioinformatic analysis of genomic sequences. Thus our hypothesis, that some proteins with a cell surface location (potential vaccine candidates) could be missed by relying solely on prediction, is supported by our findings.

Several of the membrane-associated proteins in the new cell-surface extracts prepared in the current study were selected for further evaluation as potential vaccine antigens and four targets, AAABC, NikA2, MtrF and MtrG were successfully produced in *E. coli*. Membrane proteins are often considered difficult to express. The strategy of expressing the proteins by fusion with thioredoxin may have aided the expression of the methanogen proteins as it was possible to produce sufficient quantities of all four proteins to immunise sheep for production of antibodies. High titre sheep antisera was produced against each of the four recombinant proteins as shown by ELISA and Western blotting. Western blotting was performed on the recombinant proteins using antisera previously produced against M1 antigenic fractions. Three of the recombinant *M.* sp. SM9 proteins showed reactivity with antisera produced against a crude sub-cellular fraction prepared from *M. ruminantium* M1. However, the recombinant proteins also showed reactivity to pre-immune sera and a possible explanation may be that sheep have developed natural systemic antibody responses to these methanogen proteins. Reactivity of antibody in the *M. ruminantium* M1 antisera to NikA2 was stronger than reactivity of the pre-immune sera suggesting there was some degree of cross-reactivity of antibody to *M. ruminantium* M1 proteins to the *M.* sp. SM9 NikA2 protein. The ability of the antisera produced against the four recombinant proteins to inhibit methanogen cultures was tested. Preliminary tests suggested that the various antisera to the *M.* sp. SM9 proteins were not inhibitory to *M. ruminantium* M1 cultures suggesting that the antibodies to these targets were not cross-reactive. However, these tests need to be repeated once some technical difficulties with the assay have been resolved. The ability of the antisera to the *M.* sp. SM9 proteins to inhibit *M.* sp. SM9 cultures will be determined and will provide important information about the usefulness of these proteins as potential vaccine antigens.

Analysis of the proteins in the cell extracts indicated other potential vaccine candidates, in particular Mod A and AhaI. The ectodomain (amino acids 27-275) of Mod A and either the entire protein (amino acids 2-666) or amino acids 375-666 (omitting the first part of the protein which is predicted to be intracellular) of AtpI V-type ATP synthase subunit I (AhaI) could be expressed in *E. coli.* A number of proteins with unknown function were identified in the cell-surface extracts and these could be investigated further. Further analysis of the proteins identified in the cell surface extract from *M.* sp.SM9 may reveal other potential vaccine candidates which can be tested.

In this study, the adjuvant saponin was used to formulate the recombinant proteins for immunisation of sheep and production of antisera. This adjuvant had been shown to promote antibody responses to the wide repertoire of proteins in crude sub-cellular fractions prepared from *M. ruminantium* M1 but had not been used previously to promote antibody responses to single purified proteins. In the current study we demonstrated that saponin was effective as an adjuvant when used with single antigens such as the recombinant proteins. Saponin is an inexpensive adjuvant and thus could be used for formulation of a cost effective anti-methanogen vaccine.

In summary, this research has led to the successful development of a new strategy for identifying potential vaccine candidates. A method was developed to produce sub-cellular extracts enriched for surface exposed proteins with potential as vaccine candidates, including targets not predicted by bioinformatics analysis of methanogen genomes. While these extracts are more technically challenging to produce that other fractions such as the cytoplasmic protein fractions previously produced, they have a higher proportion of membrane-associated proteins. Thus these extracts are better suited to identifying antigen candidates for a vaccine. A number of potential vaccine candidates have been identified from analysis of the new cell surface extracts prepared from *M. ruminantium* M1and several of these have been produced as recombinant proteins. Although antibodies against these targets may not be cross-reactive, only four proteins have been tested to date. Additional potential vaccine targets have been identified in other cell surface extracts prepared from *M. ruminantium* M1 and *M.* sp. SM9 and these will be investigated further. Additional cell surface extracts can be prepared from a range of rumen methanogens. Provided sufficient quantities of the cell surface extracts can be produced, antisera can be produced against the proteins in these extracts and used to further study cross-reactivity between methanogen species. The use of these new cell surface extracts to identify potential vaccine targets is complementary to predicting targets by bioinformatics analysis of methanogen genomes and will ensure potential antigens for a vaccine are not overlooked.

Acknowledgements

Justine Jacobs, Tania Wilson, and Debjit Dey for excellent technical assistance. Peter Janssen for advice and Bill Kelly, Sinead Leahy and Graeme Attwood for bioinformatic analysis, Stefan Clerens and Santanu Deb-Choudhury for LC-MS-MS analysis of proteins. We thank MPI and PGgRc for financial assistance.

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Table I. Sub-set of proteins identified in cell surface extract prepared from M1 (first extract) considered as likely to have a cell-surface location

Protein (Sequin-NCBI annotation)	**Locus tag**	**Functional classification**
Adhesin-like protein	mru_0086	Cell envelope -cell surface proteins
A1A0 archaeal ATP synthase subunit H, AhaH	mru_0695	Energy metabolism
A1A0 archaeal ATP synthase subunit A, AhaA	mru_0701	Energy metabolism
Bicarbonate ABC transporter substrate-binding protein, BtcC	mru_0949	Central carbon metabolism
Glutamylaminopeptidase, PepA	mru_1119	Transcription
Adhesin-like protein	mru_1386	Cell envelope -cell surface proteins
Nickel ABC transporter substrate-binding protein NikA1	mur_1618	Transporter
Nickel ABC transporter substrate-binding protein NikA2	mur_1710	Transporter
Amino acid ABC transporter substrate-binding protein	mru_1777	Transporter
Tetrahydromethanopterin S-methyltransferase subunit G (MtrG)	mru_1917	Energy metabolism
Tetrahydromethanopterin S-methyltransferase subunit A1 (MtrA1)	mru_1919	Energy metabolism
Adhesin-like protein	mru_2049	Cell envelope -cell surface proteins
Adhesin-like protein	mru_2090	Cell envelope -cell surface proteins

Figures

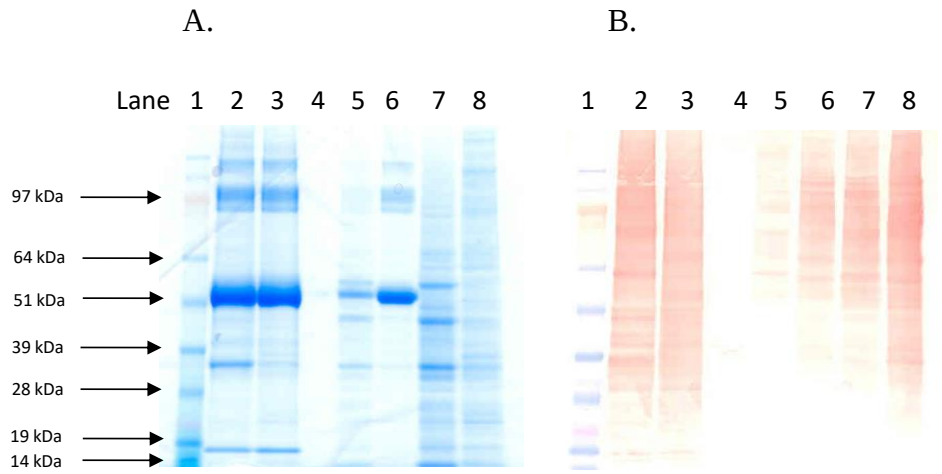

Figure 1. SDS-PAGE (A) and Western blot (B) of biotin-labelled proteins from cell surface extract prepared from *M. ruminantium*M1. Proteins were immunoblotted with streptavidin, HRP conjugated antibody (Pierce, 1:10,000 dilution). Lane 1. mol. wt. markers; lanes 2&3, proteins eluted from Dynabeads followed purification from WCLP and WCLS respectively; lanes 5&6, unbound proteins (non-biotinylated proteins); lanes 7 & and 8, whole methanogen cell lysate prepared from *M. ruminantium* M1 cells before purification with Dynabeads (lane 7: WCLP; lane 8: WCLS). Note presence of BSA in eluted fractions.

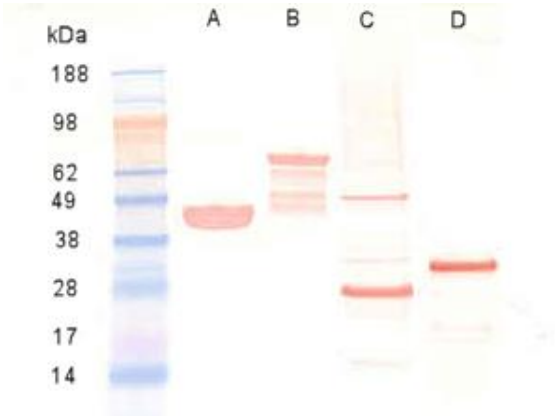

Figure 2. Western blot of recombinant proteins produced in *E. coli.* Proteins were separated by SDS-PAGE and blotted with anti-his tag antibody.Lanes A, AAABC; B, NikA2, C, MtrF, D, MtrG

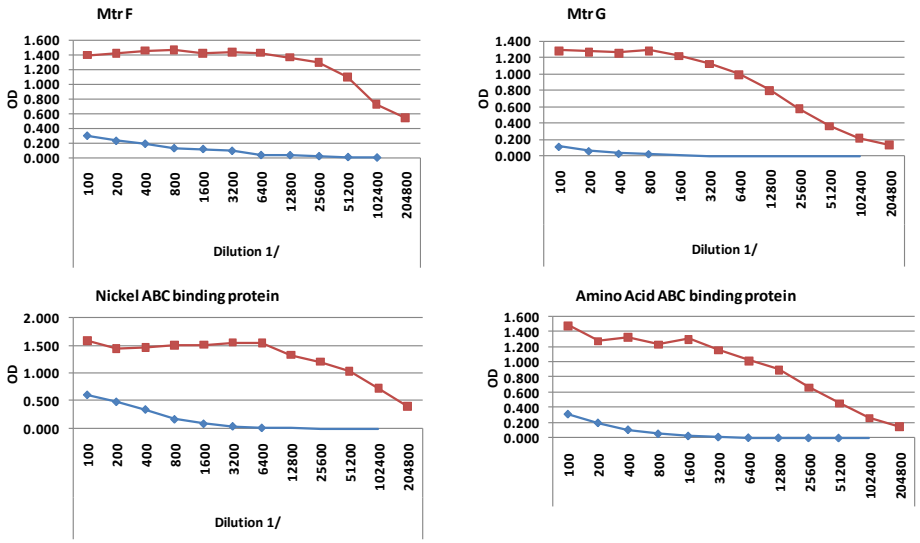

Figure 3. Antibody titres in antisera produced against recombinant proteins from *M. ruminantium* sp. SM9, MtrF, MtrG, NikA2 and AAABC. ■, antisera; ■, pre-immune sera. Antibody titres were determined by ELISA using 2-fold serial dilution of sera and depicted as optical density versus reciprocal of the serum dilution

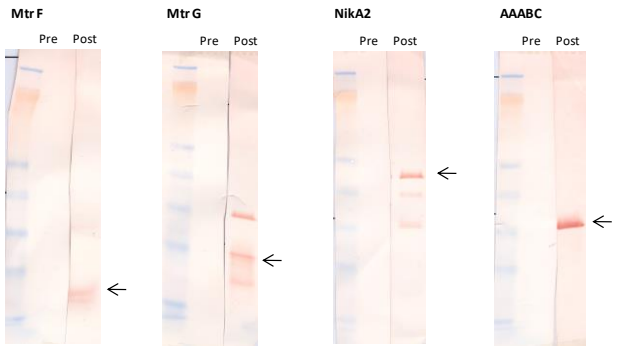

Figure 4. Western blotting with antisera produced against the recombinant proteins. Pre, preimmune sera; post, antisera. The arrow denotes expected mol. wt. of each recombinant protein.

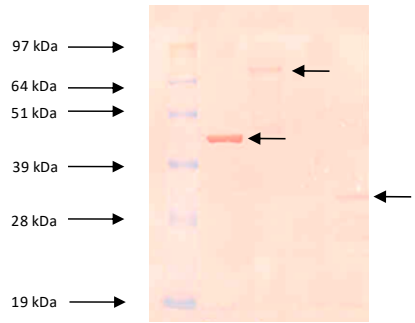

Figure 5. Western blotting of recombinant SM9 proteins with M1 antisera. Lane 1, AAABC; lane 2, NikA2; lane 3, MtrF; lane 4, MtrG. Arrows shows reactivity of antibodies with proteins of expected mol. wt.

Production of methanogen antigenic proteins in the methylotrophic yeast *Pichia pastoris*: an alternative to *Escherichia coli*

Justine Jacobs, Bryce M. Buddle and D. Neil Wedlock

AgResearch Limited, Hopkirk Research Centre, Grasslands Research Centre, Tennent Drive, Private Bag 11008, Palmerston North 4442, New Zealand.

Summary
Two methanogen proteins were selected for expression in *Pichia pastoris*, BtcC and a thermosome. Constructs were made to express the two different methanogen proteins in yeast and transformants were cultured in media to express the proteins. Secreted recombinant proteins were not detected in the culture media but Western blotting conducted on cell-free extracts of the yeast strains confirmed the presence of recombinant BtcC and thermosome proteins.

Introduction
The production of recombinant proteins can face considerable technical and biological challenges depending on the type of protein being expressed. Membrane-associated proteins are known to be difficult to express and it is possible that it may be easier to express some of the methanogen proteins in yeast than in *E. coli*. In a previous project (METH0802 PGgRc - MPI-SLMACC) we initiated work on determining the feasibility of using the yeast *Pichia pastoris* as a host for expression of methanogen proteins, particularly proteins which have transmembrane domains. Yeast is capable of glycosylating proteins and glycosylation may be important in antibody recognition and binding to the proteins. Constructs had been made to express two different methanogen proteins in yeast. These proteins were BtcC, a membrane associated protein and identified in previous methanogen fractions and also identified in the cell surface extracts prepared by biotinylation of proteins (see report above) and a thermosome. The thermosome protein was identified previously in the yeast *Methanosarcina barkerii* CM1 by Western blotting with antisera produced against a cell-wall derived fraction prepared from CM1. Thermosomes may be surface-associated proteins and have important function and thus were considered as potential vaccine targets. In this study, we expressed both proteins in *Pichia pastoris*.

Materials and Methods

Expression of proteins in yeast
Two methanogen proteins were expressed in yeast. The proteins were BtcC (mru_0949) and a thermosome (mru_1645). The *M. ruminantium* M1 amino acid sequence of each protein was used for expression. BtcC has been expressed in *E. coli* (work conducted in the NZAGRC vaccine programme) while the thermosome has not been expressed in *E. coli*. Constructs were made to express the BtcC protein (ectodomain, amino acids 24-315) and the entire protein coding region (amino acids 1-536) of the thermosome in *P. pastoris*. Genes covering the coding region of each protein/domain were synthesised with *yeast* codon bias by GeneArt

(Invitrogen) and constructs were made by cloning the genes into the *EcoR*1 and *Xba*I sites of the yeast expression vector pPICZalphaA (Invitrogen). *Pichia pastoris* SMD1168 was transformed with these constructs by electroporation and transformants selected for resistance to the antibiotic Zeocin (100 μg/ml concentration). Methods to culture *Pichia pastoris* and express recombinant proteins have been described previously (Wedlock et al., 2004).

Western blotting of cell-free extracts

Yeast transformants were resuspended in buffer containing protease inhibitors and the cell disrupted by grinding with glass beads. Following centrifugation, recombinant proteins in the supernatant (cell-free extract) were analysed by SDS-PAGE and Western blotting using mouse anti-His tag monoclonal antibody (Novagen).

Results

Expression of recombinant methanogen proteins in *Pichia pastoris*

Constructs to express two methanogen proteins, BtcC1 and a thermosome protein had been made previously using the vector pPICZαA. In these constructs, the coding sequences of the methanogen proteins are expressed as fusions with an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. The rationale was that a fusion of the methanogen protein with the α-factor secretion signal may result in the recombinant proteins being directed into the secretory pathway, resulting in secretion of the methanogen proteins into the culture medium. This may aid purification of the recombinant proteins as culture media contain few other proteins. Alternatively, the protein may localise to the yeast membrane which may assist with correct folding of the proteins (Bednarek et al., 1992). The constructs were transformed into *Pichia pastoris* strain SDM1168 for production of recombinant proteins. A series of transformants were isolated with each construct and screened for the production of recombinant protein.

SDS-PAGE analysis of culture media and staining with either Coomassie blue or silver stain did not show the presence of secreted recombinant protein, either BtcC or thermosome. SDS-PAGE analysis of sub-cellular extracts prepared by disrupting the cells showed the presence of a large number of proteins as expected. However, it was difficult to identify the recombinant proteins. Western blotting of the cell extracts of the transformants with anti-his antibody indicated the presence of recombinant protein and confirmed that both BtcC and the thermosome had been expressed by the respective transformants, albeit at low levels (Figure 1). Western blotting of the proteins in cell extracts prepared from *P. pastoris* strains transformed with plasmid expressing BtcC showed reactivity of antibody with a protein of the expected molecular weight in all the five transformants (stronger in three of the transformants). A similar result was seen with yeast transformed with the thermosome construct (data not shown).

Discussion

While *E. coli* has been shown to be an effective host for expression of methanogen proteins, we have investigated the possibility of using an alternative host particularly for membrane-associated proteins (Bednarek et al., 1992). Yeast is capable of glysosylating proteins and although the pattern of glycosylation of proteins by yeast will differ from archaea, carbohydrate moieties on proteins may be important for recognition by the immune system and influence antibody binding. To investigate yeast as an alternative expression system for

methanogen proteins, two methanogen proteins which are potential vaccine candidates, were selected to express in *P. pastoris*. Both BtcC and the thermosome were expressed in yeast but at only a low level. The proteins were produced intracellularly and were identified in cell-free extracts prepared from yeast transformants. Further optimisation of yeast will be required to produce useful quantities of recombinant protein and this may be achieved by controlling the yeast culture parameters or the choice of construct and signal sequence used to express the proteins. Both methanogen proteins had a histidine tag which will facilitate purification once further optimisation of expression has been achieved. Future studies will investigate whether the proteins expressed in yeast are glycosylated and what affect the carbohydrate moieties have on immunogenicity of the proteins.

Acknowledgements

We thank MPI and PGgRc for financial assistance.

References

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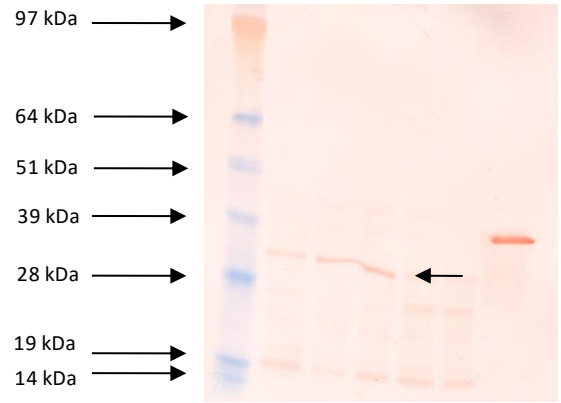

Figure 1. Western blotting (B) of cell-free extracts of *P. pastoris* strains transformed with pPICZalpha A/BtcC. Western blotting was performed with anti-His tag antibody. Lane 1, mol. wt. markers; lanes 2-6, yeast transformants; lane 7, *E. coli* expressed BtcC (included as a positive control). Arrow shows expected mol. wt. of yeast expressed BtcC. The BtcC expressed in *E. coli* has a larger mol. wt. than the yeast expressed BtcC due to fusion with thioredoxin.

Appendices

1. Research aims and goals of programme

Research aim sequence	1.1
Research aim title	Identify inhibitors and vaccine antigens
Research aim task	The project has two research aims: 1. To use methanogen enzyme structures, or structural models based on already published non-rumen methanogen structures, to develop novel inhibitory molecules that avoid existing toxicity problems. 2. To use methanogens, representative of two major groups of rumen methanogens to identify critical structures which are vaccine candidates and are conserved across a broad range of methanogens.
End Date:	30th June 2012

2. Contracted outputs

Short Title	Outputs
Description	• By June 2012, 2-3 validated target enzymes, which have been successfully expressed in *E. coli* will have been screened for initial crystal-forming conditions and optimised (if suitable crystal initial conditions are found). • 1-2 models or enzyme structures will be used for screening small molecule inhibitors *in silico* and compounds will have been ordered and tested for each target in enzyme assays and/or pure cultures and if successful, in rumen fluid-based *in vitro* assays. • Results will have been published (where appropriate) and reported to MAF. • By June 2012, 10 or more vaccine candidates will have been identified from SM9 or CM1 by bioinformatics or from Western blotting results for producing as recombinant proteins. • Three or more recombinant proteins will be produced and antisera raised against these targets will be analysed by ELISA and Western blotting. • If successful, the antisera will be tested for the ability of target-specific antibodies to reduce methane in *in vitro* pure cultures of methanogens.

3. Capability building

This project has provided early career (post-doctoral) research experience for Dr Vincenzo Carbone.

4. Outputs and publications

A paper on identifying inhibitors will be presented at an upcoming New Zealand conference subject to meeting any confidentially requirements. A new strategy for identifying potential vaccine candidates has been developed using a novel method to produce cell surface extracts from methanogens.