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Peptide-phage display libraries to discover bioactives against rumen methanogens

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Abstract

The primary goal of this project was to use bioactive peptides, discovered by screening peptide phage display libraries, to redirect fermentation end products away from methane formation, thereby reducing greenhouse gas emissions from livestock production systems. By using a specifically designed screening method, three bacteriophage-peptides were identified with inhibitory activity towards a model methanogen *Methanobrevibacter ruminantium*, as well as less dominant strains in the rumen, *Methanosarcina barkeri* and *Methanobrevibacter gottschalkii*. It is expected some modification of the peptide sequences may be required to further enhance their activity and to protect them from degradation in the rumen environment. This technology could be delivered as inhibitory peptides for use as ruminant supplements, or through a vaccination based approach where the peptides are used to identify cell surface proteins that are crucial to survival of methanogens in the rumen.

Executive Summary

The primary goal of this project was to utilise bacteriophage libraries to discover bioactive peptides capable of binding and inhibiting the growth of rumen methanogens and reducing methane emissions from livestock. Traditionally, in experiments using bacteriophage-display technologies, peptides of interest have been identified *in vitro* by screening libraries for binding against recombinant or purified versions of a target ligand. However, this strategy is only useful when valid targets have already been identified by other molecular means. In this project, more innovative strategies have been developed to utilise whole methanogen cells as binding targets. This body of work has focused on adapting phage-display techniques to an archael system, with the ultimate goal of not only discovering novel inhibitory peptides, but also identifying and characterising methanogen cell-surface proteins that could be exploited in alternative antimethanogenic strategies.

To this end an optimised, anaerobic screening protocol using the model rumen methanogen, *Methanobrevibacter ruminantium* in a medium designed to mimic the peptide-binding conditions within the rumen was developed. This protocol was employed in an effort to increase the chance that any selected methanogen binding peptides would be relevant in downstream applications.
Using two alternative screening strategies, a pool of seventy phage-displayed peptides were initially isolated and of these, three demonstrated both high binding and varied levels of inhibitory activity towards *M. ruminantium*. The best of these inhibited methane production by at least 60%. Inhibition of methane production by these phage-displayed peptides was also demonstrated to varying degrees in two other methanogen species *Methanosarcina barkeri* and *Methanobrevibacter gottschalkii*.

Because linear versions of the peptides, or some modified form of them, represent another method of delivery of bioactives to the rumen, recombinant forms of the three inhibitory phage-displayed peptides were made. However, linear peptides can behave differently to their bacteriophage-displayed counterparts so it was necessary to firstly establish their efficacy at binding methanogens under the same experimental conditions used for the phage-displayed peptides. Only after comparable levels of binding were confirmed for each of the peptides were the inhibitory activities of the peptides assayed. Two of the linear peptides mimicked the inhibition levels of their

phage-peptide counterparts against the same methanogen species, while one was considerably less active. Together they exhibited a synergistic inhibitory effect better than either peptide alone in at least one methanogen species.

As well as demonstrating inhibition of methane production in pure methanogen cultures, experiments were undertaken to demonstrate the efficacy of these peptides in mixed rumen culture replete with a broad diversity of rumen microbes. Initial experiments using single linear peptide resulted in no discernible reduction in methane production being observed. It is likely that the peptide was rapidly degraded by the proteolytic bacteria present in the mixed culture. Further work aims to mitigate degradation of the bioactive peptide/s in the rumen by using denatured phage particles or by modifications to the linear peptide such as acetylation, pegylation or concatamerisation.

The work reported so far has focused on the impact of methanogen-binding peptides on methane production in *in vitro* experiments, but other "pull-down" experiments were also devised and performed in order to identify the putative cell surface receptors responsible for binding these inhibitory peptides. The receptor molecules identified in such an approach could be used as antigens in vaccine strategies being investigated by others as a means of reducing methane emissions from livestock. While the validity of the attempted pull-down assay to recover target protein was demonstrated, insufficient methanogen protein was captured for its sequence to be determined. As identification of any captured *M.ruminantium* protein is not expected to be difficult now that the genome of this organism has been sequenced, alternative strategies need only focus on increasing the yield of protein obtained in these experiments. The identification of cell surface binding targets in the current project could productively merge with the AgResearch New Zealand vaccination program that has the capability to not only produce antibodies against target proteins but is able to undertake vaccination studies in ruminants and measure the effectiveness of different antigens against methane production in respiratory chambers.

Working toward a reduced methane production from Australia's livestock industries is considered to be beneficial for a number of reasons. Nationally it will help to reduce Australia's green-house gas pollution; and for the individual livestock producer potential economic benefits could flow from an increase in feed-conversion efficiency as well as from a reduced tax burden should methane production from livestock ever become part of a carbon tax plan to control global warming.

Contents

1 Background

1.1 Introduction

It has been well established that greenhouse gases are the major contributing factor to the anthropogenic heating of the earth's atmosphere. Advances in the technology associated with characterising, measuring and modelling greenhouse gas emissions has allowed scientists to better estimate the sources of these gases, as well as their relative warming capacities with respect to the most well known greenhouse gas, carbon dioxide (Kebreab et al., 2008; Montzka et al., 2011; Sise et al., 2011). Such studies have revealed one of the most potent and prevalent greenhouse gases is methane, with a warming capacity 21 times higher than that of carbon dioxide, making methane emissions a target for strategies to tackle climate change (Montzka et al., 2011; Solomon et al., 2010). It is estimated that the animal agriculture sector is responsible for up to 18% of global greenhouse gas emissions, with methane produced and released by enteric livestock farming being the largest contributor to this figure (O'Mara, 2011).

The methane emissions from livestock are a side-effect of enteric fermentation in the rumen that is aided by anaerobic, methanogenic archaea (Balch et al., 1979). These methanogens participate in ruminant metabolism by utilising the hydrogen and carbon dioxide that accumulate in the rumen to encourage the breakdown of cellulose into products that can be absorbed by the animal (VanNevel and Demeyer, 1996). Besides producing methane, this process is also disadvantageous because it represents a poor utilisation of the carbon source in the feed provided (Johnson and Johnson, 1995) . Consequently, various strategies to redirect fermentation end-products away from methane formation and reduce greenhouse gas emissions and increase productivity from livestock production systems have been investigated (Buddle et al., 2011; McAllister and Newbold, 2008; Patra, 2011; Shibata and Terada, 2010). These studies have tended to focus on either nutritional intervention strategies designed to reduce methane emissions by limiting the production of carbon dioxide and hydrogen (McGinn and Beauchemin, 2006; McGinn et al., 2004; Reed et al., 2004) or chemical additives intended to inhibit methanogen growth (Denman et al., 2007; Foley et al., 2009; Guo et al., 2008; NagarAnthal et al., 1996). To date, practical effective solutions have yet to be deployed to the industry on a large scale.

1.2 Approach

An alternative strategy to reduce methane emissions from livestock could potentially be based on antimicrobial peptides (AMP) directed towards the methanogens in the rumen. Investigating peptides for potential anti-methanogenic activity is an attractive option since antimicrobial peptides are universal and ubiquitous in nature and are stable in a range of conditions, from swamplands to proteolytic gut environments such as the rumen (Brogden, 2005; Gillor et al., 2011; Rossi et al., 2008). As therapeutics or antibiotics, they have many desirable traits including potency and low toxicity to mammals as well as a high selectivity and low indications of resistance when compared to current antibiotics (Cudic et al., 2002; Diep et al., 2007; Jin et al., 2005; Lien and Lowman, 2003; Vederas and Sit, 2008). Furthermore, recent advances in recombinant peptide production routes have brought the cost-effective deployment of antimicrobial peptides for agricultural applications much closer to being a viable option in the near future (Glaser, 2009; Hartmann et al., 2009; Ingham and Moore, 2007; Zorko and Jerala, 2010).

The emergence of peptide phage-display libraries has also provided researchers with a robust tool for the identification of bioactive peptides and the technology has been a major driving force behind the identification and subsequent commercialisation of peptide therapeutics, peptide antimicrobials and peptide-based biomaterials (Bratkovic, 2010; Kriplani and Kay, 2005; Pande et al., 2010; Scott and Smith, 1990; Smith and Petrenko, 1997). The libraries have been developed by engineering bacteriophage genomes to display combinatorial arrays of recombinant peptides, proteins and antibodies on predominantly the pVIII and pIII coat proteins. They are now commonly used and commercially available with one of the most prevalent being the NEB Ph.D-12 library, which has a diversity of 10^9 different 12 amino-acid peptide sequences displayed on the N-terminal of the pIII protein (see Figure 1).

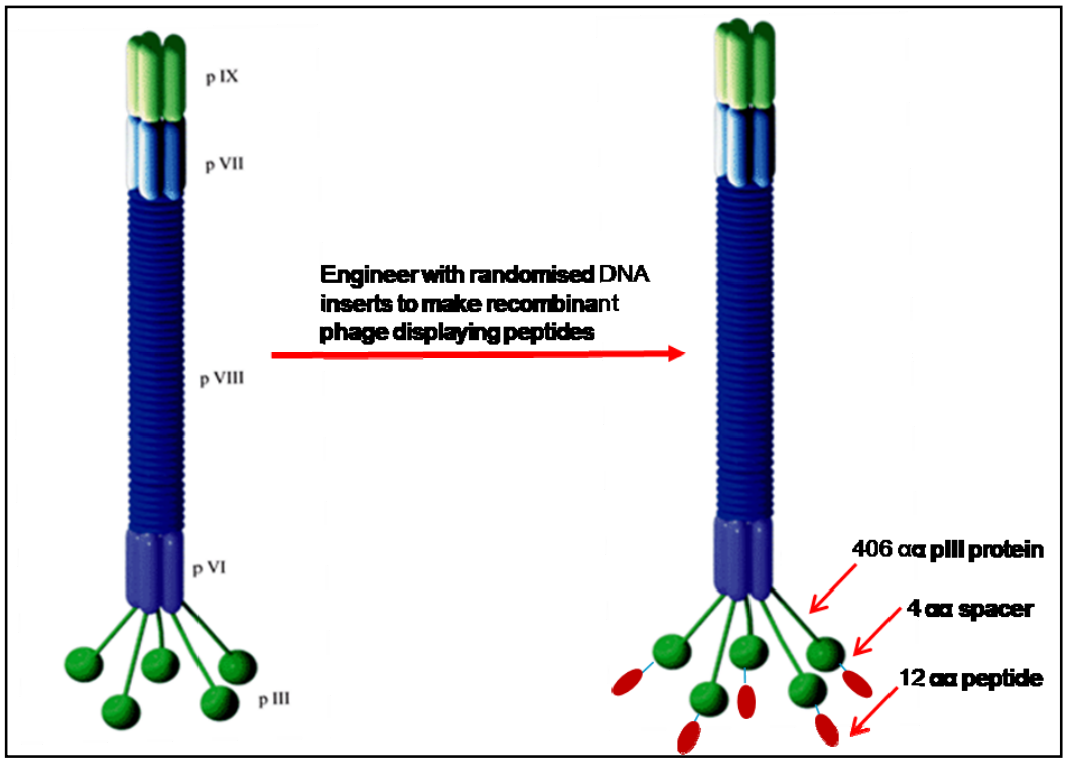

Figure 1: Schematic of native M13 bacteriophage (left) and recombinant bacteriophage in NEB Ph.D-12 library. The peptides are expressed as fusions to the N-terminii of the pIII protein. Each bacteriophage displays 5 copies of the same peptide.

Traditionally, peptides of interest were identified *in vitro* by screening the bacteriophage libraries for phage-displayed peptides that bind recombinant or purified versions of the ligand (Cudic et al., 2002; Erdag et al., 2007; Paradis-Bleau et al., 2008; Rathinakumar et al., 2009; Xie et al., 2006). However, this strategy is generally only useful when valid targets have already been identified by other molecular means. Consequently, more innovative strategies have been developed that utilise whole cells as binding targets for the phage-display libraries and these approaches have already been successfully used to identify antimicrobial peptides directed towards a range of organisms including yeasts, protozoa, fungi and bacteria (Anandakumar et al., 2011; Christensen et al., 2001; da Silva et al., 2002; Paradis-Bleau et al., 2008; Pini et al., 2007; Pini et al., 2005; Rathinakumar et al., 2009).

This body of work has focused on adapting some of these techniques to an anaerobic archaeal system, with the ultimate goal of identifying antimicrobial peptides in an *in vitro* system that are relevant in an *in vivo* environment. In order to achieve this, we developed novel "in culture" phage library screening methods with a model methanogen, *Methanobrevibacter ruminantium*, to mine for peptides capable of binding in the complex environment of the rumen. The semi high-throughput method was developed around screening the cells in a rumen-fluid enriched media designed to mimic the binding conditions of the rumen, and this theme was maintained in subsequent binding and inhibition studies (see Figure 2).

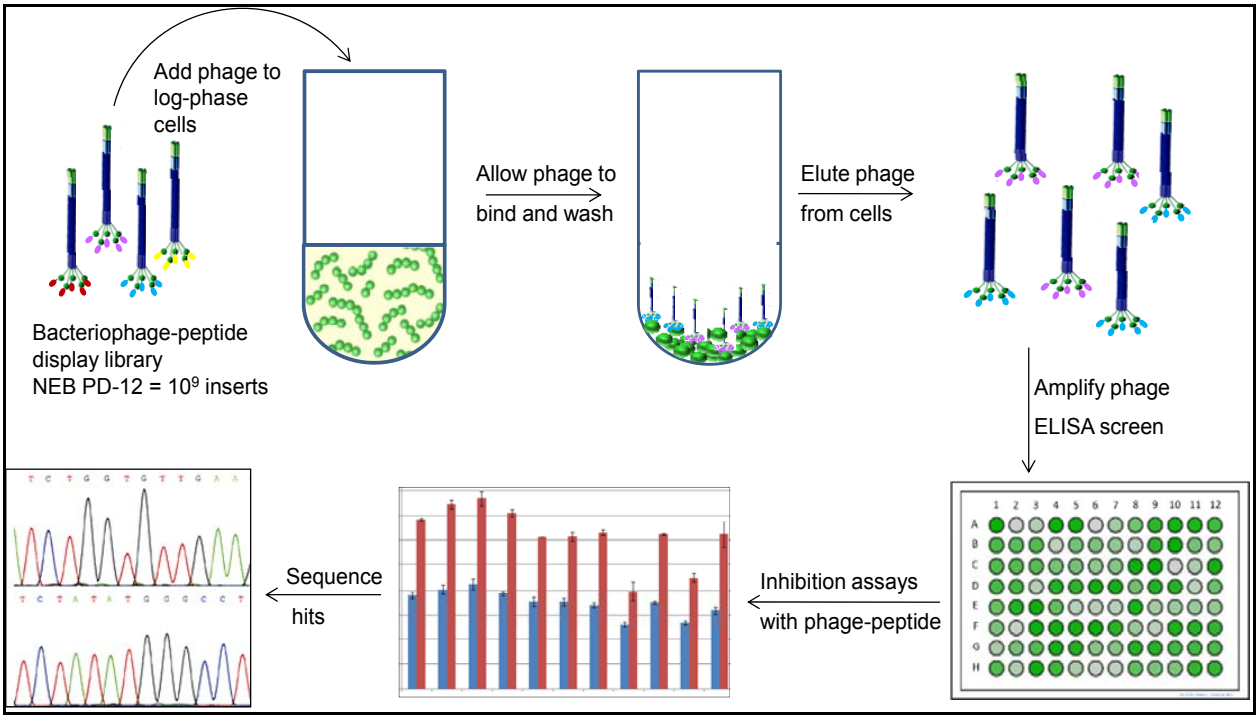

Figure 2: Schematic representation of high-throughput phage-displayed peptide screening method. The bacteriophage library is added to *M. ruminantium* cultures in rumen-fluid enriched media before elution of phage-displayed peptide binders and confirmation of binding by whole-cell ELISA. High binders are screened in inhibition assays before the putative inhibitory peptides are sequenced and identified.

2 Project Objectives

1. Establish and determine the effectiveness of *in-vitro* culturing methods to screen peptide libraries against rumen archaea
2. Screen phage display libraries for binding to a predominant rumen methanogen and recover bound phage for identification of peptides by sequence analysis
3. Screen panel of phage displayed peptides selected from objective 2 and determine the specificity and sensitivity of their inhibitory activity against predominant methanogens
4. Inhibitory peptides identified in objective 3 are synthesised and tested *in vitro* as multi-antigenic peptides against predominant archaea
5. Identify receptors on the archael cell wall that are the sites of binding by the phage peptides.
6. Test the effect of denatured phage particles or synthesised peptides identified in milestones 3&4 on methane production in a mixed rumen culture containing the natural diversity of rumen methanogens. Proof for potential use as supplements in an antimethanogenic strategy

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3 Methodology

3.1 Strain cultivation

Methanobrevibacter ruminantium M1 (Balch et al., 1979; Smith and Hungate, 1958), *Methanobrevibacter gottschalkii* (Miller and Lin, 2002) (both kindly supplied by Terry Miller) and *Methanosarcina barkeri* MS (Kluyver and Schnellen, 1947) (DSMZ800) were all initially recovered from frozen stocks by culturing anaerobically in Balch BRN media (Balch et al., 1979; Miller et al., 1982) under H_2 and CO_2 (120 kPA:30 kPa) at 39^{o}C with gentle rotation. Cultures were grown in 27 ml Balch tubes or 150 ml Balch bottles, with 18 ml or 100 ml of head-space respectively. However, all screening, inhibition-assays and ELISA experiments were performed using cultures of these strains that had subsequently been adapted by passage (3 times minimum) into Rumen Fluid Enriched-Modified BRN media (RFE-MBRN)(60 % (v/v) rumen fluid, 0.5 x Balch BRN components). Cultures were monitored by 420 nM and OD_{600} measurements (SpectraMax) (Molecular Devices, USA) in sealed optical cuvettes (Brand Scientific, Germany) and cell counts performed using Helber Counting Chambers (ProSciTech, Australia).

3.2 Screening 12-mer library for methanogen-binding phage-displayed peptides

Binding screens were performed with NEB Ph.D-12 library in conjunction with mid-log stage *M. ruminantium* cultures in RFE-MBRN media (OD600 0.1 – 0.15 corresponding to ca. 1-2 x 10^8 cells/ml). Typically, the library (or amplified stocks in subsequent steps) was added directly to the anaerobic cultures as a 100-fold excess of virions over ca. 10^9 cells during panning followed by a 30 min incubation at 39 degrees with gentle rotation. All washes were performed under anaerobic conditions with RFE-MBRN supplemented with Tween-20 (concentration specified in each method) and bound phage were eluted from the cells via incubation with Elution Buffer (0.2 M Glycine-HCl, 1mg/ml BSA, pH 2.2) followed by neutralisation with 1M Tris-HCl, pH 9.1. Phage infection, amplification, titration, DNA extraction and clone-sequencing were all performed as per the NEB instructions using the *E. coli* ER2738 host strain and -96 gIII sequencing primer supplied with the Ph.D-12 kit. Concentrations of amplified stocks of phage-displayed peptide clones were based on

physical units determined by A269 measurement (Newman et al., 1977; Petrenko et al., 2005). Wild-type M13 phage (M13-WT)(NEB, USA) was used as a control through-out. Sequencing reactions were performed using the BigDye Terminator Kit (ABI, USA) and analysed with an ABI Prism 373 Automated DNA sequencer (Applied Biosystems, USA). Two separate panning methods based on the above details were employed in this work.

Amplification method

Ph.D-12 library was added to a 9 ml culture of *M. ruminantium* (ca. OD_{600} 0.1) and allowed to bind before harvesting the pellet (P1) by centrifugation (4 k, 10 min, RT). The supernatant was collected and used to resuspend a cell pellet derived from freshly harvesting another 9 ml culture of *M. ruminantium* at the same density. After binding, the pellet (P2) was harvested by centrifugation (4k, 10 min, RT). Pellets P1 and P2 were washed 5 times with 500 µl of RFE-MBRN/0.1 % (v/v) Tween-20, and the bound phage eluted with 200 ul of Elution Buffer to give 2 eluates, which were quantified by titration and then amplified to generate phage stocks designated E1 and E2. This process was then repeated with each of the E1 and E2 stocks to generate 4 more pellets (P3 and P4 derived from panning with E1; P5 and P6 derived from panning with E2). These pellets were washed 5 times with RFE-MBRN/0.2 % (v/v) Tween-20, before elution, titration and amplification to generate phage stocks E3-E6. These phage stocks were added to 4 separate 9 ml cultures of *M. ruminantium* (ca. OD_{600} 0.1) allowed to bind before centrifugation, washing (RFE-MBRN/0.25 % (v/v) Tween-20) and elution as previously described. However, in this instance, the eluates (E3.1, E4.1, E5.1 and E6.1) were titrated out before infection to produce plates with 10 plaques or less to allow isolation of clones from individual plaques (ca. 10^{-4} – 10^{-6} dilutions required). Six plaques from each of the final eluates were picked and sequenced. Stocks of 10 novels clones (denoted 4.1 – 4.10) were amplified for ELISA and inhibition assays and stored at $4^{o}C$.

Subtractive method

Ph.D-12 library was added to a 9 ml culture of *M. ruminantium* (ca. OD_{600} 0.1) and allowed to bind before harvesting the pellet (P1) by centrifugation (4 k, 10 min, RT). This supernatant was collected and used to resuspend a cell pellet derived from freshly harvesting another 9 ml culture of *M. ruminantium* at the same density. After binding, this pellet (P2) was harvested by centrifugation (4k, 10 min, RT). Once again, the supernatant was collected and used to resuspend

a second cell pellet derived from freshly harvesting another 9 ml culture of *M. ruminantium* at the same density. After binding, the final pellet (P3) was harvested by centrifugation (4k, 10 min, RT). Pellets P1, P2 and P3 were washed 5 times with 500 ul of RFE-MBRN/0.25 % (v/v) Tween-20, and the bound phage eluted with 200 µl of Elution Buffer to give 3 eluates. The eluates were titrated before infection to produce plates with 30 plaques or less to allow isolation of clones from individual plaques (ca. 10^{-2} – 10^{-4} dilutions required). Twenty plaques from each of the three eluates (denoted 1.1-1.20, 2.1-2.20 and 3.1-2.20) were picked and amplified for ELISA and inhibition assays.

3.3 Whole cell ELISA

M. ruminantium cultures were fixed during mid-log growth (OD_{600} 0.1 – 0.15) by the direct addition of 37% formaldehyde (Amresco, USA) to the Balch bottles (anaerobically) to a final concentration of 4% (v/v), before returning the cultures to 39°C for 30 min with gentle rotation. The cultures were then further fixed by incubation at 4°C overnight with inversion. The fixed cells were harvested by centrifugation (4 k, 10 min, RT) and resuspended in PBS/formaldehyde (4% v/v) to a final OD_{600} of 1.0 before pulsing in a ultrasonicating water bath (Sanophon, Germany) for 45 s and incubating at RT with gentle rocking for 30 min. 50 µl of the fixed cells were plated out into 96-well polystyrene plates and incubated overnight at 4°C. The next day, the liquid was gently decanted from the wells and the plates air-dried before washing the wells with water and blocking with BSA (PBS/0.5 % (v/v) BSA, 2 h, RT) and air-drying, sealing and storing at 4°C for up to two weeks before use. All washing steps were performed with PBS supplemented with 0.5% (v/v) BSA, 0.1% (v/v) Tween-20, and the bacteriophage (clones 1.1 – 4.10, Ph-D-12 library and M13-WT bacteriophage), linear peptide (3.18, 4.5 and 4.7) and antibody stocks were diluted in the same buffer before use. Cells were incubated with excesses of either of phage-displayed peptide (10^{10} virions/well) or linear peptide (10 ng/well) before probing with anti-M13 (P8) MAb (AbCam, USA) or anti-HIS_6 MAb (Sigma Aldrich, USA) respectively. Primary antibodies were probed with rabbit-anti-mouse-HRP (Sigma Aldrich, USA) and the plates analysed at A_{650} (SpectraMax) after colorimetric development with TMB (Pierce, USA).

3.4 Inhibition assays

Inhibition assays with pure cultures

Empty Balch tubes were equilibrated in the anaerobic hood with 5% H_2 and 95% CO_2 before sealing and autoclaving. Recombinant peptide stocks (and the supernatant (SN) control), and phage-displayed peptide clones (and PhD-12 library) were diluted in PBS to 10µg/ml and 10^{13} virions/ml respectively prior to syringe filtering (0.45 µM filters) and equilibrating in an anaerobic hood. Cultures of methanogens (*M. ruminantium, M. gottschalkii, M. barkeri*) (5 days old maximum) were diluted down to OD_{600} 0.03 with RFE-MBRN. To 9 ml aliquots of cells, either 1 ml of phage-displayed peptide or 1 ml of peptide was added before aliquoting 2.5 ml of each mastermix into empty Balch tubes with venting. Tubes were gassed with 100 kPa of H_2 and incubated at 39°C with gentle rotation for 4 nights prior to analysis and calculation of methane production via Gas chromatography (Shimadzu GC-2014)(Rea et al., 2007).

Inhibition assay with mixed cultures

Mixed rumen culture was recovered from glycerol stocks and conditioned to grow in RFE-MBRN media supplemented with acid-treated discs of filter paper (≥0.1g) as the sole fibre source to minimise the variability observed between experimental replicates. This media was used throughout the experiment outlined in Figure 3. Conditioned mixed culture was used to inoculate media in 27ml anaerobic Balch tubes (1:50v/v), with or without 1mM bromoethanesulfonate (BES), a potent inhibitor of methanogenesis. All cultures were monitored for methane production via Gas chromatography (Shimadzu GC-2014). Cultures passaged 2 times through BES media followed by a single passage without BES, produced no methane, indicating the removal of all viable methanogens from these cultures. This culture was then inoculated with M1(*M.ruminantium*) as the sole methanogen (a 10% population), prior to the addition of recombinant 3.18-HIS peptide. Mixed cultures not treated with BES (positive control with a full complement of rumen methanogens) were also assayed with the 3.18-HIS peptide. Cells from the starter cultures (+/- BES) were counted and used to inoculate (in triplicate) anaerobic Balch tube with 5 ml of media (1:50 v/v dilution). All tubes were gassed with 50kPa H_2 to promote methanogenesis and cultures were allowed to grow for 2 days before initial gas measurements were made prior to the addition of peptide. Peptide concentration was determined by A_{280} spectrophotometry (Spectromax) and added as a PBS solution to the growing culture at an estimated 10^{10} fold excess to cells. H_2, CH_4 and CO_2 gases were monitored at 17, 26.5,43.5 and 71.5 hours after the addition of peptide.

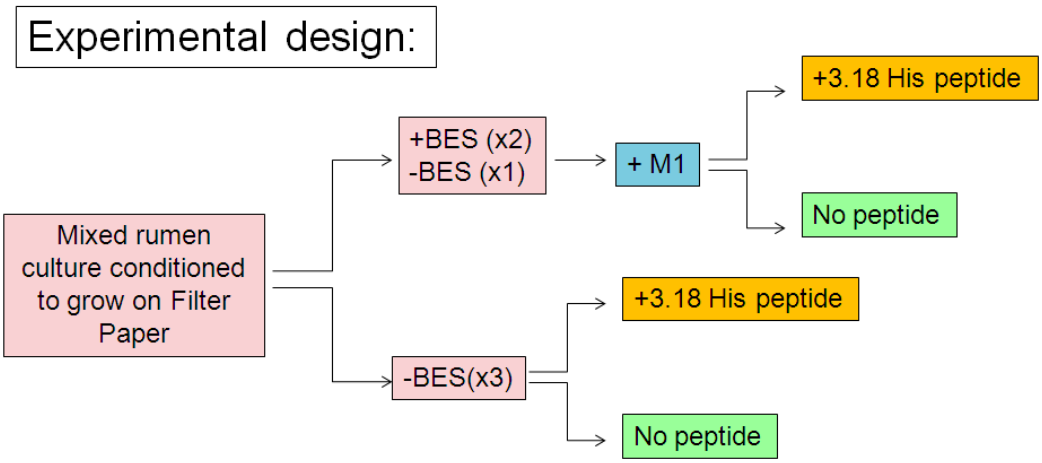


Figure 3: Schematic showing experimental design for performing inhibition assays with mixed cultures.

3.5 Recombinant peptide production

Cloning

All restriction endonucleases and ligase were supplied by NEB and oligonucleotides were from Geneworks (Australia). Expression vectors to produce peptides of interest with and without C-terminal HIS_6 tags were made using a modified version of the pET32(a)+ vector (Novagen/EMD Biosciences, Germany). The C-terminal affinity tags and MCS were digested out of the original vector using Msc1 and Xho1 restriction endonucleases and replaced with the TEVp-polylinker (see Table 1 for oligonucleotide sequences) to create the Trx-TEVp parent vector. The polylinker included novel EcoR1 and Stu1 restriction sites that allowed the insertion of annealed, double-stranded oligonucleotides corresponding to the peptides of interest into the Trx-TEVp vector in frame with a C-terminal spacer (LISAGS) and TEVp (Tobacco Etch Viral protease) consensus sequence (ENLYFQG), and an N-terminal HIS_6 tag. In order to generate expression vectors for peptides without HIS_6 tags, oligonucleotides for each peptide with 3' STOP codons (tagtaa) were inserted.

Expression vectors were also prepared to produce concatameric versions of the 3.18 peptide, with and without C-terminal HIS-tags, by adding additional copies of the peptide to the 3.18-HIS vector.

The vector was linearised with EcoR1, and double-stranded oligonucleotides corresponding to either an additional one or two 3.18 peptides, with STOP codons, were inserted to generate clones for the expression of two or three tandem repeats of the 3.18 peptide, respectively. All clones were sequenced using T7 terminator primer with BigDye Terminator Kit (ABI, USA) and analysed with an ABI Prism 373 Automated DNA sequencer (Applied Biosystems, USA).

Expression and peptide capture

Stocks of recombinant peptide were made via a Selective Solvent Precipitation method (Hartmann et al., 2009). Briefly, Trx-Peptide fusion proteins were expressed in BL21(DE3) *E. Coli* (NEB, USA) before cell harvest and physical disruption via sonication (2 x 45 s pulse, 100% Amplitude) (Vibracell) (Sonics and Materials, USA). Cell lysates were heat-treated in a water-bath for 5 min at 56 °C and then centrifuged (10 k, 10 min, RT) to remove debris, before lyophilising the soluble fraction. Cell lysates were resuspended in TEVp reaction buffer (TBS, 0.5 mM EDTA, pH 8.0) to give final protein concentrations of at least 12 mg/ml before adding TEVp (enzyme kindly supplied by Dr Waltraud Kaar – (Kaar et al., 2009) in a 100:1 (substrate:enzyme) molar ratio and incubating for 3 h at RT. Peptide was partitioned from the HMW contaminants by adding ethanol to a final concentration of 75% (v/v), vortexing and centrifuging (10K, 10 min, RT). The supernatant was collected and dried down via rotary evaporation (CentriVap) (Labconco, USA) and the peptide confirmed by mass spectrometry. A negative control ("SN control" in peptide inhibition assay) was produced by expressing the Trx fusion protein alone and processing the cell pellet in the same way as described. Analysis was performed via SDS-PAGE using the NuPAGE system (4 – 12 % gels) with MES buffer (Invitrogen, USA) and protein concentrations were estimated via Bradford Assay (Bio-Rad, USA)

Table 1: List of Oligonucleotides (Phos denotes 5' phosphorylation)

Insert	Amino Acid Sequence	Oligo Sequences (5'-3')
TEVp-Polylinker	LISAGSENLYFQGLGVGIHHHHHH	F:(Phos)actgatctccgccgggtctgagaatttgtacttccaaggcctt ggcgttg gaattcaccaccatcaccatcattga
		R:(Phos)tcgatcaatgatggtgatggtggtgaattccaacgccaaggc cttggaagtacaaattctcagacccggcggagatcagt
3.18STOP	GQLETTLALHYTP	F:(Phos)tcagctggagacgacactggccctgcattatactccgtgataa
		R:(Phos)aattttatcacggagtataatgcagggccagtgtcgtctccagctga
3.18-HIS	GQLETTLALHYTPGIHHHHHH	F:(Phos) tcagctggagacgacactggccctgcattatactccggg

		R:(Phos) aattcccggagtataatgcagggccagtgtcgtctccagctga
4.5-STOP	GVASPSTMHRPNK	F:(Phos) tgttgcaagcccgtctacaatgcaccgtccaaacaaatgataa
		R:(Phos) acaacgttcgggcagatgttacgtggcaggtttgtttactattttaa
4.5-HIS	GVASPSTMHRPNKGIHHHHHH	F:(Phos) tgttgcaagcccgtctacaatgcaccgtccaaacaaagg
		R:(Phos) aattcctttgtttggacggtgcattgtagacgggcttgcaaca
4.7-STOP	GMPAVMSSAQVPR	F:(Phos) tatgccggcggtgatgagctctgcccaagtgccacgttgataa
		R:(Phos) attttatcaacgtggcacttgggcagagctcatcaccgccggcata
4.7-HIS	GMPAVMSSAQVPRGIHHHHHH	F:(Phos) tatgccggcggtgatgagctctgcccaagtgccacgtgg
		R:(Phos) aattccacgtggcacttgggcagagctcatcaccgccggcata
3.18x2STOP	GQLETTLALHYTPGIQLETTLALHYTP	F:(Phos) aattcagctggagacgacactggccctgcattatactccgtgagg
		R:(Phos) aattcctcacggagtataatgcagggccagtgtcgtctccagctg
3.18x3STOP	GQLETTLALHYTPGIQLETTLALHYTP GIQLETTLALHYTP	F:(Phos)aattcagctggagacgacactggccctgcattatactccgggaattca gctggag acgacactggccctgcattatactccgtgagg
		R:(Phos)aattcctcacggagtataatgcagggccagtgtcgtctccagctgaatt cccggagtataatgcagggccagtgtcgtctccagctg

3.6 Binding site Identification

An outline of the experimental method designed to identify the 3.18 peptide receptors on *M. ruminantium* is shown in the Figure 4. The process employed a ca. 1000x fold excess of 3.18 bacteriophage-peptide over *M. ruminantium* cells in RFE-MBRN. The phage-displayed peptides were reversibly cross-linked to the cell surface with 1mM of the chemical cross-linker, DTSSP. The cells were then lysed via bead-beating in the presence of a non-ionic detergent, NP-40 (1% v/v/), to disrupt the cellular membranes without denaturing proteins or bacteriophage-peptide particles. The soluble fraction of this mixture was then added to Protein A/G sepharose beads that had been freshly conjugated with 10ug of M13 bacteriophage P8 protein antibody, using the Pierce Direct Immunoprecipiation Kit, as per the manufacturer's instructions. After incubation, the beads were washed thoroughly with lysis buffer before eluting the bacteriophage by reduction of the DTSSP with 50 mM DTT. The whole experiment was also performed with a WT-M13 bacteriophage control to allow exclusion of any proteins that may bind the M13 bacteriophage non-specifically. The fractions were analysed by SDS-PAGE and the released target proteins in eluates obtained from the 3.18 bacteriophage samples were subjected to in-gel digestion prior to mass-spectrometry.

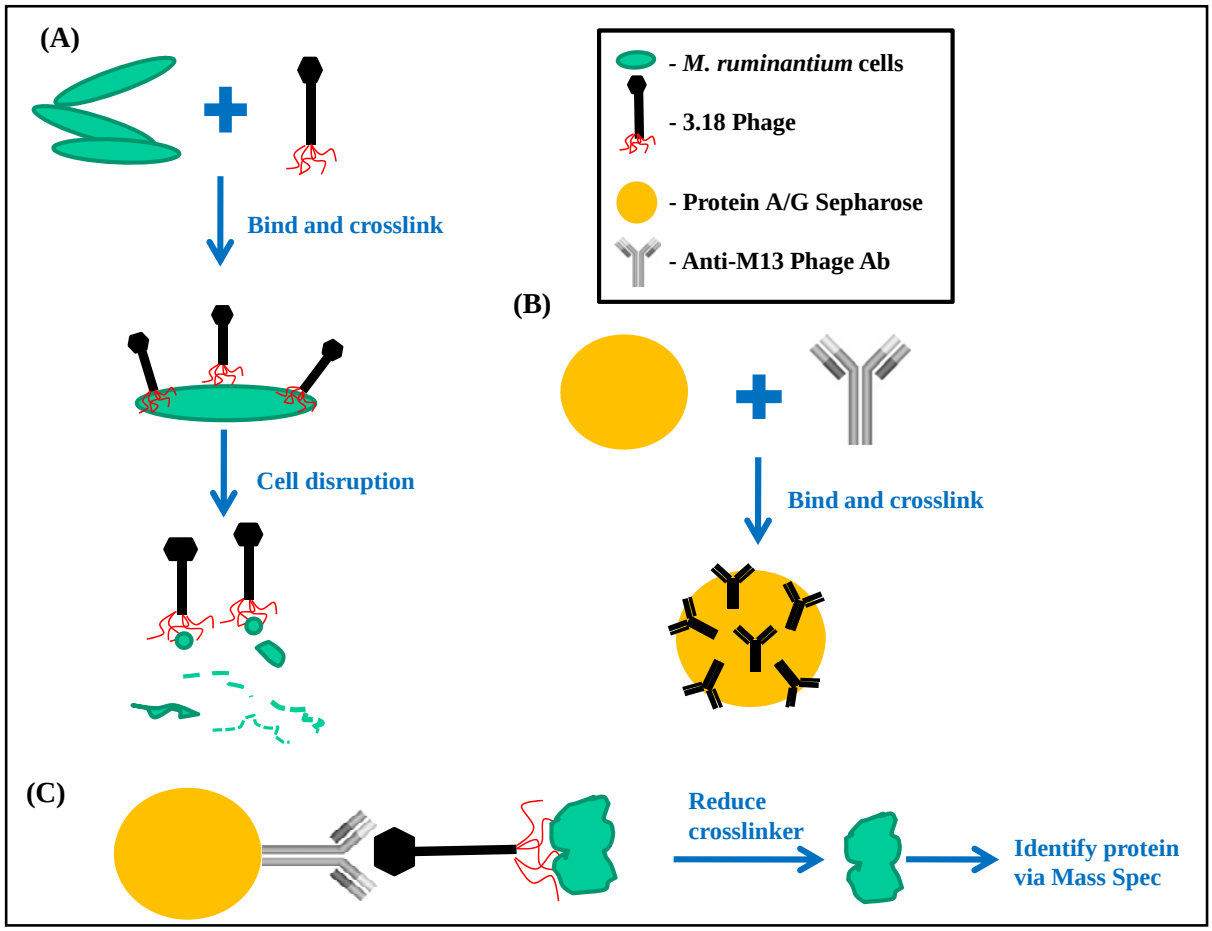


Figure 4 : Schematic showing design of binding site identification experiments. (A) The 3.18 phage is reversibly cross-linked to the *M. ruminantium* cells prior to disruption. (B) Protein A/G sepharose beads are conjugated to the Anti-M13 phage antibody. (C) Affinity capture is performed prior to release of the putative target protein for analysis.

4 Results and Discussion

4.1 Culturing and screening for inhibitory phage-displayed peptides

Preliminary binding screens (known as “panning”) using the NEB Ph.D-12 phage-displayed peptide library were performed using whole cells of a model methanogen strain, *Methanobrevibacter ruminantium*. There have been very few bovine rumen methanogen strains cultured in laboratory conditions and this organism was chosen as the model cell line as it is a robust, consistent and a prevalent species of anaerobic methanogens in the rumen (Balch et al., 1979; Janssen and Kirs, 2008). In order to increase the chances of identifying peptides that would be active in the rumen, it was deemed crucial to perform the initial binding screens in an environment that was closely related to the native one (Watt, 2006). This was an important consideration since peptide activity is dependent on peptide-cell interactions, and this interface can be heavily influenced by factors such as ionic strength and pH as well as the profile and confirmation of cell-surface proteins (Silver, 2006; van Merode et al., 2006). Consequently, rather than perform conventional panning experiments in buffered saline, we developed a method to allow direct screening using log-phase cells cultured in a rumen-fluid enriched media. Attempts made to sub-culture the *M. ruminantium* cells in cleared rumen fluid alone (with or without sodium bi-carbonate buffering) were unsuccessful. However, studies revealed that excellent growth could be sustained in a modified media containing 60 % rumen fluid (as opposed to 10%) supplemented with all of the other components of BRN media at half the conventional concentration (results not shown). This media, referred to as RFE-MBRN (Rumen fluid-enriched modified BRN) was deemed capable of sufficiently mimicking the *in vivo* binding conditions of the rumen.

The compatibility of the RFE-MBRN with the Ph.D-12 library was also assessed to confirm that the media would not interfere with the screening protocol. Comparative infection and titration studies performed with Ph.D-12 samples made up in either Tris-buffered saline (TBS), RFE-MBRN media or spent RFE-MBRN media demonstrated that the media did not interfere with the infectivity of the bacteriophage, which were stable in all 3 conditions for up to 48 hours. Binding studies conducted using a non-recombinant (empty) stock of M13 bacteriophage in both TBS and RFE-MBRN confirmed that the media did not encourage non-specific binding of the empty bacteriophage when compared to the TBS (results not shown).

To obtain a varied pool of binding phage-displayed peptide clones, two different types of panning experiments were designed, each with subtractive screens to compensate for cell-binding competition between phage-displayed peptides (Eisenhardt et al., 2007; Guo and Chen, 2006). Method 1 was designed with one subtractive screen and multiple rounds of amplification of mixed pools of eluted bacteriophage to enrich for high-binders by reselection. Method 2 was designed to be high-throughput and included two subtractive screens after which individual clones were selected and only amplified once before using in ELISA studies. This method was designed to select for phage-displayed peptides that may have been sterically hindered from binding by other phage particles, whilst also ensuring that phage-displayed peptide clones that may be less robust during amplification were selected against during enrichment. When Method 1 was utilised, the final 4 eluates were titrated out and 6 plaques were chosen from each. As it was expected that high-binding clones would be amplified preferentially, these clones were sequenced prior to the ELISA to avoid screening clones with identical inserts. The 24 bacteriophage clones yielded 10 novel peptide insert sequences and these were used in the ELISA screen. Alternatively, the three eluates obtained from Method 2 were titrated to produce plates with less than 30 bacteriophage plaques and 20 clones were selected from each for further screening. The overall phage-displayed screening for Method 2 is represented in Figure 2, showing that recombinant bacteriophage from the Ph.D-12 library were only sequenced after demonstrating both binding and inhibitory activity towards the model organism, *M. ruminantium*.

Despite the stringency of the original panning experiments, there is always potential for low/non-binding phage-displayed peptides to be selected, making it necessary for an intermediate step to assess the binding of the clones that have been amplified. Often, this is achieved by performing laborious titration assays, but these are based on the inaccurate assumptions of complete elution after binding and a 100% infectivity rate for the eluted bacteriophage (Bratkovic, 2010; Willats, 2002). Consequently, we designed a whole-cell ELISA to assess the binding of the 70 phage-displayed peptide clones. In line with the culture-based screening method, we developed this protocol using log-phase *M. ruminantium* cells fixed directly in RFE-MBRN and immobilised in 96-wells plates. This was in contrast to standard methods that involve the resuspension of cultured cells in buffered formalin mixtures. Such methods were rejected here on the premise that they would likely disturb the native display of cell-surface proteins that the phage-displayed peptides were originally screened against.

The ELISA revealed that there was only minimal non-specific binding of both the Ph.D-12 library and the wild-type M13 bacteriophage sample (see Figure 5). It also demonstrated that there was substantial binding variation amongst the different phage-displayed peptides. In order to cull the number of phage-displayed peptide clones to be assessed, an arbitrary cut-off point of 0.6 was set which resulted in 23 clones progressing through to the inhibition assay stage.

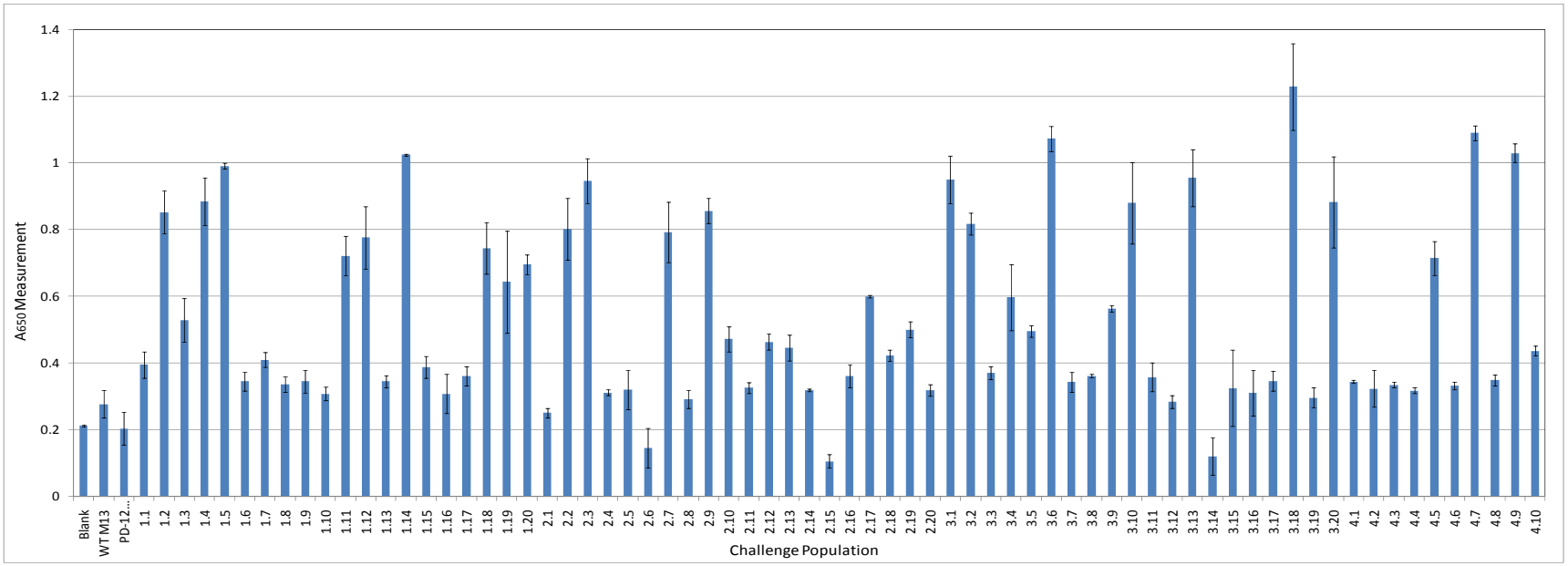


Figure 5: Whole-cell ELISA using isolated and amplified phage-displayed peptide clones directed against *M. ruminantium*. Ten phage-displayed peptide clones from Panning Method 1 (4.1 – 4.10) and sixty phage-displayed peptide clones from Panning Method 2 (1.1 – 3.20) were assessed for binding capacity by ELISA via probing with anti-M13-P8 protein antibodies. Blank; cells only. Empty; cells screened against wild-type M13 bacteriophage. Ph.D-12; cells screened against complete NEB Ph.D-12 library. 1.1-4.10; cells screened against isolated and amplified phage-displayed peptide clones.

There were many obstacles to overcome when designing this inhibition assay, with the most significant being the anaerobic growth conditions required and the slow growth rate of the methanogen cultures. Based on our requirements, we optimised an inhibition assay using a uniform starting culture (in RFE-MBRN) that could be aliquoted, supplemented with a 10^5-10^6 fold excess of a phage-displayed peptide clone and then added to equilibrated Balch tubes via vents. Initially, this assay was performed only with the model organism, *M. ruminantium,* and these results suggested that there were three clones that had a negative impact on the methane production of this strain (see Figure 6). This data reveals that clones 3.18, 4.5 and 4.7 reduced the methane output, when compared to the control cultures, by approximately 65%, 61% and 37% respectively. Consequently, these 3 clones were also tested against 2 other rumen methanogen strains, *M. barkeri* and *M. gottschalkii.* In this instance, the methanogenesis inhibition was not as substantial for each of the phage-displayed peptides, but clones 3.18 and 4.5 still demonstrated some inhibitory activity towards all three strains of methanogen. Overall, the *M. barkeri* strain was the least susceptible to the inhibitory activities of the phage-displayed peptides, but this was not entirely surprising considering that this cell-line tends to grow in grape-like clumps when in culture. It was also deemed possible that the bulkiness of the bacteriophage could be limiting the activity of the recombinant peptide fusions.

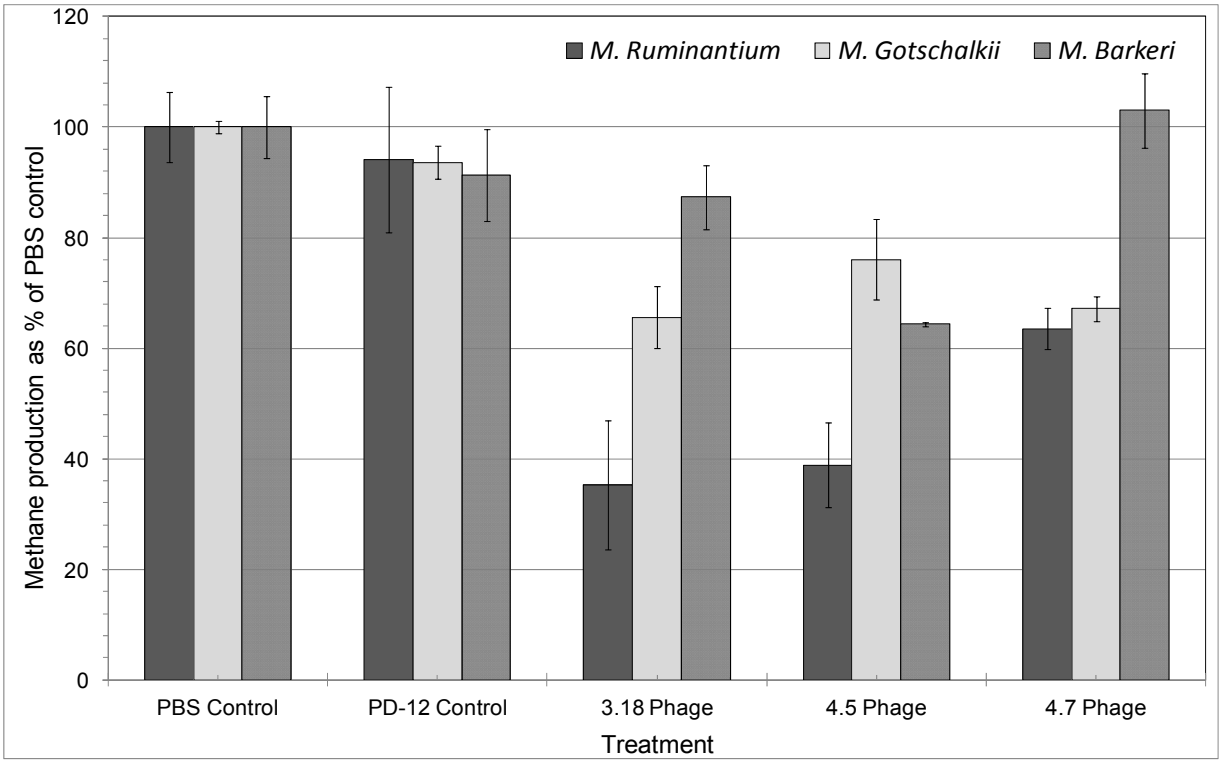


Figure 6: Methane production of REF-MBRN cultures of *M. ruminantium*, *M. barkeri* and *M. gottschalkii* in the presence of the NEB PhD-12 library and phage-displayed peptide clones, (3.18, 4.5 and 4.7). Production is displayed as percentage of the methane produced by the PBS control cultures.

After sequencing, the three phage-displayed peptide clones, 3.18, 4.5 and 4.7 were found to have peptide inserts with the following amino acid sequences:

Bacteriophage-peptide 3.18: **Q L E T T L A L H Y T P**

Bacteriophage-peptide 4.5: **V A S P S T M H R P N K**

Bacteriophage-peptide 4.7: **M P A V M S S A Q V P R**

There was no significant homology detected between the sequences and each has different properties as a consequence. The 3.18 peptide has a net negative charge and is slightly hydrophobic, while the 4.7 peptide is also slightly hydrophobic, but has a net positive charge. The 4.5 peptide has a net positive charge and is hydrophilic.

4.2 Assessment of putative inhibitory activity of linear peptides in pure cultures

Linear peptide counterparts to the phage-displayed peptides 3.18, 4.5 and 4.7 were made recombinantly via a Selective Solvent Precipitation method described previously by Hartmann et al 2009. This method was chosen because the purity requirements for our applications did not warrant the use of Solid Phase Peptide Synthesis (SPPS). Also using this process demonstrates that antimicrobial peptides could potentially be made at scale in a cost-effective manner. The parent plasmid for a high-throughput cloning strategy for Trx-peptide expression vectors was made to allow the insertion of double-stranded oligos (corresponding to peptides of interest) with and without STOP codons which would generate peptide products without and with a C-terminal HIS-tag, respectively (see Figure 7(A)). The peptide-encoding oligos were cloned into the parent vector at the C-terminal of the Trx protein, immediately downstream of the TEV protease consensus sequence such that recombinant peptide would be produced with only a single additional glycine residue on the N-terminal and a native C-terminal. This was an important consideration since the activity of small peptides can be subject to fluctuations as a consequence of even minor sequence variations. On average, 400 ml shake-flask cultures of transformed BL21(DE3) cells resulted in the generation of 2-4 mg of peptide using the production route summarised in Figure 7(b). This process was ultimately dependent on the solubility and heat-stability of the Trx-Fusion protein, and this is demonstrated in Figure 7(C) using the Trx-4.5 expression product as an example. The 15 kDa fusion protein was expressed at levels in excess of 50 % total cell protein, as seen in Lane 1. The retention of the fusion protein in the cell lysate supernatant can be seen in Lane 3, while the fusion was equally present in the heat-treatment supernatant in Lane 4. The peptide was released from the fusion protein by TEVp cleavage and is demonstrated by the presence of the free Trx protein (13.6 kDa) in Lane 5. The TEV protease was used sparingly in these instances, and as such, the proteolytic cleavage efficiency was only approximately 50%. However, as yield was not a priority for this body of work, the process was not optimised. The effectiveness of the solvent precipitation step is apparent by inspection of Lane 6, which shows that the vast majority of the protein product is in the pellet.

The partitioning capacity of the ethanol precipitation is even more evident in Figure 7(D), which shows the mass spectroscopy chromatogram obtained after analysis of the crude supernatant product. This chromatogram clearly demonstrates that the 4.5 peptide is the major low molecular weight product, with very little contamination from other protein products.

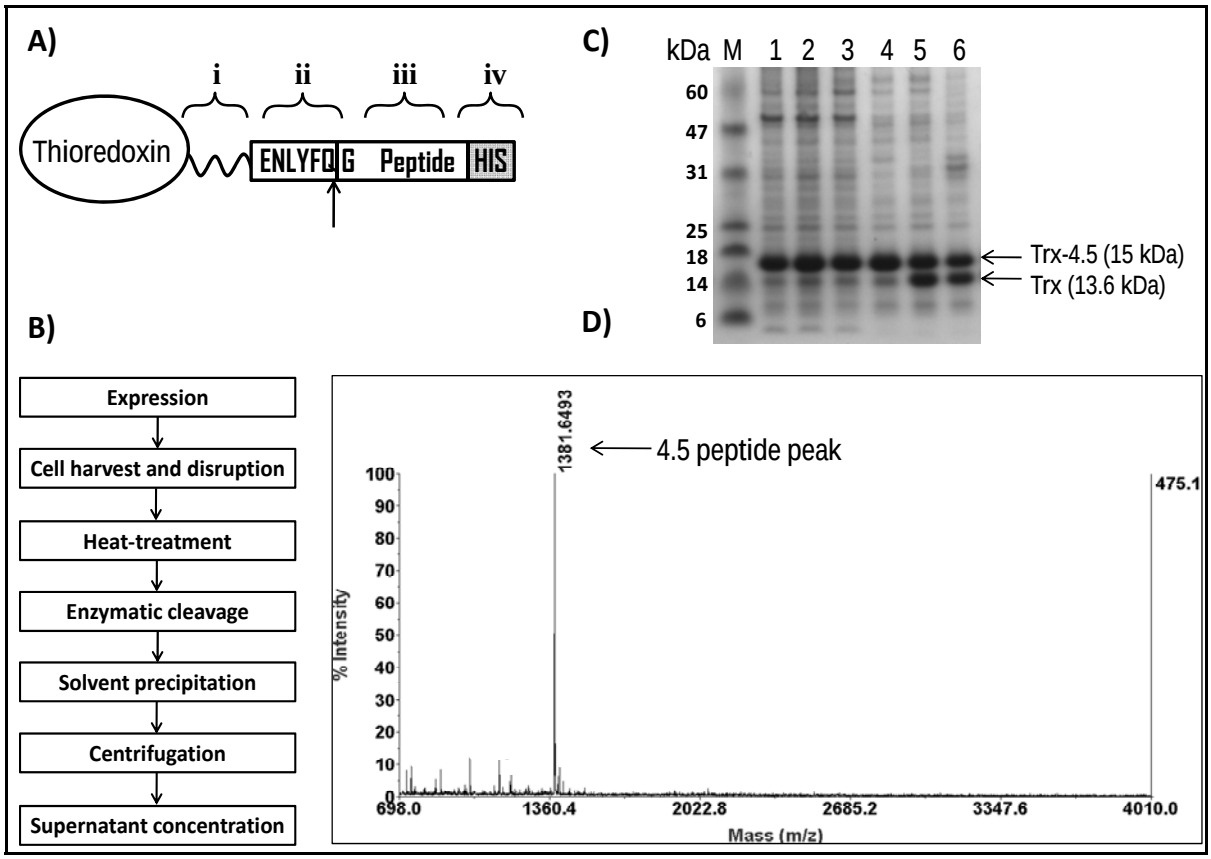


Figure 7: Recombinant peptide production. A) Schematic of Trx-peptide expression product showing i) spacer, ii) TEVp consensus sequence, iii) putative inhibitory peptide sequence and iv) HIS_6 tag. **B)** Block diagram summarising the recombinant peptide production route utilised to generate the linear peptides. **C)** SDS-PAGE showing expression and release of the 4.5 peptide. Lane 1; Induced BL21(DE3) cells expressing Trx-4.5 fusion protein. Lane 2; Cell lysate. Lane 3; Soluble fraction from cell lysate. Lane 4; Soluble fraction from heat-treated cell lysate. Lane 5; Trx-4.5 fusion protein after TEVp cleavage. Lane 6; Pellet derived from precipitation of TEVp cleaved Trx-4.5 with 75% (v/v) ethanol. All lanes were loaded with amounts equivalent to 5µl of OD 10 cells. Lane M; Prestained broad-range MWM (Gibco). **D)** Mass spectrum of the supernatant generated from the recombinant peptide production route for peptide 4.5. The monoisotopic mass of 1381.6493 Da corresponds to the hypothetical mass of 1381.57 Da for the 4.5 peptide.

Prior to testing the putative inhibitory activities of the 3.18, 4.5 and 4.7 peptides, our optimised whole-cell ELISA protocol using the model organism, *M. ruminantium*, was utilised to confirm that the peptides retained binding activity in linear form. The primary motivation for the production of the HIS-tagged versions of the peptides was for ELISA applications, and to this end, a spacer had been included between the C-terminal of the peptide and the HIS-tag during the cloning process in order to minimise any interference the HIS-tag might have on the peptide-cell interaction. The results of this ELISA can be seen in Figure 8, and as expected, the non-HIS-tagged versions of the three peptides of interest did not generate a significant signal when probed with the anti-HIS monoclonal. However, when the immobilised cells were incubated with the HIS-tagged peptides, the signal measurements were quite substantial, indicating that the recombinant linear peptides were capable of binding the methanogen. Interestingly, the variation in the binding capacity was similar to the results obtained for the phage-displayed peptide counterparts (see Figure 5) which also revealed that, out of these 3 clones, 3.18 was the strongest binder, followed by 4.5 and 4.7. This result supported the progression of all three (non-HIS-tagged) peptides into the inhibition assay stage.

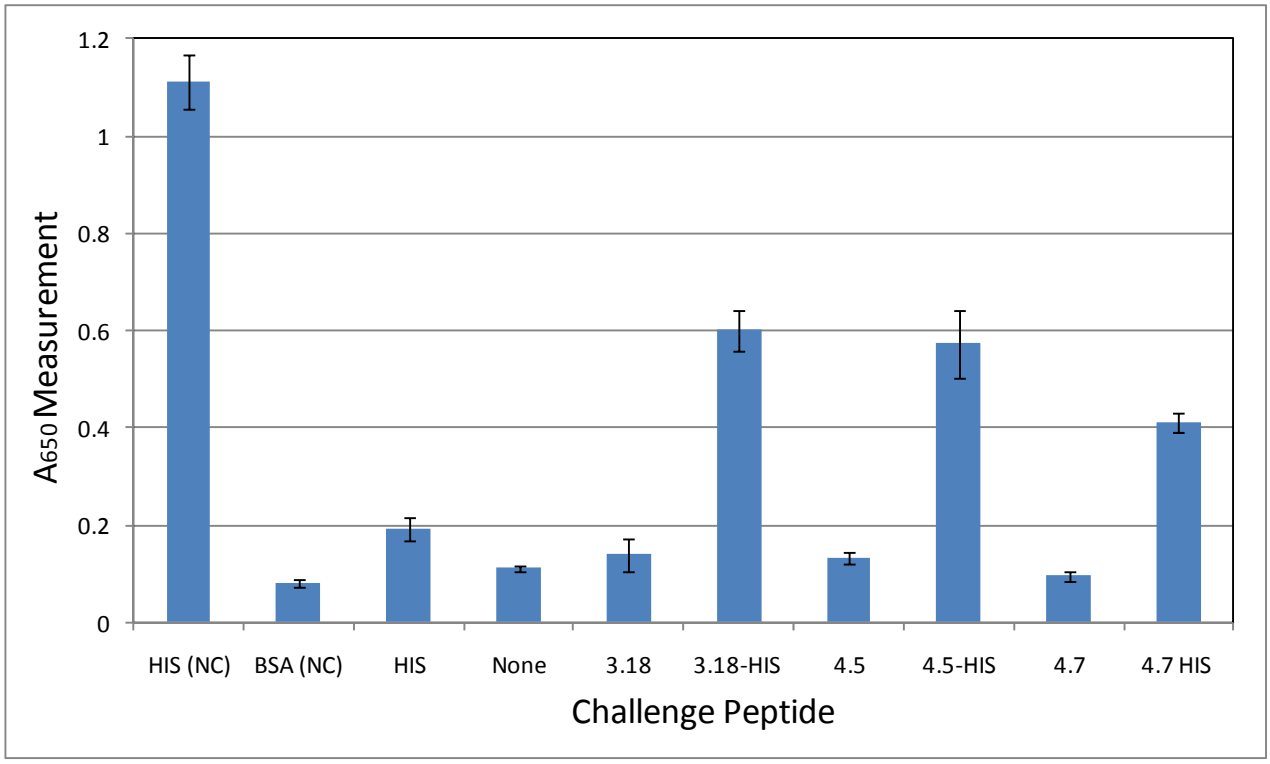


Figure 8: Whole-cell ELISA using recombinant linear peptide directed against *M. ruminantium*. Three putative inhibitory peptide were assessed for binding capacity by ELISA via probing with anti-HIS_6 antibodies. Controls included HIS-tag alone and BSA wells (no cells), as well as untagged 3.18, 4.5 and 4.7 peptides.

Inhibition assays using all three methanogen strains, *M. ruminantium*, *M. barkeri* and *M. gottschalkii,* were performed with 10^6-10^7 peptide:cell excesses of the recombinant, native 3.18, 4.5 and 4.7 peptides, along with a supernatant (SN) control. This control was included in addition to the PBS control, to assess whether any inhibition detected was a consequence of carry over cellular contamination from the recombinant peptide production route. The results of this assay (see Figure 9) clearly show that the SN control was comparable with the PBS only control, indicating that the methane production of each strain was not influenced by these low-level background contaminants. Overall, the most effective peptide and most susceptible strain was 3.18 and *M. ruminantium*, respectively. The 3.18 peptide inhibited methane generation in the *M. ruminantium* cultures by approximately 70%. This was not surprising considering the panning and binding screens used this as the model organism.

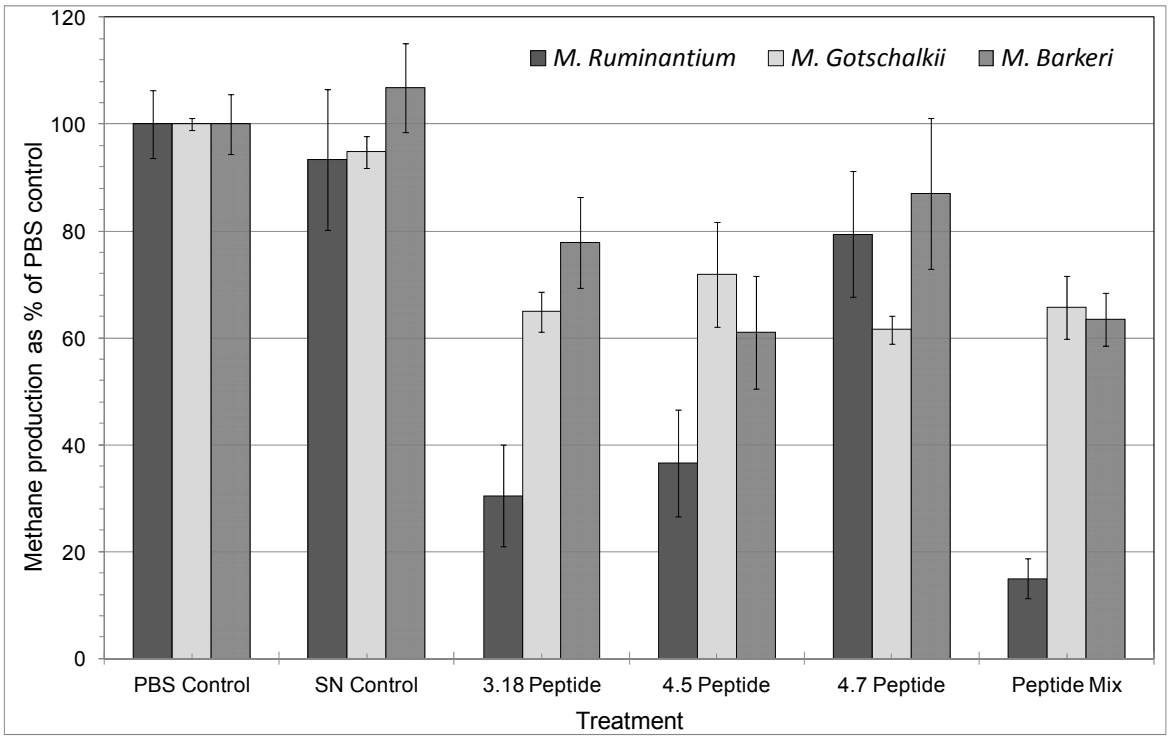


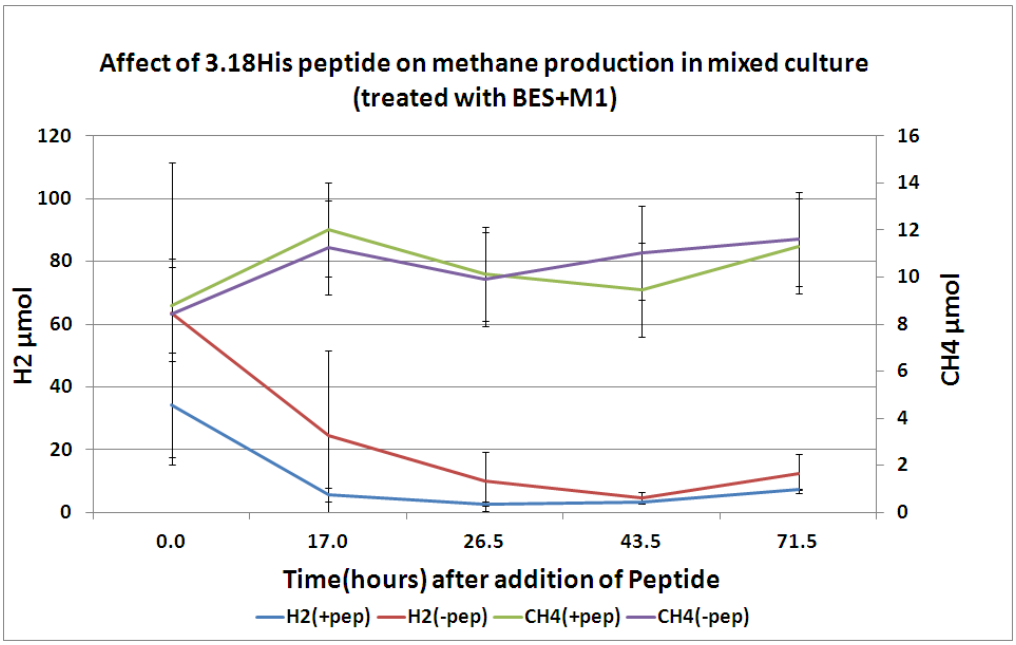
Figure 9: Methane production of REF-MBRN cultures of *M. ruminantium*, *M. barkeri* and *M. gottschalkii* in the presence of a supernatant (SN) control, linear peptides 3.18, 4.5 and 4.7, and an equimolar peptide mixture. Production is displayed as percentage of the methane produced by the PBS control cultures.

The results obtained for the 3.18 and 4.5 peptides closely mimicked the inhibition levels achieved by the phage-peptide counterparts originally assayed (See Figure 6), though the 3.18 linear peptide was slightly more active against the *M. ruminantium* than the 3.18 bacteriophage (70% reduction for the peptides versus a 64% reduction for the phage-displayed peptide), but this was only a minor variation. However, the 4.7 linear peptide was, on average, less active than the 4.7 bacteriophage.

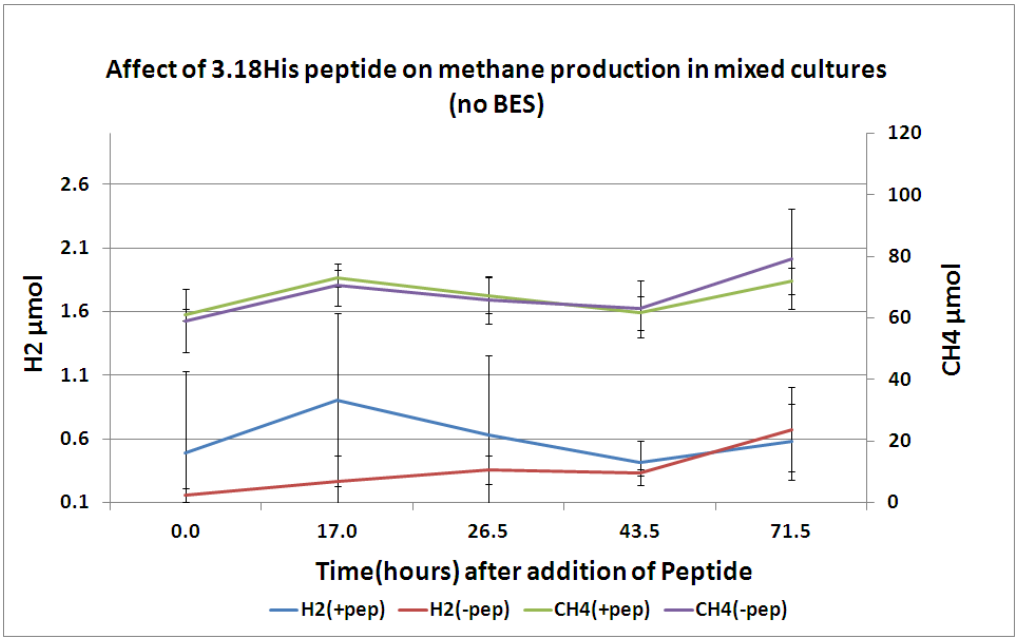
This also suggested that the increased inhibition seen for the peptide mixture was most likely a consequence of the 3.18 and 4.5 peptide's activity working in tandem. The peptide mixture had a substantial effect on the *M. ruminantium* cultures, with methane generation being reduced by close to 85%. This result indicates that the linear peptides, in particular 3.18 and 4.5, are indeed binding in culture and exerting an inhibitory effect on the methanogens.

4.3 Assessment of putative inhibitory activity of linear peptides in mixed cultures

The inhibition assays with the mixed cultures demonstrated large degrees of variability between technical replicates and there was no observable inhibition of methane production by the 3.18His-peptide. This was the case whether the *M.ruminantium* was present alone (Figure 10(i)) or all rumen methanogen species were present (Figure 10(ii)). A slight increase in H_2 post-peptide addition to the mixed culture (Figure 10(ii)) may have indicated some reduced ability to remove hydrogen from the system but this is difficult to interpret as a corresponding reduction in methane production was not observed. As these assays were performed with the single, linear 3.18 peptides, it is likely the peptide has been rapidly degraded by the proteolytic bacteria in the mixed culture and any effect has been transitory and not detected under our experimental conditions. Further work would aim to mitigate degradation of the bioactive peptide/s in the rumen. Modifications such as acetylation, pegylation or concatamerisation of the 3.18 linear peptide may make it less susceptible to proteolytic degradation (Jenssen and Aspmo, 2008; McGregor, 2008; Pini et al., 2005). Expression vectors for concatameric versions of the 3.18 peptide have been made and will form the basis of future experiments. Experiments will also be performed with the recombinant 3.18 phage, which may be more robust in mixed culture and its effect on methane production under these conditions needs to be investigated.



(i)



(ii)

Figure 10: Inhibition assays performed with mixed cultures and 3.18 peptide.

Graphs showing the amount of H_2 and CH_4 (µmol) present 17, 26.5, 43.5 and 71.5 hours after the addition of the recombinant inhibitory peptide 3.18His to mixed rumen cultures either treated with BES and supplemented with *M.ruminantium* (M1)(i) or mixed cultures not treated with BES(ii).

4.4 Putative binding site identification

Investigations into identifying the cell-surface receptor targets for the inhibitory peptides were based on the 3.18 peptide and the *M. ruminantium* strain, as it was expected that this combination was the most likely to yield relevant information considering the genome of this strain is known. The results of the pull-down assay are shown in Figure 11. The experiment did result in the appearance of unique protein bands captured by the 3.18 phage, as seen in Lane 2. These bands were excised and "in gel" digested with trypsin (using standard protocols) and the digested products extracted for analysis on the Triple TOF^{TM}5600 (AB/Sciex,Foster City,CA USA). The quantity of protein extracted was too little to give good spectra so the assay needs repeating to improve the yield of captured protein.

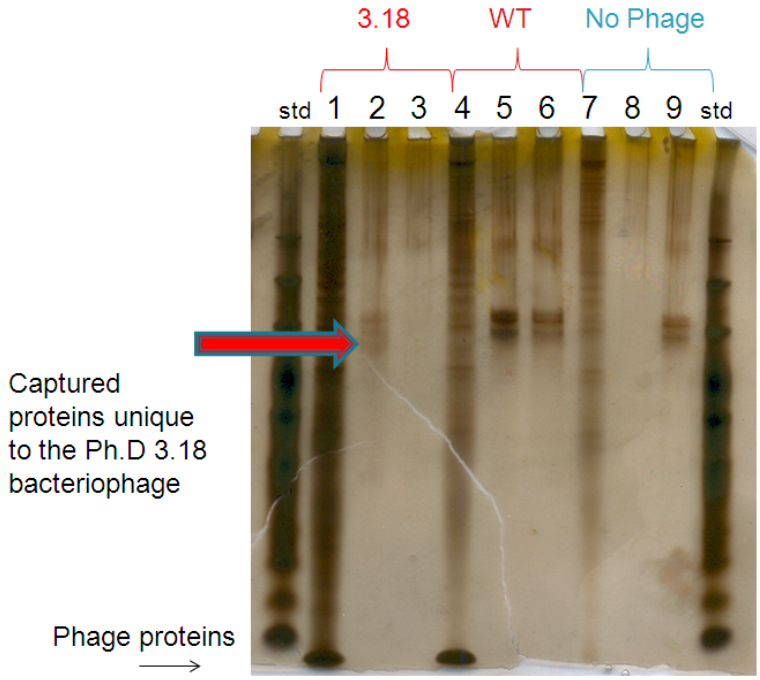


Figure 11: Silver stained SDS-PAGE gel of fractions and eluates collected from pull-down assay using *M. ruminantium* and 3.18 bacteriophage. Lanes 1, 4 and 7 represent the *M ruminantium* cell proteins with proteins from Ph.D 3.18 bacteriophage, wild type (M13-WT) bacteriophage and without phage respectively; Lanes 2-3, 5-6 and 7-8 are respectively the first and second elutions of proteins from these mixes after sepharose bead capture and reduction of the cross-linker (Figure.4)

An alternative pull-down assay using the recombinant His tagged 3.18-HIS linear peptide as the capture agent and Ni-NTA resin for purifying the captured proteins from the complete cell mix has been devised to improve the efficiency of recovery of the *M ruminantium* cell-surface binding protein (See Figure 12). Once sufficient protein is captured for sequencing, identification will be possibly because the complete genome of *M ruminantium* has been sequenced (Leahy SC et al).

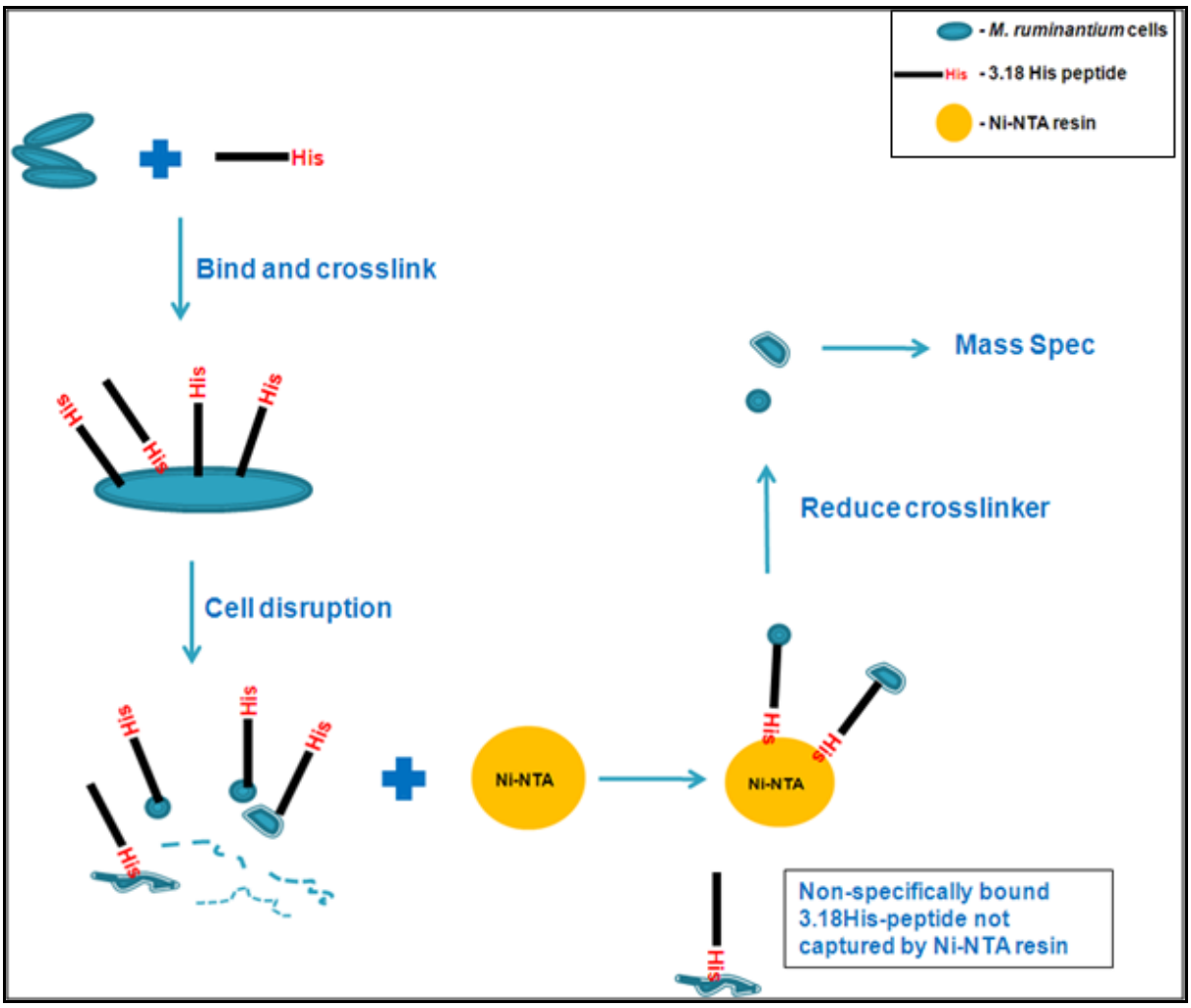


Fig. 12 Schematic showing alternative design of binding site identification experiment. The 3.18His-recombinant protein is reversibly cross-linked to the *M. ruminantium* cells prior to disruption. Ni-NTA resin is used for the affinity capture of bound *M. ruminantium* proteins prior to their release for analysis.

5 Success in Achieving Objectives

5.1 Establish in-vitro culturing methods to screen peptide libraries against rumen archea

The methanogen strain *Methanobrevibacter ruminantium* was chosen as the model cell line as this is a consistent and prevalent species of anaerobic methanogen in the rumen. Media was selected for both optimal growth of the model organism as well as optimal mimicry of the *in vivo* environment, so as to replicate the eventual binding conditions that any inhibitory peptides will be subjected to in the rumen. Minimal BRN media supplemented with a higher percentage of rumen fluid was used since research into peptide behaviour suggested that fluctuations in pH and salt concentrations can have significant affects on peptide affinity, which would influence any subsequent inhibition. The second area of exploration was the development of suitable assays to measure methane production and cell viability of cultures in a semi high-throughput and quantitative way. A number of approaches were explored utilising optical density (OD_{600}) measurements as indicators of cell numbers, an alternative method utilising gas-chromatography (GC) to measure methane production and fluorescence (F_{420}) assays to assess cell viability. After a series of growth tests, it was determined that media containing 60% rumen fluid and based on 0.5 x concentrations of the standard BRN media components was sufficient to sustain *M. ruminantium* in culture with methane production as the index of activity.

5.2 Screen phage display libraries for binding to a predominant rumen methanogen and recover bound phage for identification of peptides by sequence analysis

The suitability of using growth media for the binding assays was also tested by performing experiments which confirmed that these conditions did not result in non-specific binding, instability or a decrease in activity of the bacteriophage. A binding screen was designed that involved the addition of bacteriophage virions to live *M. ruminantium* cultures, including a subtractive screen that is repeated 3 times to achieve phage enrichment. The media conditions were maintained throughout the whole binding assay, with the washing steps performed with media supplemented

with increasing concentrations of anionic detergents. Two alternative protocols were designed based on sequential panning experiments with subtractive screens included, to allow for the selection of predominant binders in semi-native conditions. The NEB Ph.D-12 bacteriophage library was added directly to live cells growing at log-phase in a rumen fluid enriched media that was designed for these experiments to better mimic the binding conditions of the rumen. The subtractive screens, using the non-binding bacteriophage recovered from the preceding screen, were included such that any peptides that are initially sterically inhibited from binding can also be identified. The compatibility of this modified media with the NEB library components was tested and this confirmed that the media components did not interfere with the infectivity or stability of the bacteriophage throughout the assay. Using these principles applied to two different screening methods, we amplified and purified 70 phage-displayed peptide clones which entered subsequent rounds of binding and inhibition screening. These extra binding screens were performed and bacteriophage that bound persistently were isolated, amplified and sequenced to reveal the peptide identities. In one of the screens, a pool of 14 bacteriophage-peptide sequences was identified. Many of these peptides displayed a range of characteristics, such as charge, hydrophobicity and an over-representation of prolines, which are considered typical of antimicrobial peptides that bind bacterial membranes. There were also peptides identified which had different properties to these, and they may prove to be the ones with a more target specific mode of binding.

5.3 Panel of phage displayed peptides selected from objective 2 screened against predominant methanogens and specificity and sensitivity of inhibitory activity determined

ELISA results showed that approximately half of the 70 phage-displayed peptides identified in the binding screens above were superior binders and these were then tested in inhibition assays that were also designed in-house to better mimic the native conditions within the rumen. Three bacteriophage-peptides demonstrated inhibition against the model rumen methanogen, *Methanobrevibacter ruminantium*, while their inhibitory activity has also been detected against less dominant strains, *Methanosarcina barkeri* and *Methanobrevibacter gottschalkii*.

5.4 Inhibitory peptides identified in objective 3 are synthesised and tested *in vitro* as multi-antigenic peptides against predominant archaea as well as denatured phage-displayed peptides

The three phage-displayed peptides demonstrated both high binding and inhibitory activity towards the model methanogen, *M.ruminantium* and oligonucleotides representing these peptide sequences were cloned into *E. coli* expression vectors for the recombinant production of linear peptide with and without HIS-tags. These linear peptides were tested in the same binding and inhibition assays that had been developed for the phage-displayed peptides. Studies showed that the three putative antimicrobial peptides were capable of binding methanogens, and had varied, but specific, inhibitory activities towards three rumen methanogens, *M. ruminantium*, *M. gottschalkii* and *M. barkeri*. Additional inhibition studies with the three strains and higher doses of the recombinant peptides were performed which demonstrated the increased dosage had a marked affect on the inhibitory activity of the 3.18 peptide, which was capable of reducing the methane production on *M. ruminantium* to ca. 30% of the control levels, but was not as significant in the cases of the 4.5 and 4.7 peptides. All of the peptides tended to be most inhibitory towards the model methanogen, which was expected as this was used in the original screens. The 3.18 peptide has also been expressed as a dimer and it is predicted that this peptide concatamer may be more stable and potent that the 3.18 peptide monomer, making it a better candidate for activity and persistence in the rumen.

5.5 Receptors identified on the archael cell wall that are the sites of binding by the phage peptides

Investigations into identifying the cell-surface receptor targets for the inhibitory peptides were based on the 3.18 peptide and the *M. ruminantium* strain, as this combination is the most likely to yield relevant information because the genome of this strain is known. The process employed binding the phage-peptide clone to the target cells, reversibly cross-linking the peptides to the cell surface then lysing the cells via bead-beating. A/G sepharose beads coated in M13 bacteriophage P8 protein antibody were then added to the lysed mixture to capture the 3.18 phage-peptide clones. After incubation and capture, the cell-surface target protein/s bound to the 3.18 phage-peptides are reduced to release the target protein/s for fragmentation and identification via mass

spectrometry. The initial experiments did not yield enough target cell surface proteins for mass spectrometry analysis but the method is being optimised at present both using steps to scale up the initial experiment with increased volumes of starting *M. ruminantium* cultures, and increased excesses of the phage-peptide and antibody or using another strategy outlined in Fig.12. It is expected that once an appropriate balance between these major components and the stringency of the binding and washing steps is elucidated, the target proteins in the eluates will be readily identified by mass spectrometry.

5.6 Effect of denatured phage particles or synthesised peptides identified in milestones 3&4 on methane production in a mixed rumen culture containing the natural diversity of rumen methanogens. Proof for potential use as supplements in an antimethanogenic strategy

To date only the 3.18His-peptide has been evaluated in a mixed rumen culture with natural methanogens and there was no discernable inhibition of methane production. These assays were performed with the single, linear 3.18 peptides and it is likely that the peptide has been rapidly degraded by the proteolytic bacteria in the mixed culture. Further work will aim to mitigate degradation of the bioactive peptide/s in the rumen by using denatured phage particles or by modifications to the linear peptide such as acetylation, pegylation or concatamerisation which should reduce susceptibility to proteolytic degradation. Expression vectors for concatameric versions of the 3.18 peptide have been made and will form the basis of future experiments.

6 Impact on Meat and Livestock Industry – Now and in Five Years Time

The immediate impact of this project on the meat and livestock industries will be the ongoing opportunity to have an impact on reducing methane emissions from livestock with potential for further development through round 2 of the MLA/DAFF Reducing Emissions from Livestock Research Program that will commence in 2012.

The Australian livestock sector which produces the greatest amount of enteric methane is the beef cattle industry. There are approximately 22 million beef cattle in Australia and about half of this population is found in northern Australia. Northern Australian production systems are typified by low rates of gain (< 1 kg/day), high turn-off ages (~ 3.5 years old), lack of grain for finishing, and an almost complete reliance on low quality forage based diets. It has been estimated that as little as a 5% increase in the efficiency of digestion could yield an economic benefit of at least A$100 million to the cattle industry. The excretion of methane from the rumen can represent a loss of 8-10% of the energy of the diet depending on the type of diet. A reduction in methanogenesis in the rumen can be associated with improvements in feed conversion efficiency without affecting intake. Therefore reducing methane production could benefit the ruminant energetically provided the efficiency of ruminal metabolism is not compromised.

Research from the current project is likely to have impact on both the intensive and extensive sectors of the ruminant livestock industries particularly if there are productivity benefits associated with a reduction in methane emissions. Direct manipulation of ruminal metabolism by bioactives will be more appropriate under intensive production conditions; but it is also possible that a vaccine to reduce methane emissions could be developed from this work that would have more relevance for the extensive grazing sector.

7 Conclusions and Recommendations

7.1 Conclusions

- Phage display technology has proven to be a valuable approach for identifying bioactive peptides that are inhibitory to methanogenic activity of rumen archaea
- Several peptides have been identified which are active as both linear molecules and as phage displayed clones which means they could be delivered by both modes to the rumen
- Inhibitory peptides will probably need to be modified to make them resistant to degradation in the rumen which may initially be achieved by providing peptide as phage clones
- Phage display peptides provide an opportunity to identify cell surface proteins that are crucial to the survival of methanogens in the rumen environment. Antibodies can then be raised against these proteins in a vaccination approach to reduce methane production.

7.2 Recommendations

- The project has demonstrated that phage displayed peptides can bind methanogens and reduce methane production in pure cultures. There is thus an opportunity to discover more inhibitory peptides by further library screening as the methodology is now well established for screening and selecting potentially inhibitory peptides in a high throughput format.
- Current and future work will focus on (1) identifying the peptide binding sites on the methanogen cell wall, and (2) modifying the linear peptides to increase resistance to rumen proteolytic activity
- The opportunity exists to collaborate with AgResearch NZ on the development of a vaccine against methanogens. AgResearch NZ have a genomics enabled program and pipeline for evaluation of methanogen-cell wall proteins and peptides as antigens in a vaccination approach to reducing methane emissions from livestock. The identification of cell surface binding targets in the current project could productively merge with the New Zealand vaccination program that has the capability to not only produce antibodies against target proteins but can evaluate those antibodies in an *in-vitro* fermentation system. Finally, the program is able to undertake vaccination studies in ruminants and measure the effectiveness of different antigens against methane production in respiratory chambers.

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