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Archaeaphage Therapy to Control Rumen Methanogens

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Abstract

Phage therapy is becoming increasingly important as a means of eradicating or controlling microbial populations and has been raised as a potential strategy to reduce methane emissions from ruminants. To date, very little is currently known about phages which may infect the methane-producing archaeal strains (methanogens) dominant within the rumen of Australian cattle, such as the *Methanobrevibacter ruminantium*. This project aimed to assemble a collection of phages to be employed in phage therapy. A range of animal-derived and environmental source samples were tested using culture-based methodology, however no lytic phages of methanogens were isolated. Given the dearth of knowledge regarding phages of rumen methanogens, this project established that these naturally-occurring phages may be present in very low concentrations within the rumen and this will need to be considered in future methanogen-phage isolation investigations. The project has begun the process of developing and adapting new methodologies for detecting and examining these phages.

Executive Summary

Phage therapy strategies usually employ naturally occurring viruses, termed lytic phage, which specifically infect and eliminate the target (host) micro-organism. To date, very little is known of the phages which may infect the micro-organisms (methanogens) responsible for the majority of enteric methane production and very few lytic phages able to infect methanogens have been isolated. A recently published paper describing the genetic material of all the viruses (virome) present in the rumen of dairy cows (Berg-Miller *et al.* 2011), verified that the rumen contains genetic material directly related to phages of methanogens (archaeaphage) and established that these phages may occur naturally in the rumen, albeit in very low concentrations.

The primary objective of this project was to establish a collection of archaeaphage to target a range of rumen methanogens, including those known to be dominant in Australian cattle and sheep. This objective utilized a culture-dependant approach or assay system, for isolating archaeaphage, similar to that used previously for the isolation of phage of rumen bacteria (Klieve *et al*. 1991), adapted to provide the growth conditions required by the strictly anaerobic, hydrogen-utilising methanogen host strains (Gilbert *et al*. 2010). This assay system was designed to allow the detection of phage-induced clearings (plaques) in confluent layers of methanogen host strain growth.

Initially, we contacted a research group in the USA, to obtain a methanogen host strain and the lytic phage PG to be used as a positive control in plaque assay experiments. Phage PG was first isolated in the 1980's from rumen fluid and registered with the International Committee on Taxonomy of Viruses database (ICTVdB code 02.066.0.86.007). It later transpired that this phage:host system was unavailable for distribution and the methodology was validated using an alternative, relatively slow-growing rumen bacterial host (*Ruminococcus albus* AR67) and associated lytic phage (φRa02) (Klieve *et al*. 2004).

A large number of environmental and animal-derived source samples were collected for the purpose of phage isolation. Source samples chosen for examination were either from (1) environments similar to those from which the host strains were originally isolated, such as bovine and ovine rumen fluid; or (2) environments known to contain highly concentrated, diverse populations of phage, such as untreated sewage effluent, waste-water treatment plant samples and agricultural waste ponds. Screening of environmental and animal-derived source samples for lytic phage was undertaken using the plaque assay methodology developed, however none of the source samples tested provided evidence of lytic archaeaphage able to produce clear plaques in confluent layers of methanogen host strains. To address this, the conventional phage isolation methodology was adapted to encourage lytic phage infection of methanogens, including pre-enrichment of source samples with methanogens and 100-fold concentration of phage populations present within source samples. In addition, several new methanogen strains were isolated from animal-derived source samples to be employed in the plaque assay system.

The structural integrity and viability of phage particles was also ascertained using Transmission Electron Microscopy (TEM) and by employing the rumen bacterium *R. albus* AR67 in the plaque assay system. In this way, the bovine and ovine rumen fluid source samples were shown to contain a diverse population of structurally intact phage particles and several novel phages of *R. albus* AR67 were successfully isolated. These results indicated that the plating methodology was effective when

using a rumen bacterial host strain and that the source samples did contain viable, infective phage particles.

The project also investigated the presence of lysogenic phage of methanogens. Lysogenic phage may infect the host methanogens and integrate their DNA into the host genome, enabling the phage genetic material to be passed through successive generations of the host. The chemical inducing agent mitomycin C was used to facilitate the detection of lysogenic phage in methanogens but did not result in increased concentrations of phage being detected in any of the methanogens tested. Lysogenic phage may however be produced in very low concentrations during normal culture growth and four of the eleven methanogens tested, including three new methanogens isolated during the course of the project, were found to produce extracellular DNA similar in size to the average genome length of tailed phages. Whether this DNA is viral in origin requires further analysis, with initial tests proving inconclusive. The presence of remnants of phage DNA sequences, specifically Clustered Regularly Interspaced Short Palindromic Repeat Sequences (CRISPR) and associated *Cas* proteins, in the genome of several rumen methanogens was also investigated. All methanogens examined in this way were found to have regions of CRISPR sequences in their genome, providing evidence of previous phage infection events. Unfortunately these CRISPR sequences may also confer host immunity to further infection by closely-related phage.

Towards the end of the project, alternative methods for the enumeration and detection of lytic phages in culture and in environmental samples were investigated. Methods investigated included the use of epifluorescence microscopy and nucleic acid (SYBR Gold) staining (Chen *et al*. 2001) and PCR-based detection of archaeaphage-specific genes including the *Methanobrevibacter ruminantium* M1 type strain endoisopeptidase protein (*Pei*R) and the terminase large subunit genes of the type strains *Mbb. smithii* PS type and *Mbb. ruminantium* M1. The latter phage genes are utilised in the production of DNA phage particles and are highly conserved, therefore the presence of these genes in methanogen cultures may indicate the presence of an integrated phage, particularly phage related to methanogens of the *Methanobrevibacter* genus.

The results of this project were unexpected as methodology previously employed for the isolation of phage particles from the environment and animal-derived samples was not effective when applied to rumen methanogens and methanogen type strains. The lack of phage isolates obtained and the absence of a suitable control methanogen phage:host system therefore did not allow the second project objective to be addressed; that is, to establish “proof of concept” evidence that archaeaphage therapy has viable potential to reduce methane emissions from ruminal fermentation. The project did however result in the collection of a large number of animal-derived and environmental source samples for further analysis, the introduction of new rumen methanogen strains into culture and the establishment of new methodology for phage-detection and isolation. The project also increased the scientific understanding of what may be required for the successful isolation of phages of rumen methanogens and emphasized the importance of undertaking a metagenomic approach to understanding the biology of methanogens and their respective phages, within the rumen microbial ecosystem.

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1.0 Background

Phage therapy is becoming increasingly important in agriculture as a means of eradicating or controlling microbial populations, providing an effective, naturally-occurring alternative to antibiotics and chemical treatments (Monk *et al*. 2010; Mahony *et al*. 2011). Phage therapy has therefore been raised as a potential strategy to reduce enteric methane emissions from ruminants (Buddle *et al*. 2011; Cottle *et al*. 2011). Phages could be used to reduce populations of methanogenic archaea (methanogens) in the rumen *per se*, or they may be utilised to establish alternative microbial populations which will out-compete methanogens for hydrogen, such as reductive acetogens.

This project aims to assemble a collection of archaeal viruses (archaeaphage) for the purpose of phage therapy. Phage therapy strategies usually employ naturally occurring phage, termed lytic phage, which replicate using the lytic cycle of phage reproduction, which involves specifically infecting and eliminating the target (host) micro-organism. To date, very few lytic phage able to infect methanogenic archaea have been isolated (Pina *et al.* 2011) and very little is known of the phages which may infect methanogens commonly found in the rumen. A recently published paper describing the rumen virome (Berg-Miller *et al.* 2011) verified that the rumen of dairy cows did contain viral genetic material directly related to known archaeaphages. This finding indicates that phages of methanogens occur naturally in the rumen, albeit in very low concentrations.

The project utilized a culture-dependant approach for isolating archaeaphage, similar to that used previously for the isolation of phage of rumen bacteria (Klieve *et al*. 1991) and adapted to facilitate the use of the strictly anaerobic, hydrogen-utilising methanogen host strains (Gilbert *et al*. 2010). This methodology was designed to allow the detection of phage-induced clearings (plaques) in confluent layers of methanogen host strains. Initially we contacted a research group in the USA, led by Professor Larry Baresi, in regards to obtaining a *Methanobrevibacter smithii* host strain and the lytic phage PG, to be used as a positive control in screening experiments. Phage PG was first isolated in the 1980's from rumen fluid and registered with the International Committee on Taxonomy of Viruses database (ICTVdB code 02.066.0.86.007). It later transpired that this phage:host system was unavailable for distribution.

A large number of environmental and animal-derived source samples were collected for the purpose of phage isolation. Source samples chosen for examination were either (1) from environments similar to those from which the host strains were originally isolated, such as bovine and ovine rumen fluid; or (2) from environments known to contain high concentrations of phage, such as untreated sewage effluent, waste-water treatment plant samples and agricultural waste ponds (Appendix 1.). Several new methanogen strains were also isolated from environmental source samples to be employed in phage isolation experiments. Towards the end of the project, alternative methods for the enumeration of lytic phages in culture and in environmental samples were also investigated, including the use of an epifluorescence microscopy method. This method was originally developed for the enumeration of viruses in aquatic ecosystems (Chen *et al*. 2001) and has been more recently been employed in metagenomic studies for enumerating phages in environmental samples (Thurber *et al.* 2009).

In the environment, phages may also undergo an alternative replication strategy, termed lysogeny. In this strategy, host attachment and phage infection is followed by

the integration of phage genomic DNA into that of their microbial host (Klieve *et al*. 1989). In this way, the phage genome may be passed on to daughter cells during the normal processes of host replication and persist within the microbial population. Lysogenic phage integrated into the host genome may however, be triggered into the lytic cycle of phage replication, resulting in the formation and release (blooms) of progeny phage particles. The recent sequencing of the *Methanobrevibacter ruminantium* M1 type strain genome indicated the presence of a lysogenic phage integrated into the genome of this rumen methanogen (Attwood *et al*. 2008). The sequence of this phage designated φmru (Figure 1.), contains distinct functional modules encoding phage integration, DNA replication and packaging, capsid proteins and lysis functions (Attwood *et al*. 2008).

In addition, the recent whole genome sequencing of several new isolates of the dominant human gut methanogen species, *Methanobrevibacter smithii* (Hansen *et al*. 2011), revealed that 35 % of the *Mbb smithii* isolates examined (7 of 20 isolates), contained integrated prophages. The presence of prophages within the genome of methanogens is of interest as they (1) indicate that a successful phage infection event has occurred; (2) provide a valuable resource of methanogen phage genes, including host-specific, phage-encoded lytic enzymes (phage lysins) which may be utilized in alternative bio-control strategies (reviewed by O'Flaherty *et al*. 2009); and (3) the sequence of integrated phage genes may be utilized for the development of molecular tools, for example, sequences for PCR detection of related archaeaphage (including lytic phage) within pure microbial cultures or a mixed microbial, environmental samples.

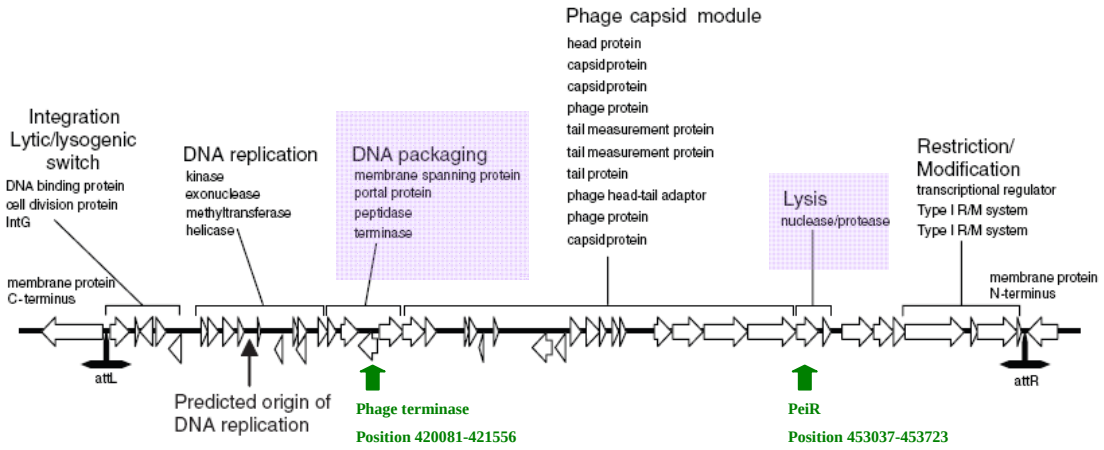

Figure 1. Predicted functional modules and gene structure for φmru, the prophage present within the *Methanobrevibacter ruminantium* M1 sequence (Accession NC_013790.1), showing the completed chromosome position of the *Pei*R and terminase large subunit genes (highlighted) used for PCR primer design (Figure adapted from Attwood *et al*. 2008).

The genome sequence of φmru was used to develop PCR primers for detection of prophages which may be integrated into the genome of the methanogen strains available to us in our laboratory, and for ascertaining the presence of these genes in environmental source samples. Prophage φmru genes investigated included the φmru endoisopeptidase gene *Pei*R. The protein encoded by this gene is related to

other phage-encoded lysis proteins (Visweswaran *et al*. 2010) and has been identified as a potential inhibitory agent for the control of methanogenic archaeal populations (Leahy *et al*. 2010). Archaeaphage terminase genes were also chosen for investigation as they have been shown to be one of the most highly conserved DNA phage genes (Sewer *et al*. 2004), therefore the presence of these genes is more likely to indicate the presence of integrated prophage, particularly those more closely related to the methanogens of the *Methanobrevibacter* genus.

Another aspect of methanogen and phage biology investigated during the course of the project involved ascertaining the presence of Clustered Regularly Interspaced Short Palindromic Repeat Sequences (CRISPR) within methanogen host strain sequences. Recent publications have indicated that microbes including methanogenic archaea, protect themselves from viruses and other invasive mobile genetic elements through a genetic interference pathway that is sequence-directed, similar to the well-known RNA intereference (RNAi) pathway that operates in the cells of plants and animals (Horvath and Barrangou, 2010; Gottesman, 2011). The CRISPR locus consists of short repeats that are separated by non-repetitive sequences called spacers. The sequence of these spacers match the sequence of phage genomes, plasmids and other previously encountered foreign DNA. CRISPR loci are also flanked by a set of CRISPR-associated (*Cas*) genes that encode the protein components of the interference machinery. Once the CRISPR sequences are transcribed and processed to CRISPR RNA molecules (crRNAs) they can combine with the *Cas* proteins to create an effector complex which cleaves invading DNA (Sontheimer and Marraffini, 2010).

Archaeal species, such as rumen methanogens, have been shown to have a relatively high proportion of CRISPR in their genomes (Sorek *et al*. 2008). The relative abundance of these sequences may indicate which methanogen strains would be better suited as hosts in phage isolation experiments, where a lower resistance to phage infection would be preferred. It is anticipated that any future analysis of these sequences will verify the suitability of individual methanogens for use in phage screening experiments and further analysis of the CRISPR spacer regions, will provide genetic evidence about the type of phages able to infect rumen methanogens.

Findings described in this report encompass the various areas of investigation undertaken during the course of the project including:
(a) lytic phage isolation experiments: plaque assays;
(b) plaque assay method validation;
(c) the isolation of new methanogens;
(d) methanogen host strain analysis: *in vitro* detection of lysogenic phage and CRISPR/Cas sequences;
(e) method development such PCR methods for the detection of known archaeaphage genes and epifluorescent microscopy for the improved detection of phage particles.

2.0 Project Objectives

1. By 1st March, 2011 establish a collection of archaeal viruses that infect the spectrum of rumen methanogens known to be dominant in cattle and sheep on typical feed types through Australia

2. By 1st December 2011 establish “proof of concept” evidence that archaeaphage therapy has viable potential to reduce methane emissions from ruminal fermentation.

3.0 Methods

3.1 Lytic phage isolation experiments: plaque assays

Culture of Archaea

Nine strains of methanogenic archaea were routinely cultured in our laboratory, including isolates of, *Methanobrevibacter sp.* (designated YE300, YE301, YE302, YE303 and YE304)*, Mbb. smithii* (DSM861), *Mbb. ruminantium* (YE286, ATCC35063), *and Methanobacterium bryantii* (YE299). Methanogens were cultured in either small (10 ml in Hungate tubes) or large (100 ml in Wheaton bottles) volume cultures of BY medium, supplemented with Methanogen Vitamin mix (media and supplements described by Joblin, 2005). Culture manipulations and inoculations were undertaken using anaerobic methods (Hungate, 1969) in an anaerobic chamber (Coy). Following culture inoculation, the headspace of the respective culture vessel was filled with hydrogen gas to a pressure of approximately 207 kPa and incubated with a rocking motion at 39 ºC.

Screening of environmental source samples for phage

In an anaerobic chamber, 3 d cultures of methanogen hosts were transferred into 15 ml volume Falcon tubes, transferred from the chamber and cells pelleted by centrifugation at 3 000 x *g* for 5 min (Eppendorf 5702 Centrifuge), then transferred back into the anaerobic chamber. Cell pellets were then each resuspended in 3 ml BY medium and 200 µl test sample added. Test samples may be, for example, a bovine rumen fluid source sample that had been clarified by low speed centrifugation and filtered through a 0.22 µm low protein binding filter unit (Millipore) to remove contaminating bacteria. Treated host cells were then left at room temperature for approximately 15 min to allow for phage adsorption, then 4.5 ml molten BY 0.7 % agar supplemented with Methanogen Vitamin mix, added and this mixture plated as an overlay onto a pre-set 1.2 % BY agar plate. Plates were placed in an anaerobic canister together with a plate of $CaCl_2$ pellets as a desiccant, filled with H_2 to a pressure of 80 kPa and incubated at 39 °C for 3 to 5 days. In order to increase the efficiency of phage isolation, variations of this standard isolation protocol were undertaken as detailed below:

(1) *Enrichment with host strains*

In order to increase phage numbers in environmental source samples prior to screening experiments, an aliquot of source sample, for example a 1 ml volume of bovine rumen fluid, was added to 10 ml volume cultures of individual methanogens which had been incubated at 39 °C for 2 d (including *Methanobrevibacter* sp. YE301, YE303, *Methanobacterium* sp. YE299, *Mbb. smithii* DSM861 and *Mbb. ruminantium* ATCC35063) and incubation re-commenced for a further 3 d at 39 °C. Cultures enriched in this way were then centrifuged at 3 000 x *g* for 10 min (Eppendorf 5702 Centrifuge) to remove cells and the supernatant filtered through a 0.45 µm filter unit then through a 0.22 µm filter unit (Millipore). The filtered supernatant was then stored at 4 °C prior to screening with the respective methanogen host species.

(2) *Virus concentration from environmental source samples*

In order to concentrate the phage population present in environmental source samples, for example, bovine rumen fluid and anaerobic digester effluent, 50 ml

volumes of source sample were centrifuged at 3 000 x *g* for 10 min (Eppendorf 5702 Centrifuge) to remove debris and microbial cells and the supernatant filtered through a 0.45 µm filter unit (Millipore), then a 0.22 µm filter unit (Millipore). Filtered supernatant was then centrifuged at 26 861 x *g* for 2 h at 4 ºC (SS34 rotor, Sorvall RC5B centrifuge, DuPont). The resultant pellet was then resuspended in 500 µl Phage Storage Buffer (PSB) (Klieve, 2005) and stored at -80 °C prior to screening with methanogen host species.

(3) Changes in phage adsorption time

To allow phage to attach more effectively to host methanogens, an aliquot of source sample, for example 50 µl of virus concentrate (100x) was added to 10 ml volume cultures of individual methanogens which had been incubated at 39 °C for 2 d (including *Methanobrevibacter* sp. YE301, YE303, *Methanobacterium* sp. YE299, *Mbb. smithii* DSM861 and *Mbb. ruminantium* ATCC35063) and incubation re-commenced for a further 4 to 6 h at 39 °C. These cultures were then transferred into the anaerobic chamber and poured into 15 ml volume Falcon tubes. These tubes were then transferred from the chamber and cells pelleted by centrifugation at 3 000 x *g* for 5 min (Eppendorf 5702 Centrifuge). The tubes were then transferred back into the anaerobic chamber and cell pellets each resuspended in 3 ml BY medium. The resuspended cell pellet was then suspended in an agar overlay in the plating procedure described above.

3.2 Plaque assay method validation

Screening of animal-derived source samples for phages of *Ruminococcus albus* AR67

Ruminococcus albus AR67 was used in a plaque assay and exposed to animal-derived source samples of (1) bovine faecal samples (virus concentrate prepared as detailed above); (2) bovine rumen fluid virus concentrate; and (3) ovine rumen fluid virus concentrate. Plaque assays were undertaken in an anaerobic chamber to maintain anaerobic conditions. Briefly, a 0.5 ml volume of an early-log culture (5 ml volume RF medium [Klieve *et al.* 1989] incubated for 6 h at 39°C) of AR67 was infected with a 50 µl volume of virus concentrate or a dilution or virus concentrate, prepared in RF medium from 10^{-1}, 10^{-2} or 10^{-3}. Treated host cells were then incubated at room temperature for approximately 15 min to allow for phage adsorption, then 4.5 ml molten RF 0.7% agar was added. This mixture was then quickly plated as an overlay onto a pre-set 1.2 % RF agar plate. Plates were placed in an anaerobic canister together with a plate of $CaCl_2$ pellets as a desiccant and incubated at 39 °C for approximately 18 h. Phage infections were visualized as clearing zones or plaques, in the otherwise confluent layer of bacterial growth. Seven plaques were picked and stored in 50 µl PSB at 4°C. The infectivity, purity and the titre of a single, representative phage designated φ149-112-5 was determined by repeated plaque assays using plaque material picked from successive assay plates.

Transmission electron microscopy

Subsamples (10 µl volume) of concentrated phage stocks were deposited on copper square mesh grids (200 mesh, Proscitech) coated with nitrocellulose and left for 5 min before removing excess fluid. Grids were then negatively stained with 1 % ammonium molybdate, pH 6.8 (Sigma-Aldrich) for 5 min. Images were obtained using a JEOL 1400 transmission electron microscope. Micrograph images were obtained with a digital camera (Gatan).

3.3 Isolation of new methanogens

Methanogenic archaea were isolated from (1) an *in vitro* Fermenter Apparatus (Infors-HT) inoculated with bovine rumen fluid and culture maintained with the addition of Mitchell grass (*Astrebla* spp.) hay; (2) a sample from a waste water plant anaerobic digester (Elanora Waste Water Treatment Plant, Gold Coast Water; (3) Bovine rumen fluid from a steer maintained on a low quality hay diet.

For each source sample, a selection enrichment culture was prepared by adding a 10 ml volume of sample to 100 ml BY medium contained in a Wheaton Bottle, supplemented with Methanogen Vitamin mix, Penicillin/Streptomycin (12 $mgml^{-1}$ /2.0 $mgml^{-1}$) and Vancomycin (2.0 $mgml^{-1}$) (media and supplements described by *Joblin*, 2005). The headspace of the Wheaton bottle was then filled with H_2 gas to a pressure of approximately 207 kPa and incubated at 39 °C for 4 days.

In an anaerobic chamber, a 1 ml aliquot of selection enrichment culture was serially diluted in BY medium and 0.5 ml volumes of the 10^{-3} to 10^{-7} dilutions added to molten 0.7 % BY agar (supplemented as above with Methanogen Vitamin mix, Penicillin/Streptomycin and Vancomycin) and plated as an overlay, onto a pre-set 1.2 % BY agar plate. Plates were placed in an anaerobic canister together with a desiccant plate of $CaCl_2$ pellets, filled with H_2 to a pressure of 80 kPa and incubated at 39 °C for 3 to 5 days. Maintaining anaerobic conditions, individual colonies were then picked from isolation plates and inoculated into Hungate tubes containing BY Medium (supplemented as above with Methanogen Vitamin mix, Penicillin/Streptomycin and Vancomycin). Hungate tubes were then filled with H_2 and incubated with a rocking motion at 39 °C for 4 days.

Culture purity was checked using microscopy and cultures maintained under antibiotic selective pressure. Genomic DNA was extracted from a selection of pure cultures using a boil cell lysis method (Gilbert *et al.* 2005), partial16S rRNA gene amplified by PCR using archaea-specific primers (46F primer, Øvreas *et al.* 1997; 1017R primer, Barns *et al.* 1994) and the product sequenced at the Australian Genome Research Facility (AGRF) using the BigDye™ sequencing kit (Applied Biosystems). Sequence data corresponding to each culture was then tested for homology in public databases. On occasion, an alternative 16S rRNA gene PCR procedure (Wright and Pimm, 2003) was employed and PCR products digested using the restriction enzyme Hae III (Roche). This enabled a fingerprint of the 16S rRNA gene to be obtained and compared to reference strains and other published data (Wright and Pimm, 2003).

3.4 Methanogen host strain analysis: detection of lysogenic phage and CRISPR/Cas sequences

Mitomycin C induction experiments

Small (10 ml) and large (100 ml) cultures of archaea including new isolates of *Methanobrevibacter sp.* were incubated at 39 °C for 2 d, including isolates of *Methanobrevibacter sp.* (YE301, YE303), *Methanobacterium bryantii* (YE299), *Mbb. smithii* (DSM861), and *Mbb. ruminantium*, (ATCC35063). The cultures were treated with mitomycin C (final concentration of 1 μgml^{-1}) and the incubation recommenced at

39 °C for up to 5 d. Depending on culture volume (10 ml archaeal cultures were usually incubated for a further 48 h, 100 ml archaeal cultures were incubated for up to 5 d). In most induction experiments, a culture of *Prevotella ruminicola* ss *brevis* AR29 was included in parallel in order to provide a positive control for the phage induction activity of mitomycin C (Klieve *et al.* 1989). Negative control cultures of each organism, to which no mitomycin C was added, were also incubated for a total of 7 d.

Isolation of phage like particles or extracellular DNA

Extracellular DNA or phage like particles (PLPs) were isolated from cultures using the methods outlined by Klieve (2005). Briefly each culture was opened and poured into a disposable tube and cells pelleted by centrifugation at 4 400 rpm for 20 min and the supernatant transferred to a second sterile tube. The supernatant was then filtered through a 0.45 µm HV low protein binding filter unit (Millipore) and then a 0.22 µm HV low protein binding filter unit (Millipore) and filtrate transferred into sterile centrifuge tubes. PLPs were then pelleted by centrifugation at 26 861 x *g* for 2 h at 4 ºC (SS34 rotor, Sorvall RC5B centrifuge, DuPont). The supernatant was then discarded and the pellet resuspended in 200 µl TE buffer pH 7.6. The method used for extraction of DNA from this pellet differed according to the electrophoresis method to be used.

DNA extraction for conventional electrophoresis

DNA extraction for conventional electrophoresis followed the protocol outlined by Klieve and Gilbert (2005). Briefly, PLP samples (resuspended centrifuge pellets described above) were incubated with RNase (2 µl of 10 $mgml^{-1}$ RNase), and the protein coat removed by digestion for 1 h at 50 ºC with proteinase K (2 µl of 20 $mgml^{-1}$ Proteinase K) and 1 µl of 20 % (w/v) SDS. Phage DNA was then extracted by phenol/chloroform extraction and DNA precipitated using 3 M Na acetate (pH 5.2) and ethanol. DNA pellets were resuspended in a 25 µl volume of sterile H_2O and stored at – 20 ºC. The presence of phage DNA was verified using 1 % agarose, 1 x TBE gel electrophoresis. Digestion of DNA with the restriction endonuclease *Hind* III (Roche) followed the manufacturer's protocol.

DNA extraction for PFGE

DNA extraction for PFGE followed the protocol outlined by Klieve and Swain (1993). Briefly, this involved embedding PLP samples (resuspended centrifuge pellets described above) in low melting temperature agarose (1.5 % wt/vol.) fashioned into plugs suitable for PFGE. Plug samples were treated to remove the particle protein coat (1 $mgml^{-1}$ proteinase K, 1 % SDS) and incubated at 50°C overnight, followed by two washes at room temperature for 30 min in TE buffer (10 mM Tris, 1 mM EDTA pH 7.6). Residual protein was then removed by incubation with 1 mM PMSF in TE buffer pH 7.6 at 55 °C for 1 h and washed for 30 min in TE buffer pH 7.6. Plugs were then washed in 20 % Isopropanol for 1 h and stored in 0.5 M EDTA pH 8.0 at 4°C.

Electrophoresis

The presence of extracellular DNA from small volume cultures was verified using conventional 1 % agarose, 1 x TBE gel electrophoresis. Extracellular DNA from large volume cultures was separated using a 1 % agarose, 0.5 x TBE PFGE gel, with buffer circulated at 14 °C using two consecutive programs at 6 Vcm^{-1}. The first program had an initial switch time of 60 s and final switch time of 90 s, continuing for 15 h and the second program had an initial switch time of 1 s and final switch time of 5 s, completing after 3 h. Gels were post-stained with GelRed™ (3 x working stock, Biotium) and visualized using a UV transilluminator and gel documentation system (Quantity One software, Bio-Rad).

CRISPR analysis of methanogen genomes

Genome sequences for type strain methanogens were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) and two draft genome sequences for the methanogen strains *Mbb. ruminantium* YE286 and *M. bryantii* YE299 were obtained from Dr Zhang (CRC for Beef Genetic Technologies). CRISPR regions were identified in completed type strain genomes, the draft genomes using the longest available contigs (for YE286) or a draft concatenated sequence (for YE299) using the CRISPR finder program (http://crispr.u-psud.fr/ Grissa *et al.* 2007a). Sequences were also obtained from those listed in a database of CRISPR arrays for published microbial genomes (CRISPRdb http://crispr.u-psud.fr/crispr/ Grissa *et al.* 2007b). Sequences of interest were further identified using BLAST (http://blast.ncbi.nlm.nih.gov/ Altschul *et al.* 1990). On occasion, sequences identified by the CRISPR program as questionable were translated and examined using blastp (Altschul *et al.* 1990). Questionable CRISPR sequences are either small CRISPRs, i.e. structures having only two or three direct repeats or similar structures (like particular kinds of tandem repeats) or structures where the repeated motifs (direct repeats in CRISPR) are not 100 % identical. These sequences are confirmed if the direct repeat consensus is found elsewhere in the database (i.e. present in another confirmed CRISPR).

3.5 Method development: PCR methods for the detection of archaeaphage genes and epifluorescent microscopy

PCR method for the detection of the φmru *PeiR* gene

Two PCR primer pairs (Table 1.) were developed using the Primer 3 design program (Rozen and Skaletsky, 2000) and checked for sequence homology using BLAST (www.ncbi.nlm.nih.gov). The first primer pair (PeiR453059F and PeiR453394R), were designed to amplify a 356 bp fragment at the start of the *PeiR* gene, covering the putative active site of the protein. The second primer pair (PeiR453393F and PeiR453555R), were designed to amplify a shorter 163 bp fragment covering a section of the gene encoding for protein with similarity to conserved domains of the peptidase C39-like superfamily. The PCR was performed (Biorad C1000 Thermal Cycler) with the following PCR conditions used for both sets of primers: an initial step of 95 °C for 5 min followed by 25 cycles of 60 °C for 30 sec, 72 °C for 30 sec and 95 °C for 30 sec and a final extension step of 72 °C for 5 min.

Table 1. PCR primer sequences for amplification of the φmru *PeiR* gene. Sequence position based on the numbering of the *Mbb. ruminantium* type strain M1 (Accession NC_013790.1).

Primer Name	Sequence Position	Primer Sequence
PeiR453059F	453059-453079	TGCTCCAGGACGGAGCGAAGA
PeiR453394R	453414-453394-	TGCAGGGCTTGTTCCACTGCC
PeiR453393F	453393-453413	GGGCAGTGGAACAAGCCCTGC
PeiR453555R	453533-453555	TCCCTTGCAGTTCCTGCTCTGGT

PCR method for the detection of prophage terminase large subunit genes

Four pairs of PCR primers (Table 2.) were developed using the Primer 3 design program (Rozen and Skaletsky, 2000). Two sets of primers were designed to amplify a 926 bp and 192 bp fragment within the *Mbb, smithii* terminase large subunit protein Msm1671. A further two sets of primers were designed to amplify a 618 bp and 295 bp fragment of the *Mbb. ruminantium* terminase large subunit protein mru_0285. The PCR was performed (Biorad C1000 Thermal Cycler) with the following PCR conditions: an initial step program used for both sets of primers was 95 °C for 5 min followed by 25 cycles of 95 °C for 30 sec 60 °C for 30 sec, 72 °C for 30 sec and a final extension step of 72 °C for 5 min.

Table 2. PCR primer sequences for amplification of the φmru and *Mbb smithii* prophage terminase large subunit genes. Sequence position was based on the numbering of the *Mbb. ruminantium* type strain M1 (Accession NC_013790.1) and the *Mbb. smithii* type strain PS (Accession NC_009515.1).

Primer Name	Sequence position	Primer Sequence
Msmterm 1F	1706778-1706798	gcaattgcagaccgccacaca
Msmterm 1R	1707703-1707703	gcagctgcaggctgatactcaa
Msmterm 2F	1706778-1706799	gcaattgcagaccgccacacaa
Msmterm 2R	1706947-1706969	tggttctgtccaatggtccaggt
Mruterm 1F	420492-420511	agtgcaggtgctggcggttc
Mruterm 1R	421090-421109	accagcgtctccgcttgcac
Mruterm 2F	420214-420233	agggacgggtgagcggcata
Mruterm 2R	420489-420508	ccgccagcacctgcactcaa

Epifluorescent microscopy to determine phage-particle concentration and purity

Samples for virus enumeration of less than 10 ml volume were either partially purified by filtration through a 0.22 µm HV filter unit (Millipore) to remove bacteria and culture debris, or if bacterial counts were also required, used directly. Using methods described elsewhere (Chen *et al.* 2001; Suttle and Fuhrman, 2010), samples (approximately 200 µl volume) were filtered through a 25 mm diameter, 0.02 µm Anodisc filter with support ring (Whatman), underlain with a pre-wetted 0.8 µm pore size, 25 mm diameter filter (Millipore) supported by a glass frit in a 25 mm, 15 ml volume vacuum assisted filter apparatus (Millipore).

Following filtration to capture virus particles, the 0.02 µm filter was removed from the filter apparatus and stained with 2 x SYBR Gold (Invitrogen) for 15 min. The filter was then mounted onto a 10 µl drop of polyvinyl alcohol mounting medium with DABCO anti-fading (Fluka Analytical) on a clean glass slide and covered with an additional 10 µl drop of mounting solution and a clean glass coverslip. Prepared slides were examined using a blue-green light excitation using an epifluorescence microscope (Nikon Eclipse 80i) and visualized and recorded using NIS-Elements BR 3.2 software (Nikon).

4.0 Results

The majority of experimental work undertaken during the course of the project focused on the primary project objective: to establish a collection of archaeal viruses that infect the spectrum of rumen methanogens known to be dominant in cattle and sheep on typical feed types throughout Australia. Environmental and animal-derived samples were collected as sources for the isolation of phage able to infect methanogens (Appendix 1.). Source samples were collected from ruminants on different diets and from different farming systems for example, cattle (steers), sheep and dairy cows in experimental diet trials. Source samples were also obtained from environmental sources with high concentrations of methanogens, for example, human waste water treatment plant effluent (including the anaerobic digester) and a commercial piggery effluent pond.

The plaque assay methodology usually used for the isolation of lytic phage from environmental source samples (Klieve, 2005) was employed. Although plaque-like clearings were detected in several experiments, these results were not reproducible and no methanogen-specific lytic phage isolates were obtained using the standard plaque assay approach. The plaque assay experiments and variations on the plaque assay methodology undertaken in order to increase efficiency of the method, are detailed below. In addition, experiments undertaken to validate the methodology and test the integrity of phage particles contained within the environmental source samples are also described.

4.1 Lytic phage isolation experiments: plaque assays

The plaque assays undertaken using standard methodology utilised a total of 14 types of source sample including rumen fluid obtained from various animals (bovine and ovine) on a range of diets and environmental samples collected from a commercial piggery, Luggage Point Wastewater Treatment Plant (Brisbane City Council) and the Elanora Waste Water Treatment Plant (Gold Coast Water). Several methanogen strains were employed as potential hosts in the majority of plaque assay experiments, with additional methanogen strains isolated during the course of the project, also utilised in plaque assay experiments. Towards the end of the project, when virus concentration and increased phage adsorption times were introduced into the methodology (detailed below), the number of methanogens tested was reduced, to allow more variables to be examined in each plaque assay experiment.

Despite a wide diversity of sample types and methanogens utilised in the lytic phage isolation experiments, no clearly visible phage plaques were obtained (Table 3.). On several occasions, plaque-like clearings were observed and material from these clearings re-tested with the respective methanogen host strains for plaque formation activity. In all cases, repeated testing could not reproduce the formation of plaque-like clearing zones in confluent layers of methanogen host strains.

These results suggested that lytic archaeaphage may be present in very low concentrations in animal-derived and environmental samples, and may be undetected using the standard plaque assay methodology. Additional steps were therefore introduced into the plaque assay methodology, to increase the numbers of phage particles present in the samples employed in the testing procedure and increase the chances of successful phage infection and replication. Variations included in the plaque assay methodology included: (1) pre-enrichment with host strains; (2) virus concentration from environmental source samples; and (3) increased length of phage adsorption time.

Variations of plaque assay methodology:

(1) *Pre-enrichment with host strains*

An additional step was introduced to the standard plaque assay methodology to pre-incubate source samples with methanogen host stains and therefore enrich or increase the concentration of any phages able to infect the respective methanogen hosts. Source samples initially employed in the standard plaque assay methodology were tested using this approach (Table 4.) however no clearly visible phage plaques were obtained.

(2) *Virus concentration from animal-derived and environmental source samples*

In order to increase the concentration of virus particles utilised in the standard plaque assay, environmental samples were first clarified by low speed centrifugation and filtration to remove microbial cells and particulates, then concentrated 100x using ultracentrifugation. Aliquots of these virus concentrates were then added to host cells and plated using the standard plaque assay procedure. Source samples employed in this approach and the methanogen host strains tested in this way are listed in Table 5. As the project progressed, once a methanogen plaque assay returned a negative result with untreated source sample, virus concentrates were prepared and utilised in any further plaque assays. In addition, as the project progressed, the number of methanogens utilised in plaque assay experiments was reduced to enable more source samples to be tested more comprehensively.

On occasion, virus concentrates were also tested for particle integrity and viability by TEM and a plaque assay with a rumen bacterial host undertaken (results detailed in the plaque assay method validation section below).

(3) *Increased length of phage adsorption time*

The testing protocol was modified to increase the length of time allocated to enable phage adsorption (phage: methanogen-host attachment). This additional step was introduced to allow for the slow growth rate of the methanogen host strains and addresses published findings which have indicated that for slower growing micro-organisms, phage adsorption times may be considerably longer than those required for relatively fast growing microbes such as *Escherichia coli*. The phage adsorption time was therefore increased in the experimental protocol when testing both source samples and source sample virus concentrates.

Of all the plaque assay experiments undertaken during the course of the project (Table 6.), virus concentrate samples obtained by pooling rumen fluid collected from 10 dairy cows (DPI VIC Tannin trial, control diet) and sludge material obtained from the Gold Coast Water anaerobic digester produced the most plaque-like clearings in confluent layers of methanogen host strains (example depicted in Figure 2.). Plaque-like clearings were excised from several isolation plates and utilized in further plaque assays with the respective host, however on all occasions, the subsequent plaque assays could not replicate the plaque-like clearing activity observed in the original isolation experiment.

Table 3. Results of standard lytic phage isolation experiments detailing source sample description and methanogens tested (neg = negative result for phage isolation). Plaque-like clearings were excised from the isolation plates indicated (*) and further testing undertaken to reproduce this result.

Source sample	Methanogens tested	Phage isolation
Bovine rumen fluid (single steer, Mitchell grass diet, 10/6/09)	*Mbb*. *sp*. YE301, *Mbb*. *sp*. YE303*, *Mbb*. *sp*. YE286, *M. bryantii* YE299*, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861*	neg
Bovine rumen fluid (single steer, Mitchell grass diet, 22/6/09)	*Mbb*. *sp*. YE301, *Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Grantham Piggery effluent pond (sludge)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Grantham Piggery effluent pond (composite animal waste)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303*, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Luggage Point Wastewater Treatment Plant, anaerobic digester (sludge)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303*, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Luggage Point Wastewater Treatment Plant, influent (raw sewage)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303*, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Elanora Wastewater Treatment Plant (Gold Coast Water), anaerobic digester sludge	*Mbb. sp*. YE301*, *Mbb*. *sp*. YE303*, *M. bryantii* YE299, *Mbb. smithii* DSM861*	neg
Elanora Wastewater Treatment Plant (Gold Coast Water), influent (raw sewage)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb*. *sp*. YE286, *Mbb. smithii* DSM861, *Mbb.* spp. YE314, *Mbb.* spp. YE315, *Mbb.* spp. YE316	neg
Bovine RF pooled from 4 steers, Hay diet	*Mbb. sp*. YE301, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861, *Mbb.* spp. YE314, *Mbb.* spp. YE315, *Mbb.* spp. YE316	neg
Fermenter, Bovine RF Inoculum, Mitchell Grass diet	*Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb.* spp. YE315	neg
Ovine RF, single sheep, fishmeal + speargrass *ad lib* diet	*Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb.* spp. YE315	neg
Ovine RF, pooled from 4 sheep on 2 diets (2 sheep on lucerne and 2 sheep on speargrass diets)	*Mbb*. *sp*. YE303, *M. bryantii* YE299	neg
Bovine RF pooled from 10 dairy cows (DPI VIC Tannin trial, control diet)	*Mbb*. *sp*. YE303, *M. bryantii* YE299	neg
Bovine faecal samples pooled from 10 dairy cows (DPI VIC Tannin trial, control diet)	*Mbb*. *sp*. YE303, *M. bryantii* YE299	neg

Table 4. Results of experiments including a pre-enrichment step detailing source samples used for culture enrichment and methanogens tested with enriched material (neg = negative result for phage isolation). Plaque-like clearings were excised from the isolation plates indicated (*) and further testing undertaken to reproduce this result.

Source sample	Methanogens tested	Phage isolation
Bovine rumen fluid (single steer, Mitchell grass diet 10/6/09)	*Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063	neg
Bovine rumen fluid (single steer, Mitchell grass diet, 22/6/09)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861*	neg
Grantham Piggery effluent pond (sludge)	*Mbb. sp*. YE301, *Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861	neg
Elanora Wastewater Treatment Plant (Gold Coast Water), anaerobic digester	*Mbb. smithii* DSM861	neg
Bovine RF pooled from 4 steers, Hay diet	*Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *Mbb. ruminantium* ATCC35063*	neg

Table 5. Results of experiments to test viral concentrates, detailing source samples from which concentrates were prepared and methanogens tested (neg = negative result for phage isolation). Plaque-like clearings were excised from the isolation plates indicated (*) and further testing undertaken to reproduce this result.

Source sample (concentrate)	Methanogens tested	Phage isolation
Bovine rumen fluid (single steer, Mitchell grass diet)	*Mbb*. *sp*. YE303*, *Mbb*. *sp*. YE286, *M. bryantii* YE299*, *Mbb. ruminantium* ATCC35063	neg
Bovine rumen fluid (single steer, Mitchell grass diet, 22/6/09)	*Mbb*. *sp*. YE301, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. ruminantium* ATCC35063, *Mbb. smithii* DSM861*	neg
Elanora Wastewater Treatment Plant (Gold Coast Water), anaerobic digester	*Mbb*. *sp*. YE303*, *M. bryantii* YE299, *Mbb*. *sp*. YE286, *Mbb. smithii* DSM861*, *Mbb. ruminantium* ATCC35063	neg
Elanora Wastewater Treatment Plant (Gold Coast Water), influent (raw sewage)	*Mbb*. *sp*. YE301, *Mbb*. *sp*. YE303, *Mbb*. *sp*. YE286, *M. bryantii* YE299, *Mbb. smithii* DSM861, *Mbb.* spp. YE314, *Mbb.* spp. YE315, *Mbb.* spp. YE316	neg
Ovine RF, pooled from 4 sheep on 2 diets (2 sheep on lucerne and 2 sheep on speargrass diets)	*Mbb*. *sp*. YE303, *M. bryantii* YE299	neg
Bovine RF pooled from 10 dairy cows (DPI VIC Tannin trial, control diet)	*Mbb*. *sp*. YE303*, *M. bryantii* YE299*	neg

Table 6. Results of experiments incorporating an increased phage adsorption time, to test source samples (with or without virus particle concentration), and methanogens employed in the testing procedure (neg = negative result for phage isolation). Plaque-like clearings were excised from the isolation plates indicated (*) and further testing undertaken to reproduce this result.

Source sample	Methanogens tested	Phage isolation
Ovine RF, pooled from 4 sheep on 2 diets (2 sheep on lucerne and 2 sheep on speargrass diets)	*Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb*. spp. YE315	neg
Ovine RF, pooled from 4 sheep on 2 diets (2 sheep on lucerne and 2 sheep on speargrass diets), virus concentrate	*Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb*. spp YE315	neg
Bovine RF pooled from 10 dairy cows (DPI VIC Tannin trial, control diet)	*Mbb*. *sp*. YE303, *M. bryantii* YE299, *Mbb*. spp. YE315	neg
Bovine RF pooled from 10 dairy cows (DPI VIC Tannin trial, control diet), virus concentrate	*Mbb*. *sp*. YE303, *M. bryantii* YE299*, *Mbb*. spp. YE315	neg
Bovine faecal samples, pooled from 10 dairy cows (DPI VIC Tannin trial, control diet)	*Mbb*. *sp*. YE303*, *M. bryantii* YE299*	neg
Bovine faecal samples, pooled from 10 dairy cows (DPI VIC Tannin trial, control diet) virus concentrate	*Mbb*. *sp*. YE303, *M. bryantii* YE299	neg

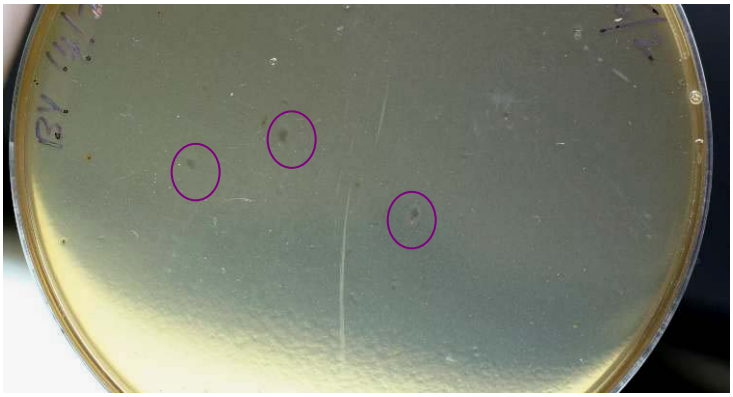

Figure 2. Example of plaque-like clearing zones (circled) observed in layers of confluent growth of the methanogen host strain *Methanobacterium bryantii* YE299. Source sample tested was a bovine RF sample pooled from 10 dairy cows (DPI VIC).

4.2 Plaque assay method validation

The concentration and structural integrity of phage particles from animal-derived source samples was checked using Transmission Electron Microscopy (TEM), to ensure that phage particles were not being damaged or excluded during steps such as filtration (for example, through 0.45 µm and 0.22 µm HV filter units) and concentration by ultracentrifugation. TEM showed that many intact phage particles, representing various viral morphotypes were present in samples used for plaque assays, such as bovine rumen fluid samples obtained from DPI VIC (Figures 3. and 4.). Virus or phage particle morphotypes observed by TEM included tailed phage, icosahedral particles and filamentous particles. On occasion, damaged particles were observed, particularly tailed phage particles where the nucleic acid was absent from the particle head structure with the phage tail structure still attached. Particles damaged in this way were easily distinguished as the negative stain pooled in the cavity of the empty head structure resulting in heavy (dark) staining of the head. Undamaged virus particles and head structures were visualised as brighter structures against the darker, negatively stained background. Some particle damage would be expected to occur in phage preparations obtained directly from the environment and was not considered to be occurring as a consequence of sample preparation.

In the absence of an archaeal phage:host system to be used as a positive control for the phage plating methodology, a rumen phage:bacterial host system (φRa02 of *Ruminococcus albus* AR67) was employed (Klieve *et al.* 2004). Use of this phage:host system verified that there were no simple technical problems being experienced with our plating methodology, with φRa02 plaque formation clearly observed within layers of confluent growth of *R. albus* AR67 (Figure 5).

To further test the viability of source samples, an experiment was undertaken to isolate viable phage from source samples previously tested for the presence of archaeaphage. This was undertaken using the alternative bacterial host *R. albus* (AR67). Three phage concentrate samples were chosen for investigation, a sample of rumen fluid pooled from 10 dairy cows during the control period of the DPI VIC Tannin-feeding trial (obtained from Peter Moate, Tannin-feeding trial, control adaptation period sample point, 1/9/2010); a sample of faeces pooled from the afore-mentioned dairy cows; and a sample of rumen fluid pooled from 4 sheep (obtained from Simon Quigley, BSC 0071 Sheep feed intake regulation trial, 29/6/2010). While more plaque-like clearing zones were observed for the sheep rumen fluid sample, both rumen fluid-derived samples produced phage plaques in confluent layers of *R. albus* AR67 and seven plaques were picked for further analysis. One example, designated φAR67 149-115-2, was purified by repeated plaque assay (Figure 6.) and high titre stocks prepared and stored for future analysis and identification.

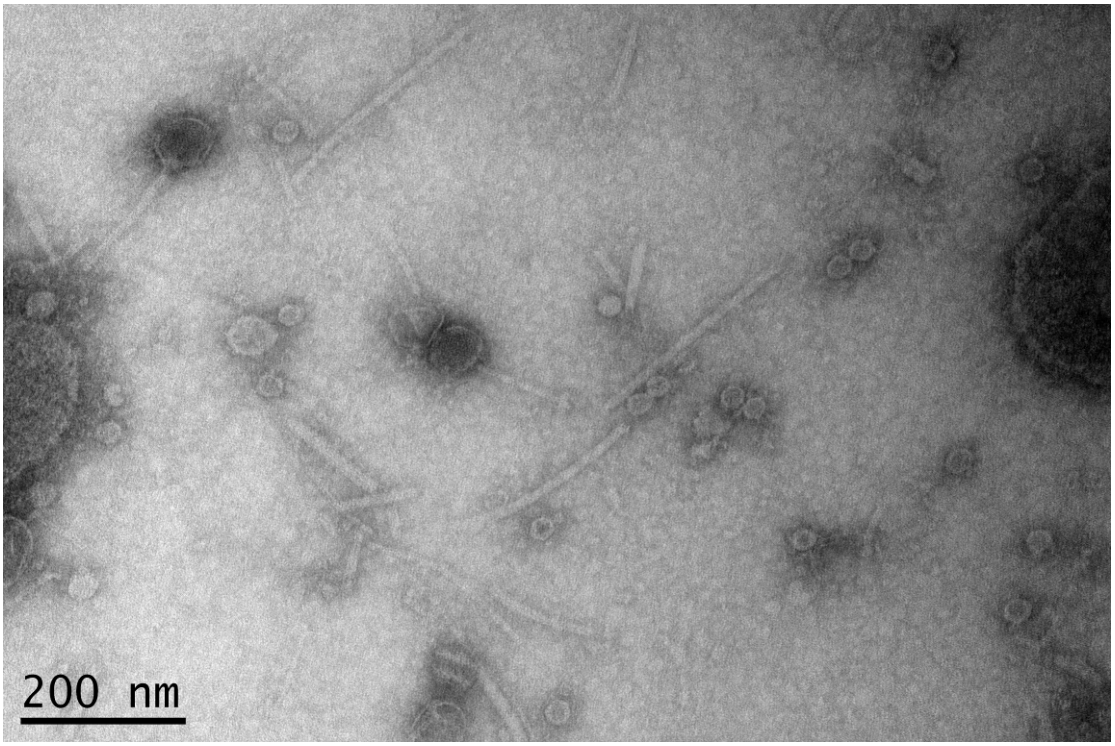

Figure 3. TEM image of bovine rumen fluid concentrate sample showing virus particle integrity and diversity, size bar 200 nm. (Image obtained with assistance from Dr Kathy Parmenter, DEEDI).

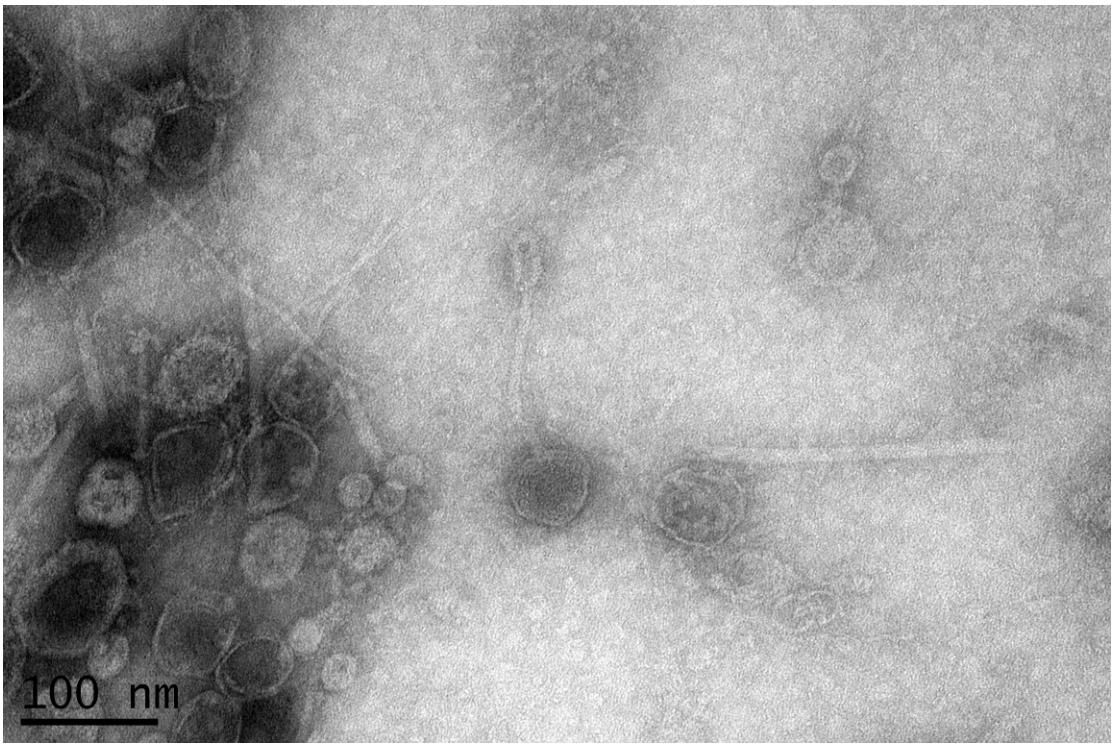

Figure 4. TEM image of bovine rumen fluid concentrate sample showing virus particle integrity and diversity, size bar 100 nm. (Image obtained with assistance from Dr Kathy Parmenter, DEEDI).

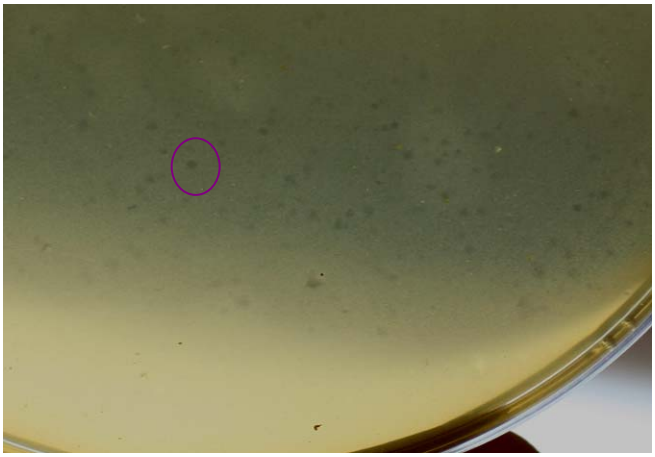

Figure 5. Plaque formation by φRa02 infecting the bacterial host *Ruminococcus albus* AR67 within an agar overlay (10^{-5} dilution of high titre phage stock, example plaque circled).

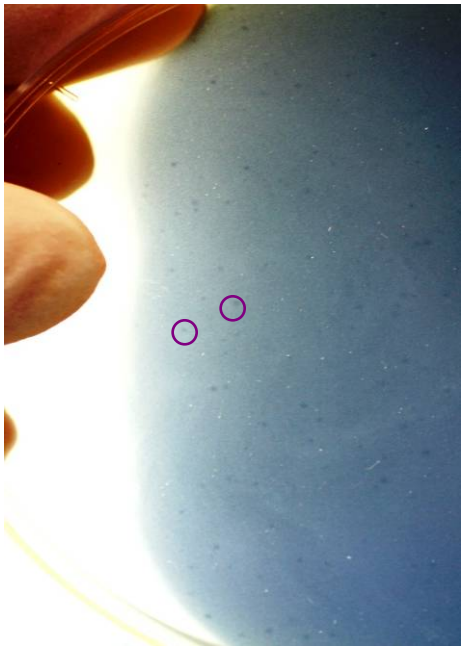

Figure 6. Plaque assay of the ovine rumen fluid-derived phage φAR67 149-115-2, producing small plaques in a confluent layer of *R. albus* AR67, example plaques circled.

4.3 Isolation of new methanogens

Several new methanogen strains were isolated from environmental source samples to be used as host strains in phage isolation experiments. Of these strains, three new methanogen isolates obtained from bovine rumen fluid collected from a steer maintained on a low quality hay diet, were characterized using molecular techniques to enable species identification. These strains, laboratory designation YE314, YE315 (Figure 7.) and YE316, were found to be closely related strains of *Methanobrevibacter* spp., related to both the *Mbb. smithii* PS and *Mbb. thaueri* CW type strains (98.1 % and 97.2 % similarity respectively over 873 bp). Further characterisation using molecular fingerprinting (Wright and Pimm, 2003) indicated that the *Hae* III digests of partial 16S rRNA gene from the new isolates (Figure 8.) were identical to that obtained for the *Methanobrevibacter sp*. isolate NZSM9 (Dr Li Hua Zhang, *unpublished results*). All new isolates were found to be able to grow as confluent layers within agar overlays and were employed in plaque assay experiments and tested for the presence of integrated phage by mitomycin C induction (results detailed in detection of lysogenic phage section below).

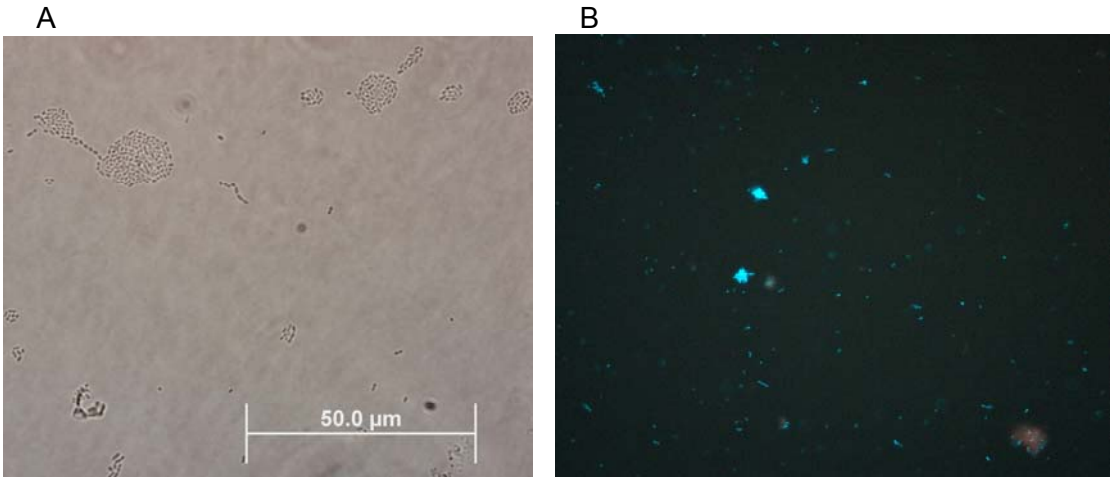

Figure 7. Phase contrast microscopy of *Methanobrevibacter* sp. isolate YE315 displaying a clumping growth habit; **A** visualized using an Olympus BX41 Microscope, 1000x oil immersion objective (image obtained with assistance from Dr Lisa Gulino); and **B** showing autofluorescence (appearing as light blue) typical of methanogenic archaea 400x (image taken using a Nikon Eclipse 80i Microscope, DAPI filter with UV excitation).

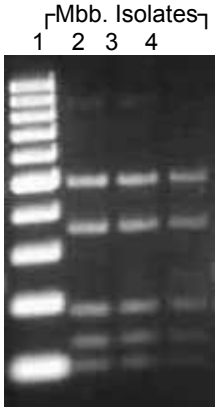

Figure 8. Molecular fingerprinting using *Hae* III digestion of 16S rRNA gene fragments amplified from genomic DNA from new isolates, Lanes 2, 3 and 4 represent new bovine isolates of *Methanobrevibacter* spp. designated YE314, YE315 and YE316, respectively. Lane 1, GeneRuler™100 bp DNA ladder.

4.4 Methanogen host strain analysis: detection of lysogenic phage and CRISPR/*Cas* sequences

To determine the extent to which lysogenic phage exist within methanogens employed in phage isolation experiments, cultures of known methanogens were treated with the inducing agent mitomycin C and DNA from extracellular particles less than 0.22 µm in size extracted. In addition, a phage induction bacterial control was employed, to verify that the mitomycin C-based methodology and following phage extraction protocols were working effectively. This control system involved chemically inducing the phage φAR29 from the rumen bacterial host, *P. ruminicola* ss *brevis* AR29 with mitomycin C.

The majority of methanogens tested did not produce extracellular DNA and in contrast to experiments with the rumen bacterial lysogenic phage:host system of *P. ruminicola* ss *brevis* AR29, there was generally little evidence of mitomycin C treatment resulting in the chemical induction and increased production of archaeaphage or phage-like particles. Initial experiments using small volume (10 ml) cultures and conventional agarose gel electrophoresis, did however indicate that extracellular DNA, possibly resulting from phage-like-particles (PLPs), could be isolated from several of the methanogen tested including *Mbb. sp.* YE302, YE303, YE304, and *Mbb. ruminantium* ATCC35063. DNA samples extracted from these *Methanobrevibacter* strains was tested using the restriction enzyme *Hind* III in order to determine if a distinct pattern of DNA size fragments could be obtained. Obtaining a distinct pattern could be used to indicate whether the extracted DNA arose from PLPs (resulting in a distinct size fragment banding pattern) or remnants of bacterial DNA, possibly contained in extracellular vesicles less than 0.22 µm in size (resulting in an indistinct smear of randomly-fragmented DNA). For most methanogens producing extracellular DNA, restriction enzyme treatment did not cut effectively when used according to manufacturer's instructions, indicating that the enzyme may be inhibited by contaminants within the DNA extract, the DNA obtained may be highly methylated, or the extracellular DNA extracted was not viral in origin.

In contrast to these results, cultures of the three newly isolated strains of *Methanobrevibacter* spp. designated YE314, YE315 and YE316 produced

extracellular DNA, even in the absence of mitomycin C treatment. When this DNA was tested with the restriction enzyme *Hind* III, a distinct banding pattern was obtained (Figure 9.). The Genomiphi kit (GE Healthcare) was therefore employed to randomly amplify and increase the amount of DNA available for cloning purposes, however this cloning process was not successful.

PFGE analysis (Figure 10.) of extracellular DNA extracted from large volume (100 ml) methanogen cultures, indicated that some methanogens, for example, *Methanobrevibacter sp*. YE303 and *Methanobrevibacter* spp. YE315, produced high molecular weight extracellular DNA within the expected size range for tailed phage (30-100 kbp). Whether this extracellular DNA is of viral origin requires further verification. Methanogens which produced a positive result for extracellular DNA in both small and large cultures as confirmed by PFGE analysis and/or produced a distinct banding pattern following restriction enzyme digestion were confirmed as positive for the production of PLPs or extracellular vesicles (Table 7.).

Table 7. Methanogens producing extracellular DNA (+) from PLPs or extracellular vesicles less than 0.22 µm in size, in the absence of chemical induction.

Methanogen tested	Production of extracellular DNA
Mbb. sp. YE301	neg
Mbb. sp. YE302	neg
Mbb. sp. YE303	+
Mbb. sp. YE304	neg
M. bryantii YE299	neg
Mbb. ruminantium YE286	neg
Mbb. smithii DSM861	neg
Mbb. ruminantium ATCC35063	neg
Mbb. spp. YE314	+
Mbb. spp. YE315	+
Mbb. spp. YE316	+

Interestingly, cultures of the methanogen type strains *Mbb. ruminantium* M1 (ATCC35063) and *Mbb. smithii* (DSM861), which are known to contain prophage sequences within their genomic DNA, did not produce easily distinguished DNA bands on PFGE gels. This finding suggests that the prophage integrated within the methanogen genome is not sensitive to chemical induction with mitomycin C and PLPs are produced either in concentrations undetectable with the methodology employed or PLPs with intact phage genetic material are not produced by the host methanogens i.e. the integrated prophage is defective and does not form intact phage particles.

Initial TEM of a sample prepared from the methanogen YE315 in the absence of any chemical induction, indicated the presence of low concentrations of phage particle heads, approximately 60 nm in diameter (Figure 11.). In addition darkened empty particles, characterised by their retention of the TEM negative stain were visible, again in very low concentrations. TEM was repeated with a second batch of YE315 cultures, however no phage particles were visualised using this preparation. With the development of the epifluorescent microscopy method towards the end of the project, testing of YE315 cultures indicated the presence of extremely small particles fluorescing within the culture medium (Figure 17.).

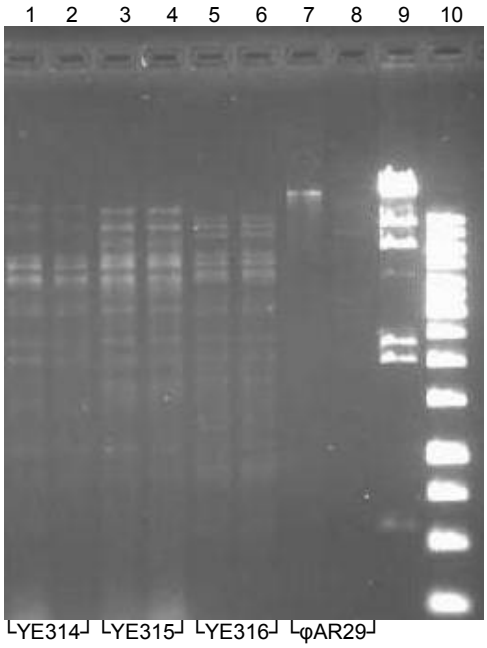

Figure 9. Restriction enzyme (*Hind* III) digests of extracellular DNA prepared from *Methanobrevibacter* spp. strains YE314, YE315 and YE316, with or without mitomycin C treatment, showing distinct banding patterns. The lysogenic phage φAR29 was used as a positive control (lanes 7 and 8). DNA size ladders, lanes 9 and 10, Lambda Hind III digest (NEB) and 10 kb ladder (Fermentas).

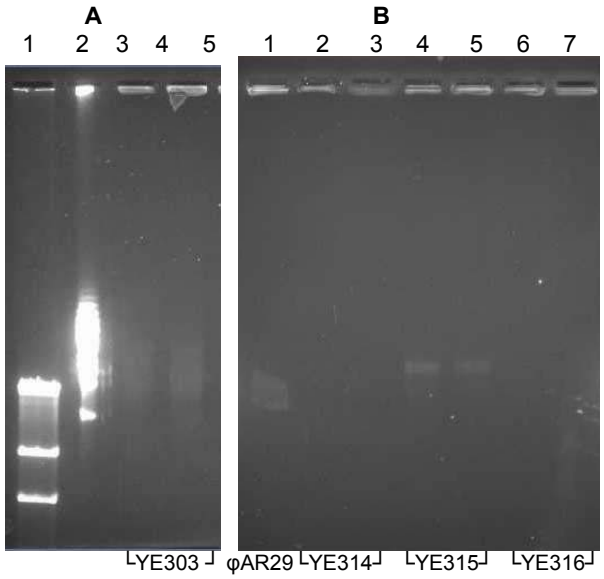

Figure 10. PFGE of extracellular or PLP DNA of approximately 25 - 100 kb size range, obtained from large cultures of **A** *Methanobrevibacter sp*. YE303. Lane 1, Lambda DNA HindIII Digest DNA size marker (NEB); lane 2, MidRange I PFG Marker (NEB); lane 3, YE303 control culture; lane 4, YE303 Mitomycin C treatment; **B** *P. ruminicola* AR29 (φAR29) control lane 1; lane 2. *Mbb*. spp. YE314 control culture; lane 3. *Mbb.* spp. YE314 mitomycin C treatment culture; lane 4. *Mbb.* spp. YE315 control culture; lane 5. *Mbb.* spp. YE315 mitomycin C treatment culture; lane 6. *Mbb.* spp. YE316 control culture; lane 7. *Mbb. sp*. YE316 mitomycin C treatment culture.

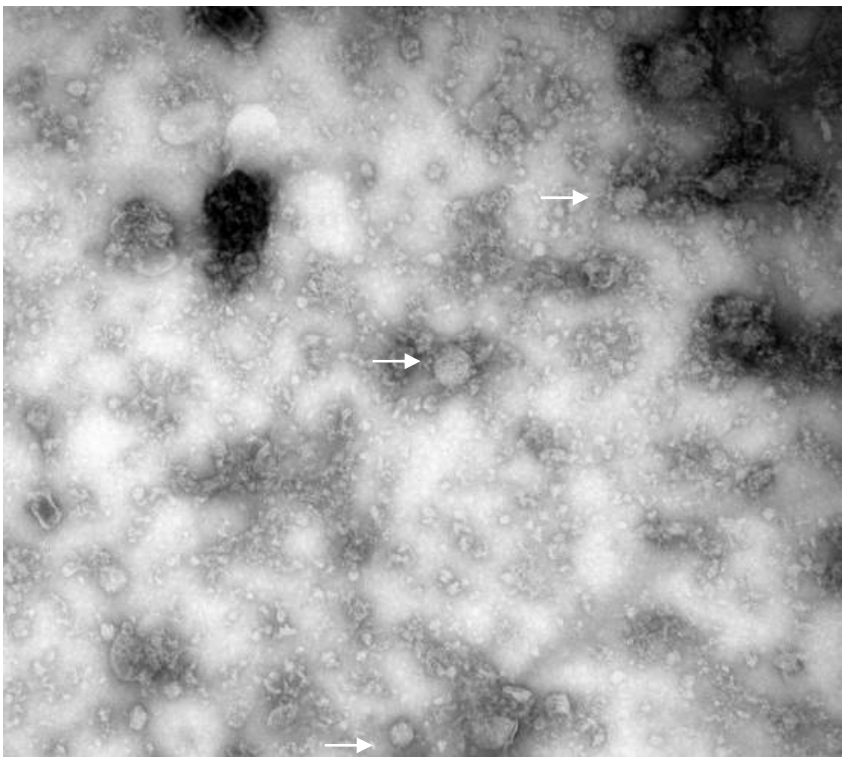

Figure 11. TEM of sample prepared from *Methanobrevibacter* sp. YE315, possible phage particles indicated with arrows. (Image obtained with the assistance of Dr Kathy Parmenter, DEEDI Plant Virology Group).

Genome sequences were only available for four of the methanogen strains being used in phage isolation experiments, however each of these strains were found to contain CRISPR sequences and many *cas* proteins (Tables 8. and 9.). Both of the methanogen type strains *Mbb. ruminantium* M1 (ATCC35063), and *Mbb. smithii* (ATCC35061) have been shown to contain prophages (Leahy *et al.* 2010; Hansen *et al.* 2011) and have extensive regions of CRISPR/*cas* loci in their genome. Complete prophage sequences have not been identified in the draft genome sequence obtained for the strains *Mbb. ruminantium* YE286 or *M. bryantii* YE299, however both genomes were found to contain CRISPR-like sequences and *cas* proteins, with YE299 having a greater number of CRISPR sequences in the genome, including sequences which were found to be similar to previously reported *cas* proteins.

In addition, using the CRISPR database, the predominance of CRISPR in other methanogens, particularly type strains similar to or representative of those found in the rumen microbial ecosystem for which complete genome sequences were publically available, were examined *in silico* (Table 10.). This analysis showed that the majority of methanogen type strains have regions of CRISPR sequences in their genomes, indicating a history of phage infection within populations of these organisms.

Table 8. Presence of CRISPR sequences and *cas* proteins within the draft genomes of methanogens employed in plaque assay experiments, using the CRISPR finder program (http://crispr.u-psud.fr/ Grissa *et al.* 2007a). Questionable CRISPR sequences are either small CRISPRs, i.e structures having only two or three direct repeats or similar structures (like particular kinds of tandem repeats) or structures where the repeated motifs (direct repeats in CRISPR) are not 100% identical. (nd = not determined)

Methanogen	Number of questionable CRISPR sequences	Number of cas proteins
Mbb. ruminantium YE286	3*	16
M. bryantii YE299	20#	nd

*YE286 CRISPR sequence 2 (position 1416859-1416948) was similar to *Methanococcus vannielii* A6UNC6_METVS CRISPR locus-related DNA binding protein.
YE299 CRISPR sequence 14 (position 3161110-3161211) translated protein sequence was similar to the hypothetical protein Metbo_1511 *Methanobacterium sp*. AL-21 conserved repeat domain protein.

Table 9. Presence of CRISPR-associated (*cas*) proteins referenced in NCBI blast (http://blast.ncbi.nlm.nih.gov/ Altschul *et al.* 1990), encoded by type strain methanogens employed in plaque assay experiments (http://crispr.u-psud.fr/ Grissa *et al.* 2007a).

Methanogen	CRISPR associated protein referenced in Genbank file	Genome sequence position	
		Start	End
Mbb. ruminantium M1 ATCC35063	CRISPR-associated protein Cas6	1023766	1024497
	CRISPR-associated protein CT1132 family	1026658	1027617
	CRISPR-associated protein Cas5 Hmari subtype	1027652	1028515
	CRISPR-associated helicase Cas3	1028497	1031322
	CRISPR-associated protein Cas4-1	1031319	1031825
	CRISPR-associated protein Cas1-1	1031886	1032854
	CRISPR-associated protein Cas2-1	1032857	1033123
	CRISPR-associated protein Cas1-2	1554122	1555126
	CRISPR-associated protein Cas2-2	1555431	1555709
	CRISPR-associated protein TIGR02710 family	1557620	1558987
	CRISPR-associated RAMP protein Csm5 family	1559080	1560282
	CRISPR-associated RAMP protein Csm4 family	1560279	1561274
	CRISPR-associated RAMP protein Csm3 family	1561365	1562132
	CRISPR-associated protein Csm2 family	1562146	1562577
	CRISPR-associated protein Csm1 family	1562582	1565239
	CRISPR-associated protein TIGR02710 family	1577736	1579025
	CRISPR-associated protein Cas4-2	2012900	2013595
	CRISPR-associated protein Cas1-3	2136838	2137113
	CRISPR-associated protein Cas1-4	2137148	2137843
	CRISPR-associated protein Cas2-3	2137856	2138131
Mbb. smithii DSM861	Putative CRISPR-associated protein	161313	162671

Table 10. Presence of CRISPR sequences within the genomes of methanogen type strains originally isolated from the rumen or similar to those found in the rumen (*) detailed in the CRISPRdb, http://crispr.u-psud.fr/crispr/ (Grissa *et al.* 2007b).

Methanogen type strain	Confirmed CRISPR sequences	Corresponding number of spacers
Mbb. ruminantium M1 (ATCC35063)	NC_013790_17	61+
	NC_013790_23	11+
	NC_013790_24	38+
Mbb. smithii (DSM861)*	NC_009514_2	43+
Methanosphaera stadmanae (DSM 3091)*	NC_007681_1	61+
	NC_007681_2	56+
	NC_007681_3	4+
Methanobacterium sp. AL-21*	NC_015216_1	7+
Methanospirillum hungatei JF-1*	NC_007796_1	30+
	NC_007796_2	7+
	NC_007796_3	37+
	NC_007796_4	3+
	NC_007796_5	79+
	NC_007796_6	43+
	NC_007796_7	65+
*Methanosarcina barkeri str. fusaro**	NC_007355_11	18+
	NC_007355_3	50+
	NC_007355_32	24+
	NC_007355_4	3+
Methanosarcina mazei Go1*	NC_003901_10	80+
	NC_003901_2	1+
	NC_003901_3	1+
	NC_003901_4	46+

4.5 Method development: PCR methods for the detection of archaeaphage genes and epifluorescent microscopy

Two sets of PCR primers were developed to amplify adjoining regions of the phage-encoded lytic enzyme *Pei*R, previously identified during sequence analysis of the integrated *Mbb. ruminantium* M1 (ATCC35063) phage, φmru (Leahy *et al.* 2010). The first primer pair (PeiR453059F and PeiR453394R), were designed to amplify a 356 bp fragment covering the putative active site of the protein. The second primer pair (PeiR453393F and PeiR453555R), were designed to amplify a shorter, 163 bp fragment, covering a section of the gene encoding for protein with similarity to conserved domains of the peptidase C39-like superfamily. PCR testing of both primer sets with genomic DNA of the *Mbb. ruminantium* M1 (ATCC35063) type strain resulted in the amplification of the DNA fragments corresponding to the predicted PCR product sizes (Figure 12.).

Genomic DNA extracted from all archaeal strains available within our laboratory, including new methanogen isolates was also tested using both sets of PCR primers. With the exception of the positive control *Mbb. ruminantium* M1 (ATCC35063), all methanogens tested were found to be PCR-negative for both fragments of the *Pei*R gene (Table 11.). This result indicates that the methanogen strains examined may not contain a phage such as φmru or phages closely related to φmru which may also utilise the *Pei*R lytic enzyme, integrated into their genome. This PCR was also applied to genomic DNA extracted from bovine rumen fluid and bovine faecal samples obtained from four individual dairy cows (obtained courtesy of Dr Peter Moate, DPI Vic), with no animal-derived samples positive for the *Pei*R gene.

PCR primer sets were also developed to amplify prophage terminase large sub-unit genes from the *Mbb. smithii* type strain (DSM861) and the *Mbb. ruminantium* type strain (ATCC35063). The PCR primers developed were all able to detect the respective prophage terminase gene fragments in genomic DNA prepared from the corresponding methanogen type strains (Figure 13.). No other methanogens tested yielded a positive PCR result for these terminase gene fragments under standard PCR conditions (Table 12.) When genomic DNA extracted from bovine rumen fluid and faecal samples obtained from four dairy cows were tested, all animal-derived samples were PCR negative for these terminase gene fragments.

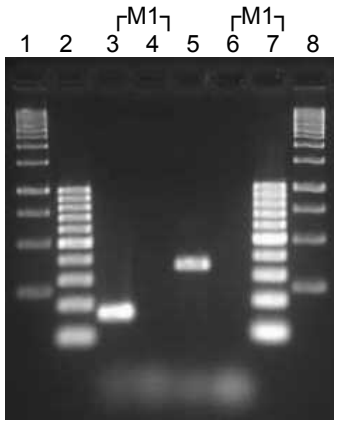

Figure 12. PCR detection used to amplify either a163 bp or 356 bp fragment of the φmru-encoded lytic enzyme *Pei*R, using *Mbb. ruminantium* M1 (ATCC35063) genomic DNA as template and the primer pairs PeiR453393F and PeiR453555R (lane 3) and PeiR453059F and PeiR453394R, (lane 5). Lanes 4 and 6 represent PCR reactions using *Mbb. smithii* (DSM861) genomic DNA as template and the primer pairs PeiR453393F, PeiR453555R and PeiR453059F, PeiR453394R, respectively. The GeneRuler™ 1kb DNA ladder (lanes 1 and 8) and GeneRuler™100 bp DNA ladder (lanes 2 and 7) were used as DNA size markers.

Table 11. PCR detection of φmru *Pei*R gene using genomic DNA extracted from pure cultures and environmental source material, positive results indicated (+).

Test sample (genomic DNA)	PCR Primer set	
	PeiR453059F and PeiR453394R	**PeiR453393F and PeiR453555R**
Mbb. sp. YE301	neg	neg
Mbb. sp. YE302	neg	neg
Mbb. sp. YE303	neg	neg
M. ruminantium YE299	neg	neg
Mbb. sp. YE286	neg	neg
Mbb. spp. YE315	neg	neg
Mbb. ruminantium M1 (ATCC35063)	**+**	**+**
Mbb. smithii (DSM861)	neg	neg
Escherichia coli K12	neg	neg
Bovine RF (4 cows)	neg	neg
Bovine Faeces (4 cows)	neg	neg

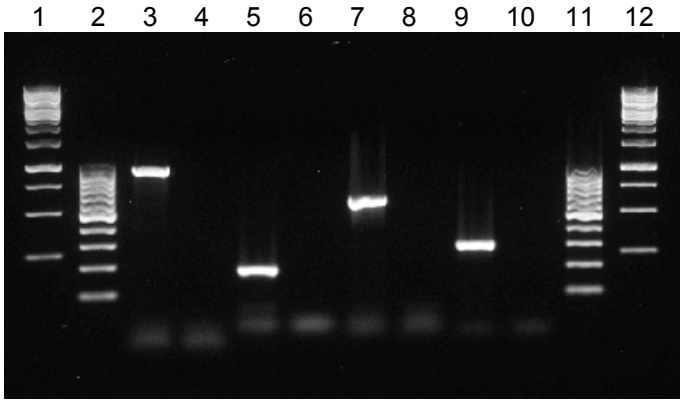

Figure 13. Example of PCR results, terminase primer sets tested with respective methanogen type strains. Lanes 3 and 5, primer sets φmruterm1 and φmruterm2 tested with *Mbb. ruminantium* M1 (ATCC35063) genomic DNA respectively and lanes 7 and 9, primer sets Msmterm1 and Msmterm2 tested with *Mbb. smithii* (DSM861) genomic DNA respectively. Lanes 4, 6, 8 and 10 PCR negative control reaction for each primer set (H_2O blank). Lanes 1 and 12, GeneRuler™ 1kb DNA ladder; lanes 2 and 11, GeneRuler™100 bp DNA ladder.

Table 12. PCR detection of *Mbb. smithii* (DSM861) prophage and φmru terminase genes using genomic DNA extracted from pure cultures and environmental source material, PCR positive results indicated (+).

	PCR Primer set			
Test sample (Genomic DNA)	**φmruterm1**	**φmruterm2**	**Msmterm1**	**Msmterm2**
Mbb. sp YE301	neg	neg	neg	neg
Mbb. sp. YE302	neg	neg	neg	neg
Mbb. sp. YE303	neg	neg	neg	neg
M. ruminantium YE299	neg	neg	neg	neg
Mbb. spp. YE315	neg	neg	neg	neg
Mbb. ruminantium M1 (ATCC35063)	**+**	**+**	neg	neg
Mbb. smithii (DSM861)	neg	neg	**+**	**+**
Escherichia coli K12	neg	neg	neg	neg
Bovine RF (4 cows)	neg	neg	neg	neg
Bovine Faeces (4 cows)	neg	neg	neg	neg

In order to better detect and enumerate phage particles in pure cultures and environmental samples, a method originally developed for the enumeration of phage particles in aquatic ecosystems (Chen *et al.* 2001) and more recently applied to the enumeration of phage and viruses in environmental samples being prepared for metagenomic analysis (Thurber *et al.* 2009), was adopted in our laboratory. This method involved capturing the phage particles onto a specialized filter which was then stained with a nucleic acid dye (SYBR gold). Phage particles may then be visualized using a fluorescent microscope and counted.

The virus:host system initially chosen for the development of this method was the rumen bacterium *P. ruminicola* AR29 which is host to a lysogenic phage, φAR29 (Klieve *et al.* 1989). Cultures of AR29 were induced with mitomycin C to provide samples containing (a) a mixture of both bacterial and phage particles (Figure 14.) and (b) phage particles only. In addition, samples from an *in vitro* fermenter were examined using conventional phase contrast microscopy to visualize micro-organisms present and UV excitation with dark field to visualize microbes (such as methanogens) with autofluorescence (Figure 15.). This same sample was then used with the epifluorescent method, firstly with whole fermenter contents (sample containing bacteria, archaea, viruses and remnants of feed material), with virus particles clearly visible. A 0.22 µm filtered preparation of the fermenter contents with the plant debris and microbes removed and containing only virus particles (usually < 0.22 µm in size) was also examined (Figure 16.) In addition, a culture of the new methanogen isolate *Mbb* spp. YE315 was examined using this methodology. For this methanogen, a milky background of DNA-containing particles (pin-pricks of SYBR-Gold stained material) derived either from extracellular vesicles or PLPs was observed (Figure 17.).

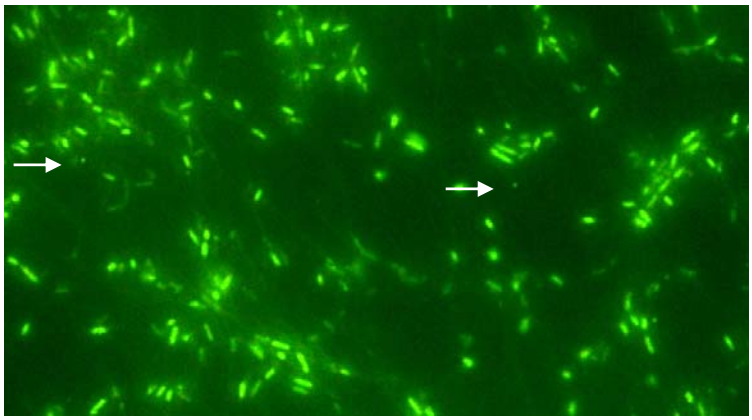

Figure 14. Epifluorescence microscopy of a *P. ruminicola* AR29 culture induced with mitomycin C, captured on a 0.02 µm Anodisc filter and stained with SYBR gold, 1000x image. Sample contains both *P. ruminicola* AR29 cells φAR29 particles (arrow indicating phage particles).

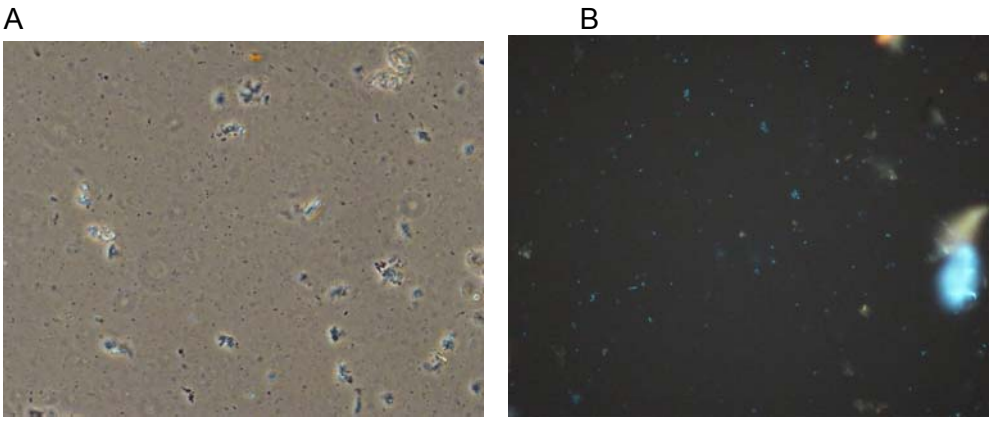

Figure 15. Development of epifluorescent microscopy methods, examination of rumen fermenter fluid; **A** microbial diversity, phase contrast 400x image; **B** same slide as image A, showing natural fluorescence of a small proportion of microbes (possibly methanogens) and plant material. UV excitation with dark field filter, 400x image, Fermenter fluid obtained from Emilio Martinez (Lipids project B.CCH.1014).

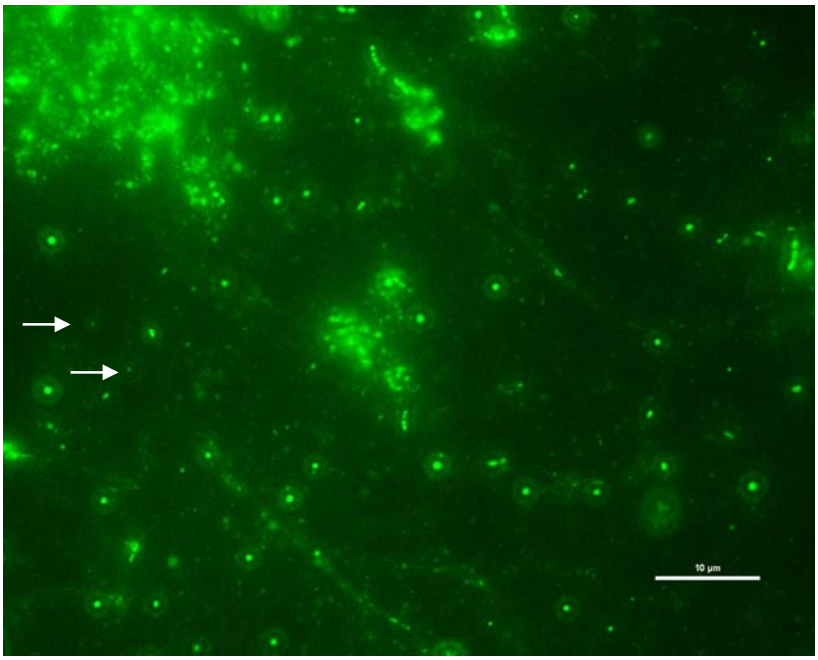

Figure 17. Epifluorescent microscopy of *Methanobrevibacter* spp. YE315 culture trapped onto a 0.02 µm anodisc filter, stained with SYBR gold showing stained microbes and possible viral particles (milky background of pin-prick phage particles, examples indicated with an arrow), FITC green excitation,100x oil immersion lens, 1000x magnification.

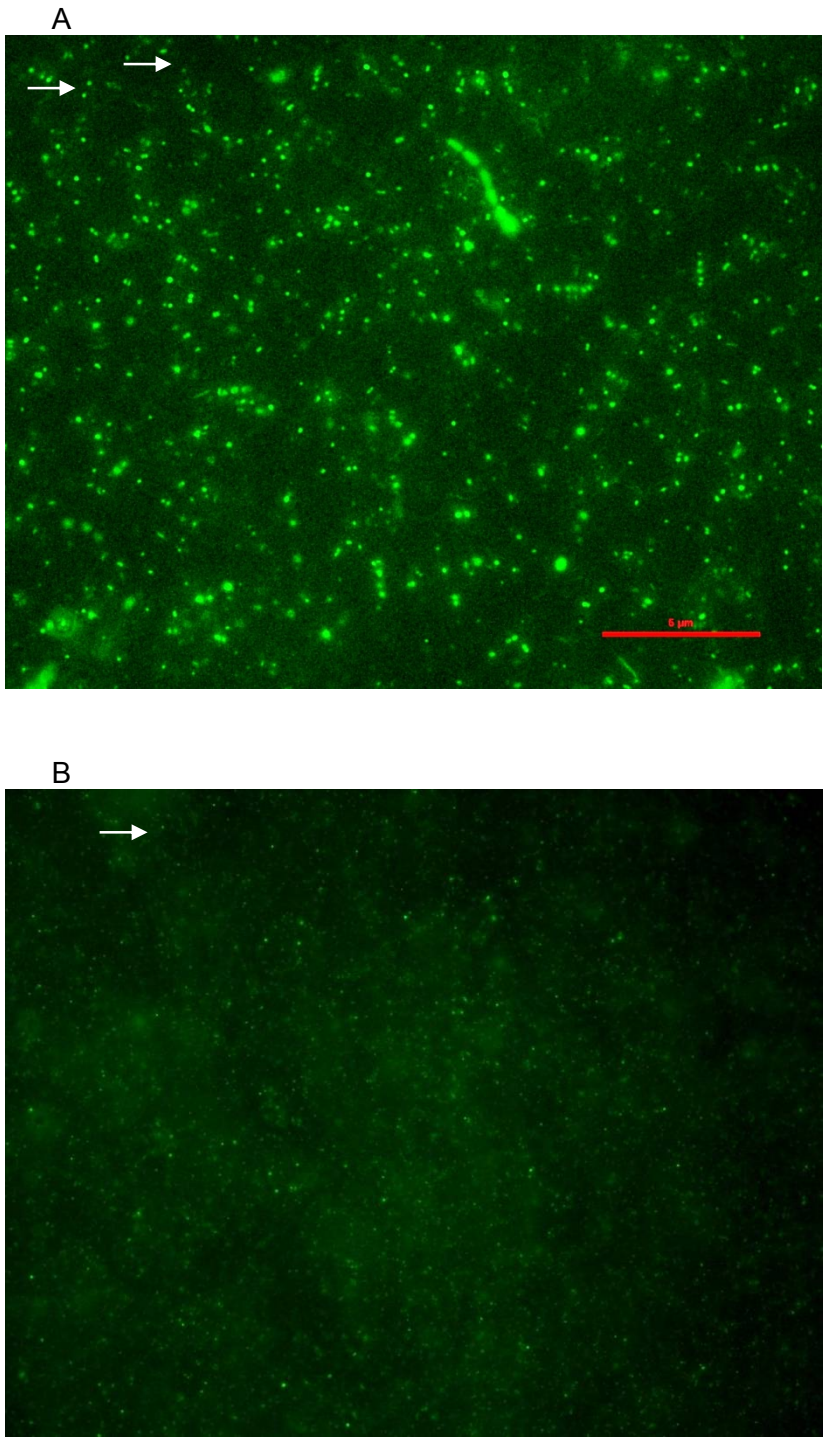

Figure 16. Development of epifluorescent microscopy methods, examination of rumen fermenter fluid; **A** 1000x image of whole fermenter fluid (100 µl) trapped onto a 0.02 µm anodisc filter, stained with SYBR gold showing stained microbes and viruses (examples indicated with arrow), FITC green excitation,100x oil immersion objective; **B** 400x image of fermenter fluid (200 µl) pre-treated by filtration through a 0.22 µm filter to remove plant material and microbes, showing many SYBR gold stained viruses, FITC green excitation. Fermenter fluid obtained from Emilio Martinez (Lipids project B.CCH.1014).

5.0 Discussion / Conclusion

The primary objective of the project, to establish a collection of archaeaphage that could infect a range of rumen methanogens, was investigated throughout the duration of the project. The technical approach used was a plaque assay technique similar to that successfully employed in previous investigations undertaken in our laboratory for the isolation of phages which specifically infect rumen bacteria, from the environment (Klieve *et al*. 1991; Klieve, 2005). This plaque assay technique was modified to take into account the specific growth requirements (Gilbert *et al*. 2010) of rumen methanogens and was used to assay a total of 14 different source samples encompassing animal-derived and environmental source samples. It was also employed to replicate the plaque-forming activity occasionally noted on original phage-isolation plates. Up to 10 different methanogens were utilised as potential host strains and the standard procedure modified with several source samples, to increase the efficiency of phage infection (for example, source sample virus concentration, pre-enrichment with methanogen host strains and increasing the length of the phage adsorption time).

Despite these modifications, the plaque assay procedure was unable to reproducibly detect zones of methanogen growth inhibition and lysis in confluent layers of host methanogens (phage plaques), and no lytic phage of methanogens were isolated during the course of the project. The integrity of phage particles contained in source samples was ascertained by TEM and intact virus particles representing a diverse range of virus structures were observed. It was also established that source samples contained populations of viable, infective phage particles, with the successful isolation of several plaque-forming phage able to infect the relatively slow growing, strictly anaerobic rumen bacterium, *R. albus* AR67.

The second objective of the project, to establish “proof of concept” evidence that archaeaphage therapy has viable potential to reduce methane emissions from ruminal fermentation, was not addressed, due to the lack of lytic phage obtained. Interestingly, during the duration of the project, to our knowledge, there were no other reports published in the scientific literature describing the isolation of lytic phage of rumen methanogens, despite this area of research gaining increasing international interest as a possible enteric methane abatement strategy (Buddle *et al*. 2011; Cottle *et al*. 2011). This also limited the further testing of the plaque assay procedure and the subsequent “proof-of concept” studies utilising *in vitro* rumen fermentation systems fitted with methane-measuring equipment, as no methanogen phage:host positive control system was available.

The project also involved the isolation of new methanogens and investigated the presence of lysogenic phage. Lysogenic phage integrate their genomic DNA into the genome of the host methanogen and may be produced in high concentrations following chemical treatment with an inducing agent, such as mitomycin C. Of the 11 methanogens tested only four methanogens were found to produce relatively high concentrations of extracellular DNA as determined by PFGE methodology. This extracellular DNA was produced by each of these methanogens even in the absence of chemical (mitomycin C) induction. In the case of the extracellular DNA extracted from cultures of the new isolates *Mbb*. spp. YE314, YE315 and YE316, digestion with a restriction enzyme produced a clear banding pattern. The formation of a distinct banding pattern suggested that this DNA was not from remnants of the methanogen chromosomal DNA, which may be contained in extracellular vesicles produced by the methanogen, similar to the extracellular DNA transfer systems (or vesicles) reported for the methanogenic archaean *Methanococcus voltae* (Eiserling *et al.* 1999).

Repeated TEM of culture material for the presence of virus particles was inconclusive; epifluorescent microscopy of culture material however, verified the presence of small particles of DNA-based material. Further investigations would be required to validate the presence of complete lysogenic phage genomes (prophage) integrated within the chromosome of the methanogen examined. Complete genome sequencing, such as that undertaken for the methanogen type strains *Mbb ruminantium* M1 (ATCC35063) and *Mbb. smithii* (DSM861), would be the most effective way to determine if the genome of these organisms contained an integrated prophage, particularly in the absence of an effective chemical phage-inducing agent.

The relative difficulty experienced in isolating phages capable of infecting rumen methanogens may be attributed to the biological differences that occur between archaea and bacteria. Relevant differences may include:
(a) the slow growth of methanogens *in vitro* and poor plaque formation;
(b) biological differences, such as differences in host cell wall and other extracellular structures (e.g. pili, flagella) which may act as phage receptors;
(c) the relatively high abundance of genetically-based host defences such as CRISPRs in methanogen host strains used for testing. It has been suggested that archaea may have considerably more CRISPR sequences in their genomes than bacteria with approximately 90% of sequenced archaeal genomes containing at least one CRISPR locus and up to 18 loci per genome (Pina *et al*. 2011; Sorek *et al.* 2008). Despite this high level of sequence-directed immunity, it is possible that when archaeal cells are challenged by an archaeaphage with no match to any CRISPR spacer, they will be unprotected and phage replication may proceed. This has recently been demonstrated in experiments with the hyperthermophilic archaeon, *Sulfolobus solfataricus* (Manica *et al*. 2011) for which a model virus:host system is available.

Genome sequences were only available for four of the methanogen strains being used in phage isolation experiments. Each of these strains was found to contain CRISPR sequences and many *cas* proteins (Tables 8. and 9.). Both of the type strains *Mbb. ruminantium* M1 (ATCC35063), and *Mbb. smithii* (ATCC35061) have been shown to contain prophages (Leahy *et al*. 2010; Hansen *et al*. 2011) and have extensive regions of CRISPR/*cas* loci in their genome. Complete prophage sequences have not been identified in the draft genome sequence obtained for the strains *Mbb. ruminantium* YE286 or *M. bryantii* YE299, however both genomes were found to contain CRISPR-like sequences and *cas* proteins, with YE299 having a greater number of CRISPR sequences in the genome, including sequences which were found to be similar to previously reported CRISPR associated proteins. These sequences will require further analysis to determine their biological and/or genetic significance.

Using the CRISPR database (Grissa *et al*. 2007b), the predominance of CRISPR in other methanogens, particularly type strains similar or representative of those found in the rumen microbial ecosystem, was examined. This analysis showed that the majority of methanogen type strains examined have CRISPR sequences in their genomes, suggesting that finding a methanogen without any pre-existing CRISPR-based immunity is unlikely. This concurs with the findings of researchers examining archaea found in more extreme environments, such as the thermal or highly acidic thermal environments of volcanic hot springs, which have been shown to contain a relatively high abundance of CRISPR in their genomes (Guo *et al*. 2011). It should be noted however, that the extreme environments in which these archaea predominate, also contain a high abundance of diverse, viable archaeaphages (Breitbart *et al*. 2004). This indicates that cycles of phage infection and replication continuously occur despite the presence of CRISPR/cas-mediated immune systems in the host archaeal

populations. Archaeaphage populations are therefore sustained in the environment and may continuously evolve at a genetic level to evade the archaeal host defences.

The phenomenon of CRISPR/*cas* mediated immunity has two main implications for the current study. Firstly, if methanogen host strains used in phage isolation studies have a high proportion of CRISPR/*cas* loci, they may be less susceptible to phage infection and consequently be less useful for the isolation of phages in a plaque assay system. Secondly, the CRISPR spacer regions represent a genetic archive of previous phage attacks, providing sequence information which could potentially be used to identify the types of phages able to infect methanogens. Sequence information of this kind may also facilitate the development of PCR assays for screening of phage infection and determining abundance in environmental samples. the majority of methanogen type strains have regions of CRISPR sequences in their genomes, indicating a history of phage infection within populations of these organisms.

In addition to the investigation of lytic and lysogenic phage of methanogens, the project also incorporated the development of new methodology for the detection and enumeration of archaeaphage in pure cultures of methanogens and environmental and rumen-based source samples. New methodology included the development of PCR primer sets for the detection of archaeaphage genes and epifluorescence microscopy for the improved detection and enumeration of virus-like particles. Archaeaphage genes utilised in the PCR methodology included the *Mbb. ruminantium* M1 φmru-encoded lysis gene *Pei*R and the phage terminase large subunit genes found in the prophage sequences of the methanogen type strains *Mbb. ruminantium* M1 (ATCC35063) and *Mbb. smithii* (DSM861). All of the PCR primer sets designed during the course of the project were able to detect the target genes in the genetic material of the methanogen host strains from which they were derived. They were not however, able to detect respective target genes in genomic DNA of other methanogens (for which genome sequence data is currently unavailable) and in several bovine-derived rumen fluid and faecal samples. Further analysis is required to determine if these archaeaphage genes may be found in the rumen phage population of other animals, including those on different diets and in the wider environment, however initial testing (including primer sequence testing *in silico*) suggests that these genes may be relatively uncommon. The advantage of this finding is that these genes may be employed in sequence-specific detection technologies such as DNA-hybridisation methods and microscopy-based hybridisation methods such as the increasingly sensitive fluorescent *in situ* hybridisation (FISH) based methodology, such as catalysed reporter deposition (CARD) and rolling circle amplification (RCA) FISH (Pernthaler *et al*. 2002; Hoshino and Schramm, 2010).

The new approaches being developed in our laboratory may yield more information regarding the presence of lysogenic phages, genetic remnants of phage infection in laboratory strains of archaea and the concentration of phage particles in environmental samples. The SYBR gold nucleic acid staining of virus particles trapped with the 0.02 µm filter, allowed virus particles to be visualized in a way usually only possible with an electron microscope. It is expected that this method may be used as an alternative to plating methods in the current project for the detection and enumeration of phage particles in (a) infected cultures; (b) chemically induced cultures; and (c) environmental samples. It may also be utilized in future viral metagenome-based projects where sample purity and concentration may need to be ascertained using a rapid, accurate method.

A shift to a metagenomics-based approach, specifically to further characterize the rumen virome, may be required to facilitate the detection and targeted isolation of lytic archaeaphages, which may then be employed in future phage therapy-based enteric methane reduction strategies. Such an approach would also (a) greatly increase the current knowledge-base of archaeaphage and rumen phage populations in general; (b) facilitate the detection of archaeaphage by identifying animal-derived or environmental source samples with high concentrations of archaeaphage; and (c) identify naturally-occurring, archaeaphage-encoded genes with potential anti-methanogen activity.

6.1 Appendix 1. Source Sample Collection

Table A1.1 Animal-derived source samples collected for phage isolation experiments. (Rumen fluid [RF]).

Sample description	Archaeaphage Screening Undertaken
Bovine RF, single steer Mitchell Grass diet, CAAS, UQ Gatton (10/6/09)	+
Bovine RF single steer Mitchell Grass diet, CAAS, UQ Gatton (22/9/09)	+
REU Fermenter, bovine RF inoculum (Mitchell grass 24/9/09)	
REU Fermenter, bovine RF inoculum (Mitchell grass 25/2/10)	
Bovine RF (strained), E. Martinez trial (23/2/10)	
4 x High quality grass	
4 x Low quality grass	+
4 x Grain	
Pooled bovine RF samples for each dietary group, EM trial (23/2/10)	
High quality grass diet	
Low quality grass diet	+
Grain diet	
Multiple bovine RF and faecal samples from Dr Peter Moate Dairy Trial (DPI VIC), various dates (September, 2009)	+
Multiple ovine RF samples (sheep from Simon Quigley BSC 0071 Sheep feed intake regulation trial, UQ), 3 diets, Lucerne, Spear grass, Spear grass + Fish meal (28/610-2/7/10)	+
REU Fermenter, bovine RF inoculum (Mitchell grass 1/7/10)	+
REU Fermenter, bovine RF inoculum (Mitchell grass 10/9/10) samples taken at various time points (methane levels recorded)	+
Bovine RF (strained) 4 steers x high grain diet (Highchester Abattoir, 21/2/11)	
Multiple bovine RF and faecal samples from Dr Peter Moate's Dairy fat/tannin feeding trial (DPI VIC) various	+

dates (February, 2011)	
Multiple bovine RF and faecal samples from Dr Peter Moate's Grapemarc feeding trial Dairy Trial (April, 2011)	
Multiple bovine RF samples from steers collected during the control period of a phosphorous supplementation trial undertaken by Dr Simon Quigley and Professor Dennis Poppi June, 2011 (CAAS, UQ Gatton)	

Table A1.2 Environmental source samples collected for phage isolation experiments.

Sample description	Archaeaphage Screening Undertaken
Grantham (Whitby) Piggery (17/11/09): Whitby effluent pond sludge	+
Whitby composite animal waste	+
Whitby pond effluent	
Luggage Point Wastewater Treatment Plant, DIG sludge	+
Luggage Point Wastewater Treatment Plant, "raw" sewage (influent)	+
Nudgee Landfill (Brisbane City Council) Soil (30 - 40 cm depth, 4/3/10 DF)	
Elanora Waste Water Treatment Plant (Gold Coast Water) influent (raw) sewage (31/3/10)	+
Elanora Waste Water Treatment Plant (Gold Coast Water) anaerobic digester (31/3/10)	+
Agricultural effluent ponds A and B (primarily piggery waste), CAAS, UQ Gatton (17/11/10)	

6.2 Appendix 2 Publications

Peer reviewed conference abstracts

Gilbert, R.A., Ouwerkerk, D., and Klieve, A.V. (2010) Isolation of viruses for bio-control of methanogenic archaea from the rumen. Proceedings of the 28th Meeting of the Australia Society for Animal Production, p. 68.

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