Journal of Animal and Feed Sciences, 13, Suppl. 1, 2004, 51–54

Examining diversity of free-living methanogens and those associated with protozoa in the rumen*

C.T. Christophersen[1,2,3], A.-D.G. Wright[2] and P.E. Vercoe[1]

[1]University of Western Australia, Faculty of Natural and Agricultural Sciences,
School of Animal Biology
35 Stirling Highway, Crawley 6009, W.A, Australia
[2]CSIRO Livestock Industries, Centre for Environment and Life Sciences,
Floreat Park Research Laboratory
Private Bag 5, Wembley 6913, W.A, Australia

ABSTRACT

The diversity of methanogens in the rumen of sheep fed three different diets was examined using denaturing gradient gel electrophoresis (DGGE). In addition, half of the sheep had pot scrubbers added to their rumen to increase flow rate. The methanogens were separated by a series of washing steps into three populations: free-living, ecto-symbiotic and endo-symbiotic.

Preliminary DGGE banding patterns demonstrated considerable differences between populations, treatment groups, and within groups. This suggests that the diversity of methanogens is influenced by diet, flow rates of digesta and the niche they occupy in relation to the protozoa.

KEY WORDS: methanogens, denaturing gradient gel electrophoresis, rumen, protozoa, pot scrubber

INTRODUCTION

It is well established that there is a large diversity of methanogens in the rumen, which is influenced by diet (Wright et al., 2004). The interaction between methanogens and rumen protozoa is also well documented (Vogels et al., 1980), but only a few studies have focused on the diversity of methanogens that associate with protozoa (Tokura et al., 1999).

In this experiment we divided the total methanogenic population into three populations: free-living, those attached to the surface of protozoa (ecto-symbionts), and those living inside the protozoa (endo-symbionts). This was done to examine the diversity of methanogens found in each of the three populations. This has important

* Supported by the Danish Research Agency
[3] Corresponding author: e-mail: cchristoph@agric.uwa.edu.au

implications for scientists trying to target specific methanogens in an effort to reduce methane emission from livestock. To our knowledge this has not been done previously. We have designed an experiment to investigate the effect of diet and flow rate of digesta in the rumen on the diversity of methanogens found in the three populations. With this design we expect to see a reduction in methane production when pot scrubbers are introduced and as grain proportion of the diet is increased. This should also increase propionate and reduce acetate production (Baker, 1997). This design is expected to give the best opportunities to examine the species composition of these 3 populations and how they change.

MATERIAL AND METHODS

Experimental design

The experiment had 4 treatment groups with 6 sheep in each. The first and second treatment groups were used as control groups, but the second group had pot scrubbers added to their rumen to increase the flow rate of digesta. The third and fourth treatment groups were given 3 different diets each for 3 weeks. The fourth group also had pot scrubbers added to the rumen. Rumen samples were collected from all 24 fistulated sheep at the end of each 3 week period and used to appraise volatile fatty acids (VFA), to measure in vitro methane production, and to extract DNA for analysis of methanogen diversity.

Diets

Three diets (oaten chaff - control, 35% oaten grain, 70% oaten grain) were fed at maintenance levels. Each diet was fed for 3 weeks. Control animals were maintained on the chaff diet.

Rumen parameters

VFA samples were analysed using a capillary gas chromatograph according to Erwin et al. (1961). Methane production was measured in vitro in 100 ml serum bottles with 30 ml of rumen fluid for 24 h. All measurements were done in triplicates. Methane was measured using gas chromatography with argon as the carrier gas.

DGGE

Methanogens were separated by a series of centrifugation and washing procedures, according to Williams and Coleman (1992), into 3 aliquots: aliquot 1: contained free-living, ecto- and endo-symbiotic methanogens, aliquot 2: contained ecto- and endo-symbiotic methanogens, and aliquot 3: contained endo-symbiotic methanogens. Samples were stored at -20ºC until DNA was extraction. Total DNA was extracted from 1 ml of the samples using an Ultra Clean Faecal

DNA kit (MoBio Laboratories). The extracted DNA was used in Polymerase Chain Reaction (PCR) targeting the 16S rRNA gene of methanogens using specific primers designed for DGGE. The PCR mixture (50 µl) contained 5 µl dNTP mixture (200 µM of each dNTP), 5 l 10X Qiagen PCR buffer (with 15 mM $MgCl_2$; Qiagen Pty Ltd), 5 µl of forward primer (1.2 nM), 5 µl of reverse primer (400 nM), 2 µl of 25 mM $MgCl_2$, and 0.5 µl of Qiagen hotstartaq (Qiagen Pty Ltd). One PCR cycle constituted: 94ºC for 1 min, 60ºC for 1 min and 72ºC for 2 min, this cycle was repeated 44 times, initiated with a 15 min hotstart and a final extension of 5 min. The DGGE gels were run for 18 h at constant 100 volts, loaded with 15 µl of PCR-product and 10 µl of loading buffer. The gels were stained with SYBR green I (Sigma Chemicals).

RESULTS

There are different banding patterns between aliquots and treatment groups, and within groups (Figure 1). Differences in banding patterns between aliquots can be seen by comparing sample 4.1, 4.2 and 4.3 (Figure 1). The differences between treatment groups is illustrated by comparing banding patterns from treatment group 4 with patterns from treatment group 3 (Figure 1). For example, the first 3 bands appear higher in 4.1 than in 3.1 and 3.1^A. Differences within treatment group are apparent when comparing the banding pattern of sample 3.1 with 3.1^A. Sample 3.1^A has more bands than 3.1 (Figure 1).

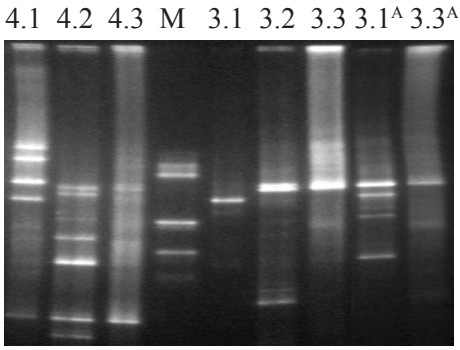

Figure 1. DGGE gel of PCR product using DNA from 8 different rumen samples. First digit corresponds to treatment group and the second digit to aliquot number. The superscript letter indicates different samples and M is a marker

Grain and pot scrubbers had the expected effect on methane production and VFA concentration. Methane production decreased with increased grain and when pot scrubbers were present, and propionate levels increased while acetate levels decreased with higher levels of grain (data not shown).

DISCUSSION

Our expectation that DGGE banding patterns would change with diet and flow rate manipulations was partially supported. However, some of the results were unexpected. For example, we expected an overlap in banding pattern between samples 4.1-4.3, with all bands being present in sample 4.1 and for bands to disappear in samples 4.2 and 4.3, because of the sequential washing and removal of different populations of methanogens in different aliquots. Instead, new bands are present in sample 4.2. However, the banding patterns for samples 4.2 and 4.3 are consistent with our expectations.This pattern of having additional bands in the second aliquot containing ecto- and endo- symbionts has been consistent between the samples analysed to date.

The explanation for the additional bands in sample 4.2 is not clear but it may be due to the bias introduced by PCR. Due to the nature of PCR, dominant species may be over represented on gels and minor species may not be amplified sufficiently to be detected on gels. It is possible that in sample 4.1 the free-living methanogens are the dominant ones and the ecto- and endo- symbionts are not in great enough numbers to be detected on the gel. However in aliquot 2, where the free-living methanogens have been removed by washing and the ecto- and endo- symbionts have been concentrated by centrifugation, the bands representing the free-living methanogens should disappear and new bands representing the endo- and ecto- methanogens should be detected.

CONCLUSIONS

These preliminary results suggest that there is diversity in methanogens between the treatment groups and that at least some of the free-living methanogen species are different to the species living attached to and within protozoa. The next step in these analyses is to identify which species of methanogens the individual bands represent, and to estimate numbers of methanogens in the different samples using quantitative real-time PCR.

REFERENCES

Baker S.K., 1997. Gut microbiology and its consequences for the ruminant. Proc. Nutr. Soc. 21, 6-13

Erwin E.S., Macro G.J., Emery E.M., 1961. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. J. Dairy Sci. 44, 1768-1771

Tokura M., Chagan I., Ushida K., Kojima Y., 1999. Phylogenetic study of methanogens associated with rumen ciliates. Current Microbiol. 39, 123-128

Vogels G.D., Hoppe W.F., Stumm C.K., 1980. Association of methanogenic bacteria with rumen ciliates. Appl. Environ. Microbiol. 40, 608-612

Williams A.G., Coleman G.S., 1992. The Rumen Protozoa. Springer-Verlag, New York, pp. 133-164

Wright A.-D.G., Williams A.J., Winder B., Christophersen C.T., Rodgers S.L., Smith K.D., 2004. Molecular diversity of rumen methanogens from sheep in Western Australia. Appl. Environ. Microbiol. 70, 1263-1270