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# Opportunities for biological control of ruminal methanogenesis

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*Abstract.* Methane is a major waste product from ruminants, contributing 53% of Australia's total emissions of the gas. A range of chemical inhibitors of methanogenesis are known to be effective but the real and perceived risks to the environment and health from chemical residues may curtail widespread application of these products in ruminant industries. As with other agricultural industries, future control of unwanted organisms (such as methanogens) is likely to lie with biocontrol agents rather than with chemicals. Opportunities exist for biological reduction of rumen methane emissions using direct means (viruses and bacteriocins specific to methanogens), and indirectly through the elimination of rumen protozoa which symbiotically support some rumen methanogens. The feasibility of these approaches and their current research position are discussed. The most promising work to date is using the common food preservative, nisin (a safe and naturally occurring bacteriocin). When ruminal contents were incubated with nisin *in vitro,* methane emissions were reduced by 36%.

*Additional keywords:* archaea, methane, viruses, reductive acetogenesis, bacteriocin, nisin, protozoa, defaunation.

## Introduction

Global warming due to increases in the atmospheric concentration of gases such as carbon dioxide and methane is an important issue. The generation of methane from livestock industries, and particularly from ruminants, is a significant contribution to the problem. Methane has a greater global warming potential than carbon dioxide and it has been calculated that 53% of Australia's anthropogenic methane emissions (or about 2.8 million tonnes annually) is from livestock (NGGIC 1997).

While control of rumen methanogenesis may be achieved by a range of chemical inhibitors (Van Nevel and Demeyer 1995; McCrabb *et al.* 1997), efficacy of many agents declines with continued or repeated use (Johnson *et al.* 1994; Sauer *et al.* 1998) and there is growing concern about using chemicals in animals destined for human consumption. This is evidenced by the need to test all Australian export beef for chemical and antibiotic residues. Biological control of pests and pathogens is a growing industry in other spheres of agriculture (Rodgers 1993). Host-specific pathogenic protozoa, bacteria, fungi, and rickettsiae are all being evaluated as potential biopesticides for agriculture (e.g. Poinar and Poinar 1998). If appropriate biological control agents for methanogens can be developed they may well aid in reducing methane emissions from the rumen.

Methane is generated in the rumen by methanogenic archaea that utilise $H_2$ to reduce $CO_2$, and is a significant electron sink in the rumen ecosystem. Recently, reductive acetogenesis has been suggested as an alternative electron sink (Mackie and Bryant 1994; Joblin 1996; Nollett *et al.* 1997). A detailed assessment on ruminal acetogens and their potential to lower ruminant methane emissions is given elsewhere (Joblin 1999). Despite methanogenesis predominating in the rumen, and having an energetic advantage over acetogenesis, acetogenesis predominates in other anaerobic gut ecosystems, such as the termite, cockroach, and rat caeca (Breznak and Switzer 1986). Not only does this reduce or nullify methane emissions, it can also supply a considerable proportion of the energy needs of the animals. In the termite, reductive acetogenesis has been calculated to produce enough acetate to account for 33% of the animals total energy requirement (Breznak and Switzer 1986). If methane was wholly replaced by acetate, propionate, or other useful fermentation products in ruminants this would represent an energetic gain of 4–15% to the animal (Joblin 1996; Nollet *et al.* 1997). Thus in ruminant-based industries (beef, lamb, wool, and dairy) a reduction in methanogenesis and corresponding increase in acetogenesis would improve productivity as well as alleviate greenhouse gas emissions.

Nollet *et al.* (1997) have argued that in order to establish acetogenesis, and allow it to compete for reducing equivalents in the rumen, it will be necessary to inhibit methanogenesis. This will allow the partial pressure of hydrogen to increase above the threshold required for reductive acetogenesis which is necessary either to allow the incumbent acetogens to be competitive or to allow introduced acetogens to


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establish in the rumen. Hydrogen accumulation in the presence of an active acetogen appears not to inhibit general fermentation; rather, Nollet *et al.* (1998) observed an increase in VFA production *in vitro* and a smaller short term positive effect *in vivo*.

This paper will investigate some biological strategies to reduce ruminal methanogenesis. Three strategies will be considered, two that directly inhibit methanogenic archaea (viruses and bacteriocins or killer particles) and one indirect method that would remove a major habitat or niche available to the archaea (protozoal removal or defaunation).

**Archaeal viruses**

Viruses are obligate pathogens that, in the lytic phase of development, infect and lyse bacteria and archaea. As such these viruses can rapidly reduce the population density of their hosts and have been viewed as possible agents of biological control.

In the rumen, bacterial viruses (bacteriophages or phages) are well known (Ritchie *et al.* 1970; Klieve and Bauchop 1988; Klieve and Swain 1993). Phages occur in dense populations, $>10^9$ particles/mL fluid (Klieve and Swain 1993), and they attack a wide variety of bacterial hosts (Klieve *et al.* 1989; Mackie and White 1990). Although knowledge of the bacteriophages of rumen bacteria has progressed in recent years it is still limited. Knowledge of archaeal viruses, on the other hand, is at best rudimentary. No viruses have been recorded from rumen archaea and few from methanogens generally. Phage-like particles are known to infect *Methanobrevibacter smithii* and *Methanobacterium thermoautotrophicum* (Nagle 1989). *M. smithii* strain PS is the host for a virus that has a morphology identical to phages of the family Siphoviridae (Knox and Harris 1986). The morphological similarities between phages and archaeal viruses, at least those that are recorded, suggests they may have similar properties such as infectivity and lysogeny.

At the present time, few serious attempts have been made to use phages as biocontrol agents. The greatest limitation to the use of rumen phages as biocontrol agents will probably be a lack of universal infectivity. Studies on the host range of phages of the common ruminal bacteria *Prevotella ruminicola* (Klieve *et al.* 1991) and *Streptococcus bovis* (A. V. Klieve, unpublished data) show that many of the phages isolated to date are strain specific or at best limited in infectivity to 3 or 4 closely related strains. If specific strains or groups of genetically similar strains of methanogens were to be targeted then viruses may be suitable as biocontrol agents and could have a significant impact on ruminal methane production, but if genetically heterogenous groups, as with the ruminal bacteria *P. ruminicola* and *Butyrivibrio fibrisolvens* (Hudman and Gregg 1989), are to be targeted then it may be difficult to locate phages with sufficient host range. However, as few studies have been undertaken on archaeal viruses it is too soon to assume that broad host-range viruses of methanogens are uncommon. In support of the use of viruses as biocontrol agents in the rumen environment, Tarakanov (1994) has reported the successful use of *S. bovis* phages as feed additives to suppress amylolytic bacteria. This treatment also increased numbers of cellulolytic bacteria, ruminal propionate and butyrate to acetate ratios, and milk fat content in dairy cows.

To date no attempt has been made to use archaeal viruses to control methanogens. Without a considerable increase in knowledge of the genetic diversity and viral susceptibility of methanogens and the host range of archaeal viruses it is difficult to assess their potential as biocontrol agents. The limited host range of phage ΨM1 of *M. thermoautotrophicum* to strain Marburg has already been suggested as a limitation to the use of this phage as a vector system for the genetic manipulation of methanogens (Leisinger and Meile 1990; McAllister *et al.* 1996). However, for a given strain there is evidence phage may cause widespread infection, with Newbold *et al.* (1996) observing substantial lysis of *Methanobrevibacter* MF1 in cell-free rumen fluid, with lysis being avoided by prior autoclaving of the rumen fluid.

The use of viruses should not be viewed as limited to biocontrol agents. Phages can be extremely important in the development of systems for the genetic modification of micro-organisms. An example is the extreme utility of phage lambda of *Escherichia coli* and the importance of this phage in the development of modern molecular biology (Cairns *et al.* 1992). The use of bacteriophages in vector systems for the genetic manipulation of rumen bacteria is being considered and developed (Lockington *et al.* 1988; Mackie and White 1990; Gregg *et al.* 1994). In particular, a study by Jiang *et al.* (1995) specifically isolated phages of rumen acetogens for future use in the genetic manipulation of acetogens. In addition, of the few viruses isolated from methanogens, ΨM1 is already being developed for the genetic manipulation of *M. thermoautotrophicum* (Leisinger and Meile 1990).

**Bacteriocins**

Bacteriocins are bacteriocidal compounds that are generally peptide or protein in nature, and are produced by bacteria (Sahl 1994). Bacteriocins are believed to be of considerable ecological importance, allowing organisms to compete for niches within an ecosystem.

The possible uses and benefits of these compounds to manipulate the rumen ecosystem have been recognised and research is currently being undertaken to isolate new bacteriocins and also on the impact of existing bacteriocins.

Recently, Kalmakoff *et al.* (1996) surveyed 50 strains of the common rumen bacterium *B. fibrisolvens* and found that 50% showed bacteriocin-like activity. Some of these bacteriocin-producing *B. fibrisolvens* strains (particularly those grouped on 16S rRNA similarity as Group II) also inhibited a wide range of other Gram+ve ruminal bacteria. No methanogens were used in the work. From this work it was

suggested that bacteriocins could be of use in manipulating the rumen ecosystem prior to the introduction of strains to be used probiotically, and that they may be effective alternatives to ionophore antibiotics as feed supplements. Callaway *et al.* (1997) specifically investigated the use of the bacteriocin nisin as an alternative to monensin. Nisin is a very well known bacteriocin, of the lantibiotic type, produced by *Lactococcus lactis*, and is widely used in the food industry as a preservative (Sahl 1994). *In vitro* incubation of ruminal contents with nisin showed similar effects to monensin on rumen metabolism and on microbial populations, including a reduction in methane emission (by 36%). Whether this reduction in emission is due to the impact of nisin directly on the methanogens or on the bacteria supplying them with substrate, as occurs with monensin (Sahl 1994; Callaway *et al.* 1997), is unknown at this time.

Major advantages of using bacteriocins are that they have a long history of safe use, can be incorporated into feed, are susceptible to proteolytic digestion (Kalmakoff *et al.* 1996), and could possibly be introduced by genetic manipulation into other organisms (e.g. protozoa and fungi). A limitation may be the degree of stability of these peptides in the rumen environment as rapid proteolytic digestion could reduce the effectiveness of these compounds.

There do not appear to be any reports of the isolation of bacteriocins specifically targeting methanogens. However, should this be undertaken in the future, ecosystems where acetogenesis outcompetes methanogenesis, such as the termite gut (Breznak and Switzer 1986), could be environments in which useful bacteriocins may be found.

## Defaunation

Protozoa provide a habitat for up to 20% of rumen methanogens (Stumm *et al*. 1982), and rumen fluid with high numbers of protozoa tends to have the highest rates of methanogenesis (Krumholz *et al.* 1983). Rumen ciliate protozoa are known to contain endosymbiotic methanogens (Finlay *et al.* 1994) and methanogens attached to the pellicle (Imai and Ogimoto 1978). These protozoa-associated methanogens have been variously reported as contributing up to 37% of total rumen methane emissions and ruminants without rumen protozoa produce less methane than do ruminants with a normal rumen fauna (Williams and Coleman 1992).

Elimination of rumen protozoa therefore offers one opportunity to reduce indirectly rumen methane emissions and this is reviewed elsewhere in these proceedings (Hegarty 1999). The animal production responses to the absence of rumen protozoa have been frequently reviewed (Kreuzer 1986; Bird 1991). Pasture production responses to defaunation are generally obtained in wool, liveweight, and milk production when these are constrained by amino acid availability. When productivity is not constrained by amino acid availability there is little response to defaunation and a small reduction in rumen degradability of DM frequently occurs (Williams and Coleman 1992). A wide range of dietary regimes and synthetic chemicals, as well as naturally occurring compounds, have been evaluated for their anti-protozoal activity (Hegarty 1999). There has, however, been no systematic assessment of the use of other organisms to biologically suppress or eliminate rumen protozoa. An overview of organisms known to be pathogenic to protozoa in the rumen or in related ecosystems is given below.

### *Fungal pathogens*

Fungi are known to infect many animal, plant, bacterial, and protozoal species. Chytrid fungi infecting rumen protozoa were first observed in 1929 and their presence and pathogenicity in a range of protozoa has been reviewed (Kirby 1964). Chytrid fungi were observed in *Entodinium* spp. (Winogradowa 1936) and Lubinsky (1955*a*) found *Sphaerita hoari* infecting *Eremoplastron bovis* but doing no apparent damage to the host protozoa. In contrast, fungi of the genus *Sagittospora* which multiplied within a range of Ophyryoscolidae protozoans caused death of the protozoa (Lubinsky 1955*b*). Cultures of free living amoeba have been reported to perish due to infection by either these cytoplasmic chytrid fungi or by *Nucleophaga* which infects the nucleolus (Kirby 1964).

### *Viruses*

Many of the blood and intestinal protozoal parasites which cause disease in domestic animals and humans are known to contain viruses (Wang and Wang 1991; Hotzel *et al*. 1995; Khramtsov *et al.* 1997). The RNA viruses present in a range of protozoa, but particularly those in species of *Giardia*, *Trichomonas*, and *Leishmania* have been well documented. However, it is unclear whether these viruses are in any way destructive of the host organism, since in some cases they appear to have coexisted for extremely long periods (Widmir and Dooley 1995).

In ecosystems outside of the animal, however, such as in the marine environment, DNA viruses have been shown to cause lysis of nanoflagellate protozoa and a decline in the population density of nanoflagellates (Garza and Suttle 1995). Specific studies on viruses in rumen protozoa have not been reported.

### *Rickettsia-like organisms*

Rickettsia-like organisms have been identified in a range of protozoa including rumen isotrichs (Prins and van den Vorstenbosch 1975) but their efficacy as biocontrol agents has not been determined.

## Conclusion

Inhibition of ruminal methane production is necessary to raise the partial pressure of $H_2$ to the threshold for initiation of acetogenesis. Chemical inhibitors of methanogenesis

which raise $H_2$ pressure have not been developed as commercial compounds and there is a clear need for livestock industries to develop biological tools for stable long-term inhibition of rumen methanogenesis. The potential of 3 biological strategies to control rumen methanogenesis are summarised below:

- Archaeal viruses could be used to directly reduce populations of rumen methanogens. No research appears to be under way to evaluate their use as biocontrol agents of methanogens. Host specificity may be a limiting factor. These viruses, and the phages of acetogens, have potential for use in the genetic manipulation of methanogens and acetogens, respectively.
- Bacteriocins could also be used to reduce methanogen populations. They appear to have a much wider host range than viruses and have a history of safe use. Some research is under way and nisin, a lantibiotic bacteriocin produced by *L. lactis*, has been shown to reduce methane emission by 36%.
- Defaunation or the elimination of mixed populations of ciliate protozoa from the rumen significantly reduces methane emissions. Potential biocontrol agents infecting rumen protozoa have already been identified in the form of fungi, and rickettsia-like organisms and viruses may reasonably be expected to be present. The efficacy of these pathogens in suppressing or eliminating protozoa and so reducing rumen methanogenesis has not been investigated. Death of rumen ciliates due to infection with chytrid fungi and lysis of marine protozoa due to virus have been observed, suggesting biocontrol of rumen protozoa may be a realistic opportunity.

While the biological agents suitable for controlling rumen methanogenesis by the above means have not even been comprehensively screened, clear opportunity exists to move from chemical to ecological modification of rumen fermentation. It is anticipated that biological control agents will provide long-term rumen modification and will be applicable in extensive production systems where chemical administration is not possible. They may also provide a means to raise $H_2$ pressure sufficiently to initiate acetogenesis in the rumen.

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