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A Vaccine against Rumen Methanogens Can Alter the Composition of Archaeal Populations▽

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The objectives of this study were to formulate a vaccine based upon the different species/strains of methanogens present in sheep intended to be immunized and to determine if a targeted vaccine could be used to decrease the methane output of the sheep. Two 16S rRNA gene libraries were used to survey the methanogenic archaea in sheep prior to vaccination, and methanogens representing five phylotypes were found to account for >52% of the different species/strains of methanogens detected. A vaccine based on a mixture of these five methanogens was then formulated, and 32 sheep were vaccinated on days 0, 28, and 103 with either a control or the anti-methanogen vaccine. Enzyme-linked immunosorbent assay analysis revealed that each vaccination with the anti-methanogen formulation resulted in higher specific immunoglobulin G titers in plasma, saliva, and rumen fluid. Methane output levels corrected for dry-matter intake for the control and treatment groups were not significantly different, and real-time PCR data also indicated that methanogen numbers were not significantly different for the two groups after the second vaccination. However, clone library data indicated that methanogen diversity was significantly greater in sheep receiving the anti-methanogen vaccine and that the vaccine may have altered the composition of the methanogen population. A correlation between 16S rRNA gene sequence relatedness and cross-reactivity for the methanogens ($R^2 = 0.90$) also exists, which suggests that a highly specific vaccine can be made to target specific strains of methanogens and that a more broad-spectrum approach is needed for success in the rumen. Our data also suggest that methanogens take longer than 4 weeks to adapt to dietary changes and call into question the validity of experimental results based upon a 2- to 4-week acclimatization period normally observed for bacteria.

Livestock are a major source of methane production in Australia, with over half of the total anthropogenic methane emissions attributed to them (1). Methane from livestock is produced as a by-product of enteric fermentation and is vented mostly via the mouth and nose by belching and exhalation, with a small amount expelled via the anus (12). There are many approaches being investigated for reducing the methane production of ruminant livestock (for examples, see references 3 and 22). Our laboratory has been working on a novel immunization approach to decrease the numbers and/or activity of the methanogenic archaea (i.e., methanogens) in the rumen. Wright et al. (30) vaccinated sheep with an anti-methanogen vaccine that was based on three strains (1Y, AK-87, and ZA-10) belonging to the genus *Methanobrevibacter* and produced a 7.7% decrease in methane production per kg of dry matter (DM) intake (DMI). Wright and his colleagues (28) later discovered that less than 20% of the different species of methanogens detected in those sheep were closely related to the methanogens in the vaccine. On the basis of these findings, it was suggested that greater methane abatement might be possible if a greater proportion of the methanogen species/strains were targeted by the vaccine.

Thus, the objectives of this experiment were to formulate a vaccine based on the key methanogens present in the sheep intended to receive the vaccine and to determine if a targeted vaccine could be used to decrease the methane output of sheep. The hypothesis was that sheep vaccinated with the targeted anti-methanogen vaccine would have decreased methane output compared with control sheep.

MATERIALS AND METHODS

Animal ethics approval and permits from the Australian Quarantine Inspection Service and the Australian Pesticides and Veterinary Medicines Authority were obtained.

Prevaccination. Thirty-six 2-year-old Merino wethers with a live weight (LW) of 56.1 ± 0.16 kg (average ± standard error of the mean) were sourced from a flock of around 230 sheep grazing at CSIRO's Yalanbee research station (Bakers Hill, Western Australia) in late March 2004. Sheep were randomly allocated to individual pens at the CSIRO animal house facility in Perth. A 14-week prevaccination period allowed sheep to be acclimatized to the pens and experimental diet and to be familiarized with the methane chambers (8).

Sheep were offered a pelletized, oaten hay-based ration as a single feed (1.60 kg day^{-1}) each morning, and they had continual access to water. The ration comprised 63 to 70% chopped oaten hay, 14 to 20% cereal grains, 10 to 12% lupines, 2% Siromin mineral mixture (23), and 2 to 5% binder ingredient. On a DM basis, it contained 93% organic matter, 9% crude protein, 42% neutral detergent fiber, and 22% acid detergent fiber. Methods used for collection and

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analysis of feed samples have been described by Williams et al. (25). After 4 weeks of acclimatization to the experimental diet, rumen fluid (30 to 50 ml) for construction of 16S rRNA gene libraries was collected from all sheep by insertion of a stomach tube and application of suction 2 to 3 h after they were fed.

Methane output over a 22-h period from each sheep during weeks 11 to 13 was measured, for use as a covariate, using open system chambers and gas chromatography (8). Thirty-two of the 36 sheep that were the most settled, as indicated by both their level of feed intake and their general behavior in the methane chambers, were selected for the experimental period.

Cross-reactivity and sequence identity between vaccine candidates. Cross-reactivity between *Methanobrevibacter ruminantium* $M1^T$, *Methanobrevibacter* strains AK-87 and 1Y, *Methanobrevibacter millerae* $ZA\text{-}10^T$, *Methanobrevibacter smithii* PS^T, *Methanobrevibacter arboriphilus* $DH\text{-}1^T$, *Methanobacterium formicicum* MF^T, *Methanomicrobium mobile* BP^T, and *Methanosarcina barkeri* MS^T was tested using the indirect fluorescent antibody test, originally described by Gnanasampanthan (4) and modified to resemble the enzyme-linked immunosorbent assay (ELISA) technique outlined by Wright et al. (30). The sources of the methanogen strains $M1^T$, $ZA\text{-}10^T$, 1Y, AK-87, PS^T, $DH\text{-}1^T$, MF^T, BP^T, and MS^T and their culture conditions have been described previously (26). Cells were prepared for use in the cross-reactivity tests by washing and resuspending them in phosphate-buffered saline (PBS). The cells were then added to wells of microtiter plates, and plasma samples containing antibodies to the different cell types were added to the cells. The plasma samples were obtained from a separate group of sheep that had been vaccinated with the different methanogen cell types. Cross-reactivity was expressed as a percentage of the titer. The results of the clone library and cross-reactivity studies were used to select methanogens for the vaccine used in the experimental period. Pairwise 16S rRNA gene sequence identity between the methanogen strains was also determined, and a linear regression of percent sequence identity and percent cross-reactivity was determined.

Vaccine preparation. The five methanogens chosen for use in the vaccine were *Methanobrevibacter* strains AK-87 and 1Y, *Methanobrevibacter millerae* $ZA\text{-}10^T$, *Methanomicrobium mobile* BP^T, and *Methanosphaera stadtmanae* $MCB\text{-}3^T$. Vaccines were prepared using aseptic techniques described by Wright et al. (30). The concentration of cells in the vaccine was equivalent to 0.4 mg ml^{-1} protein, or 2.0×10^9 cells ml^{-1}. Each milliliter of vaccine consisted of 0.12 ml of each of the three *Methanobrevibacter* strains (1 mg protein $ml^{-1} = 5 \times 10^9$ cells ml^{-1}) and 0.02 ml of both *Methanomicrobium mobile* and *Methanosphaera stadtmanae* (5×10^9 cells ml^{-1}). Prior to in vivo use, vaccines were tested for mycoplasmal, bacterial, and fungal contamination per 9CFR (USDA Code of Federal Regulations) 113.28 and 9CFR 113.27(d). The freshly prepared vaccine was stored at 4°C for less than 2 days before use. Vaccinations (1-ml dose with 18-gauge needle) were administered subcutaneously, high on the neck, behind the ear. Alternate sides of the neck were used for the second and third vaccinations. All sites were liberally swabbed with 70% ethanol prior to injection, and vaccination sites were also clipped of wool.

Experimental design and measurements. Selected sheep were allocated to treatments on the basis of their methane output prior to the first vaccination (26.1 ± 1.33 liters methane kg DMI^{-1}) and LW (59.6 ± 0.48 kg), using stratified randomization (2). There were two treatments in a randomized complete block design, with 16 sheep per treatment, and the experimental unit was an individual sheep. The control sheep received a vaccine containing only PBS and adjuvant, whereas the methane sheep received a vaccine containing the five-methanogen mixture, PBS, and adjuvant.

The experimental period began with the first vaccination (day 0) and ran for 137 days. A second vaccination occurred on day 28, and methane output was measured approximately 4 weeks after the second vaccination, between days 62 and 70. A third vaccination occurred on day 103, and methane output was again measured approximately 4 weeks after the third vaccination, between days 127 and 136.

Sheep were removed from the individual pens and returned to the research station to graze between day 77 and 97 of the experimental period. During this time, preliminary experimental results were analyzed and it was decided that the sheep would be given a third vaccination to boost specific immunoglobulin G (IgG) titers in the saliva and that the experiment would be continued. Sheep were brought back to their individual pens in the animal house on day 98 for the remainder of the experimental period.

Blood, saliva, and rumen contents were collected from all sheep 2 days prior to primary vaccination and on days 27, 55, 99, and 123 for analysis of specific anti-methanogen antibody titer (IgG). Rumen contents were also used to extract DNA for molecular analyses (real-time PCR and 16S rRNA gene libraries). Methods of sample collection and the ELISA developed to monitor the antibody responses have been described elsewhere (30). Methane output was measured over 22 h using open-system chambers and gas chromatography (8). Four sheep, two from each treatment, were measured on each day during the methane measurement periods. LW was recorded at 3- to 4-week intervals for monitoring purposes.

16S rRNA gene library construction. Rumen fluid was pooled randomly into two samples, pool A and pool B, with 18 sheep per pool. DNA was extracted from each sample by using a phenol-chloroform/bead-beating method (20) and PCR amplified using archaeon-specific primers Met86F and Met1340R (26). A 16S rRNA gene library was constructed for each sample, following the protocol of Wright et al. (29). Combined results from the two clone libraries were used to identify the dominant methanogens present after 4 weeks of acclimatization to the experimental diet for inclusion in the vaccine formulation. Two additional 16S rRNA gene libraries (control and treatment) were constructed from the pooled PCR product amplified from frozen samples collected from individual sheep on day 55 (4 weeks after the second vaccination).

For all clone libraries, the cloned PCR product was digested with the HaeIII endonuclease and separated on a 4% molecular screening agarose gel (100 V for 2 h). Restriction fragment length polymorphisms were grouped according to their riboprint patterns (26). At least five clones from each riboprint pattern were sequenced in both directions for confirmation. Sequencing was performed with an ABI Prism 3730 48 capillary sequencer using BigDye Terminator and TaqFS with two forward and two reverse methanogen 16S rRNA gene primers (26). The online chimeric detection program Bellerophon (5) was used to identify chimeric sequences from the library.

Distance data were generated for each clone library by using the Kimura-2 parameter model (7) and analyzed using the computer program DOTUR (13) to calculate the Shannon diversity index (14), with 95% confidence limits, at 98% similarity. Clones were given the prefix "CSIRO," followed by "WA" to indicate the location of collection (i.e., Western Australia) and a number to identify each phylotype.

Real-time PCR analysis. Real-time PCR was used to determine the number of methanogens per gram wet weight for all 32 sheep. External standards for methanogens were prepared according to the method of Christophersen (3a), using a mixture of pure cultures of *Methanobrevibacter ruminantium* $M1^T$ and *Methanobrevibacter smithii* PS^T, ranging from 1.0×10^3 to 1.0×10^8 cells g^{-1}. Real-time PCR amplifications were carried out with a Bio-Rad iCycler in a 25-μl volume containing the following reagents: 12.5 μl SYBR green mixture (QuantiTect SYBR green PCR; Qiagen), 400 nM each of primers Met630F and Met803R (3a), and 1.0 μl template DNA (10 ng). Real-time PCR amplification was initiated by a hot start at 95°C for 15 min, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Threshold cycles were calculated automatically by the iCycler software program (version 3.5), and PCR efficiency for each extract was calculated from the logarithmic portion of the sigmoid-shaped curve in real-time PCRs according to the methods described by Liu and Saint (9). Three dilutions of DNA were amplified, and the threshold cycle of the most efficient PCR was recorded.

Statistical analyses. The effects of vaccine treatment on methane output, LW, LW gain, and average feed intake were analyzed according to a completely randomized design using analysis of variance (Genstat V; Lawes Agricultural Trust, Rothamstead Experimental Station, United Kingdom). When a significant F value was detected, means were compared by the least-significant-difference test. Methane outputs, repeatedly measured over time, were analyzed by repeated measures of residual maximum likelihood, using an ante dependence model. Vaccine treatment and time were fitted as fixed effects in the model, while times for each animal were fitted as random effects.

Nucleotide sequence accession numbers. The new 16S rRNA sequences generated in this study have been deposited in GenBank under accession numbers EU093103 to EU093110.

RESULTS

Prevaccination: clone library and methanogen cross-reactivity. In the first 16S rRNA gene clone library (pool A), 52 clones were examined, revealing 10 different sequences, or phylotypes. In the second clone library (pool B), 84 clones were examined, also revealing 10 different phylotypes. DOTUR analysis of the two clone libraries (pool A and pool B) indicated that the Shannon diversity indices were not significantly different from each other, regardless of percent similarity cutoff (Table 1). Therefore, it was feasible to combine the data,

TABLE 1. Shannon indices for methanogen clone libraries comprising rumen fluid samples from sheep before and after vaccination with a control or treatment formulation and also for sheep from other experiments[a]

Clone library	Diet of sheep prior to sampling	Shannon index ± 95% CI[c]
Prevaccination (pool A)	Grazing of autumn pasture, followed by pelletized ration (67% oaten hay, 18% lupines, 10% barley) for 4 wk	1.76 ± 0.20*
Prevaccination (pool B)	Same as that for prevaccination (pool A) group	1.56 ± 0.19*
Prevaccination (pools A and B combined)	Same as that for prevaccination (pool A) group	1.70 ± 0.15*
Control (after second vaccination)	Pelletized ration (67% oaten hay, 18% lupines, 10% barley) for 19 wk	0.69 ± 0.27**
Treatment (after second vaccination)	Same as that for control sheep	1.38 ± 0.24*
WA expt 1[b]	Grazing of autumn and winter pastures	1.70 ± 0.12*
WA expt 2[b]	Mixed ration (67% oaten hay, 18% lupines, 10% barley) for 12 wk	0.76 ± 0.13**
WA expt 3[b]	Mixed ration (68% lucerne hay, 20% lupine grain, 10% molasses) for 6 wk	0.20 ± 0.14***

[a] Calculations were made using DOTUR (13).
[b] Data taken from Wright et al. (29). WA, Western Australia.
[c] Values marked by different numbers of asterisks are significantly different at minimum P values of <0.05.

resulting in a total of 132 clones made up of 12 different phylotypes (Table 2).

Methanobrevibacter strains accounted for more than two-thirds of the different species/strains of methanogens detected in the rumen (Table 2). Seven phylotypes were identified as new taxa on the basis of a similarity criterion of <97% (19) and accounted for almost 40% of the 132 clones. Of these seven new taxa, five phylotypes (CSIRO-WA08, CSIRO-WA09, CSIRO-WA10, CSIRO-WA11, and CSIRO-WA12) belong to the distant group of uncultured archaea, one phylotype (CSIRO-WA06) is a possible new species within the order Methanobacteriales, and one phylotype (CSIRO-WA05) represents a new genus and a new species within the order Methanosarcinales.

With the exception of *Methanobrevibacter ruminantium* M1, immunotyping of various methanogen strains by using ELISA indicated that there was high cross-reactivity between the *Methanobrevibacter* strains, indicating common antigenic epitopes (Table 3). *Methanobrevibacter ruminantium* M1 had very low cross-reactivity to other closely related strains within its genus. Therefore, *Methanobrevibacter* strains AK-87 and 1Y and *Methanobrevibacter millerae* ZA-10^T were chosen for the vaccine. *Methanosphaera stadtmanae* MCB-3^T and *Methanomicrobium mobile* BP^T were also chosen for use in the vaccine, as

TABLE 2. Methanogen 16S rRNA gene libraries constructed prior to vaccination and 4 weeks after the second vaccination

16S rRNA clone name[a]	GenBank accession no.	Nearest valid related organism	% Sequence identity	Related vaccine strain	% Sequence identity	Predicted % cross reactivity[b]	No. of clones per library		
							Before expt	Postvaccination	
								Control	Treatment
CSIRO-WA10[1]	AY351466	*Thermoplasma volcanium*	78.6	ZA-10	76.1	<0.1	31		2
CSIRO-WA04	AY615202	*Methanobrevibacter ruminantium* M1	99.0	AK87	100.0	100.0	24		
CSIRO-WA03	AY196669	*Methanobrevibacter smithii* PS	100.0	1Y	98.2	89.9	18	5	3
CSIRO-WA01[2]	AJ009958	*Methanobrevibacter millerae* ZA-10	99.1	ZA-10	99.1	94.1	18	29	22
CSIRO-WA02	AB034185	*Methanobrevibacter millerae* ZA-10	98.4	1Y	99.3	95.0	12	9	4
CSIRO-WA08[3]	AY995282	*Thermoplasma acidophilum*	74.2	AK87	76.7	<0.1	9		
CSIRO-WA07	AY196679	*Methanomicrobium mobile* BP	100.0	BP	100.0	100.0	8		
CSIRO-WA06[4]	DQ123883	*Methanosphaera stadtmanae* MCB-3	95.8	MCB-3	95.8	78.9	7		
CSIRO-WA12[5]	AY995292	*Thermoplasma acidophilum*	74.1	AK87	75.9	<0.1	2	1	
CSIRO-WA05[6]	DQ123877	*Methanimicrococcus blatticola*	89.8	BP	79.6	<5.0	1		
CSIRO-WA09[7]	AY995280	*Thermoplasma acidophilum*	72.7	AK87	75.3	<0.1	1		
CSIRO-WA11[8]	AY995300	*Thermoplasma volcanium*	73.5	1Y	75.2	<0.1	1		
CSIRO-WA13	EU093103	*Methanobrevibacter millerae* ZA-10	98.8	ZA-10	98.8	92.7		1	
CSIRO-WA14	EU093104	*Methanobrevibacter millerae* ZA-10	99.0	ZA-10	99.0	93.6		1	
CSIRO-WA15	EU093105	*Methanobrevibacter millerae* ZA-10	98.9	ZA-10	98.9	93.2		1	
CSIRO-WA19[9]	AY351494	*Methanobrevibacter millerae* ZA-10	99.0	ZA-10	99.0	93.6			1
CSIRO-WA20	EU093107	*Methanobrevibacter millerae* ZA-10	98.9	ZA-10	98.9	93.2			1
CSIRO-WA16[10]	AY351434	*Methanobrevibacter millerae* ZA-10	96.3	AK87	97.0	84.4		2	18
CSIRO-WA17[11]	AY351493	*Methanobrevibacter millerae* ZA-10	99.8	ZA-10	99.8	97.3		1	
CSIRO-WA18	EU093106	*Methanobrevibacter smithii* PS	97.8	1Y	98.7	92.2		7	
CSIRO-WA21	EU093108	*Methanobrevibacter wolinii* SH	95.4	AK87	93.5	68.4			2
CSIRO-WA22	EU093109	*Methanobrevibacter millerae* ZA-10	83.6	ZA-10	83.6	22.9			1
CSIRO-WA23	EU093110	*Methanobrevibacter wolinii* SH	95.3	AK87	93.3	67.4			1
Total[c]							132	57	55

[a] Alternative designations are represented by superscript numerals as follows: 1, CSIRO1.33; 2, SM9; 3, CSIRO-Qld09; 4, ON-CAN.13; 5, CSIRO-Qld24; 6, ON-CAN.05; 7, CSIRO-Qld07; 8, CSIRO-Qld34; 9, CSIRO3.06; 10, CSIRO3.07; and 11, CSIRO3.05.
[b] Based upon the linear regression equation $Y = 360.81284 - 4.59014X$ (where X equals the percent sequence identity).
[c] Total number of clones examined in each library.

TABLE 3. 16S rRNA gene sequence identities and percents cross-reactivity for some methanogen strains[a]

Strain	% Identity or cross-reactivity[b]								
	M1	AK-87*	1Y*	ZA-10*	PS	DH-1	MF	BP*	MS
Methanobrevibacter ruminantium M1^T	**100.0**	98.9	94.1	93.5	94.2	94.5	91.9	78.7	78.9
Methanobrevibacter sp. strain AK-87*	47.5	**100.0**	94.4	94.2	94.8	94.9	91.8	78.6	79.4
Methanobrevibacter sp. strain 1Y*	41.4	87.1	**100.0**	98.4	98.2	94.9	90.5	78.8	78.7
Methanobrevibacter millerae ZA-10^T*	39.2	82.6	94.8	**100.0**	97.9	94.4	90.5	78.5	78.5
Methanobrevibacter smithii PS^T	34.6	72.9	83.6	88.2	**100.0**	95.0	90.9	78.4	78.2
Methanobrevibacter arboriphilus DH-1^T	28.3	59.6	68.4	72.1	81.8	**100.0**	92.0	78.3	79.6
Methanobacterium formicicum MF^T	21.0	44.2	50.8	53.5	60.7	74.2	**100.0**	80.8	78.8
Methanomicrobium mobile BP^T*	0.1	0.2	0.3	0.3	0.3	0.4	0.5	**100.0**	82.2
Methanosarcina barkeri MS^T	0.1	0.2	0.2	0.2	0.2	0.3	0.4	0.6	**100.0**

[a] Asterisks indicate four of the five methanogen strains used in the vaccine formulation.
[b] Values in the upper triangle (to the right of the bolded values) are 16S rRNA gene sequence identities, and values in the lower triangle (to the left of the bolded values) are percents cross-reactivity.

they represented 5% and 6%, respectively, of the combined clone library population (Table 2). Furthermore, the immunotyping data indicated that there was no cross-reactivity between the protein cell walls of *Methanomicrobium mobile* BP^T cells and any of the pseudomurein cell walls of the *Methanobrevibacter* strains cells (Table 3). A pure culture of *Methanosphaera stadtmanae* MCB-3^T was unavailable when we were conducting our cross-reactivity experiments, so it was not immunotyped. However, it was deduced that this methanogen was likely to have different antigenic sites on its cell wall because it used different energy sources for growth (10).

The five methanogen strains selected for the vaccine formulation accounted for 52% of the different methanogen sequences recovered from a 16S rRNA gene clone library during the prevaccination period. The remaining 48% of the clone sequences were either from uncultivated strains, from strains that we did not have available, or from strains not approved for use in vaccines. Linear regression analysis of percent sequence identity and percent cross-reactivity ($Y = 360.81284 - 4.59014X$) was significant ($P < 0.001$) and produced an R^2 value of 0.90 (Table 3).

Experimental period: immune response, methanogen numbers, and diversity. Titers of methanogen-specific IgG in plasma, saliva, and rumen fluid increased after each vaccination in methanogen-vaccinated sheep but not in control-vaccinated sheep (Fig. 1).

Mean densities of methanogens (number of cells g wet weight^{-1} ± standard error of the mean) in the rumen digesta of individual sheep before vaccination, as determined by real-time PCR, were $1.58 \times 10^7 \pm 6.86 \times 10^6$ for the control group and $2.33 \times 10^7 \pm 1.96 \times 10^7$ for the treatment group, whereas after the second vaccination, the methanogen numbers were $1.50 \times 10^6 \pm 5.77 \times 10^5$ and $1.33 \times 10^6 \pm 3.39 \times 10^5$ for the control and treatment animals, respectively. There was no difference ($P > 0.05$) between the control and treatment groups in mean density of methanogens before or after vaccination.

The 16S rRNA gene clone library for control sheep generated 57 clones, comprising 10 different phylotypes (Table 2). The 16S rRNA gene library for the treatment sheep generated 55 clones, made up of 10 phylotypes. Overall, 244 clones were examined, representing 23 different phylotypes. The Shannon diversity indices (14) for the methanogen clone libraries differed ($P < 0.05$) between the control and treatment sheep after the second vaccination (Table 1). Of the 10 phylotypes from the treatment animals, four were identified as new taxa and were not detected in the other clone libraries (i.e., during the prevaccination period and in the control animals after the second vaccination). Of these four new taxa, three phylotypes

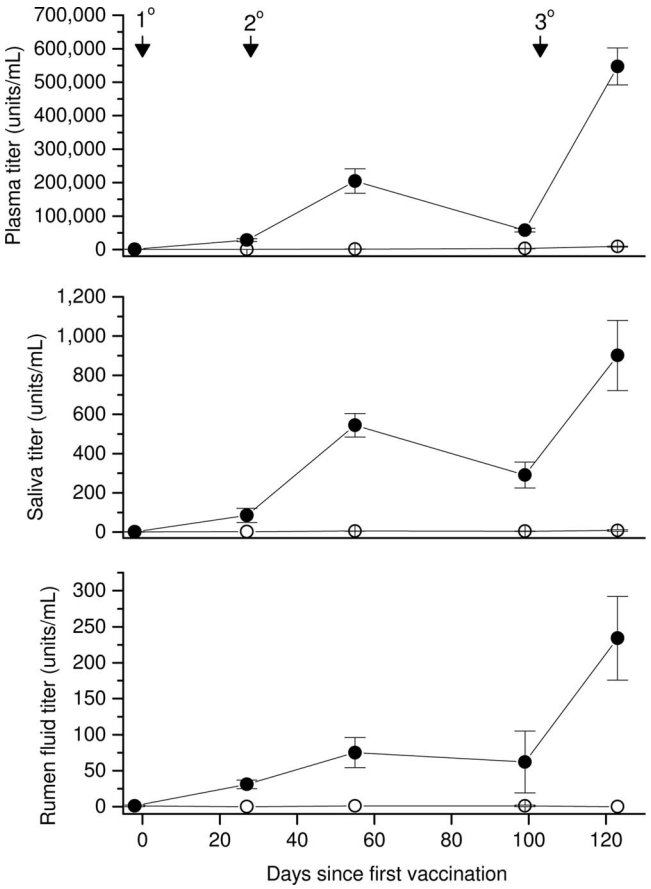

FIG. 1. Specific IgG titers over the experimental period in plasma, saliva, and rumen fluid samples of sheep vaccinated with a control (○) or anti-methanogen vaccines (●). Timings of first (1°), second (2°), and third (3°) vaccinations are indicated by arrows.

TABLE 4. Total methane outputs (corrected for DMI) and feed intakes for sheep during the prevaccination period and after the second and third vaccinations[a]

Vaccine formulation	Methane output (liters kg DMI^{-1})			Feed intake (kg DM)		
	Prevaccination	After second vaccination	After third vaccination	Prevaccination	After second vaccination	After third vaccination
Control	23.0 ± 1.65	25.0 ± 2.83	23.9 ± 2.22	1.41 ± 0.016	1.30 ± 0.069	1.50 ± 0.011
Anti-methanogen	24.2 ± 1.67	30.2 ± 3.00	28.2 ± 2.58	1.44 ± 0.007	1.25 ± 0.079	1.44 ± 0.052

[a] Values shown are means ± standard errors.

(CSIRO-WA16, CSIRO-WA21, and CSIRO-WA23) represent possible new species within the order Methanobacteriales, and one phylotype (CSIRO-WA22) represents a new genus and a new species. With the exception of CSIRO-WA16, all clones recovered from the control animals were markedly decreased (CSIRO-WA01, CSIRO-WA02, and CSIRO-WA03) or undetectable (CSIRO-WA12, CSIRO-WA13, CSIRO-WA14, CSIRO-WA15, CSIRO-WA17, and CSIRO-WA18) in the treatment animals.

Overall, *Methanobrevibacter* strains made up approximately 98% and 95% of the rumen methanogen clones recovered from the control and treatment sheep, respectively (Table 2). The five methanogens used in the vaccine formulation accounted for 67% of the methanogen clones recovered from control sheep but only 47% of the methanogen clones recovered from sheep vaccinated against these same five methanogens.

The Shannon index for the clone library prior to the experiment was not different ($P > 0.05$) from that for the clone library of treatment sheep after the second vaccination but was different ($P < 0.05$) from that for control sheep after the second vaccination (Table 1). With the exception of clone CSIRO-WA01, all clones found in animals prior to the first vaccination were either markedly decreased in number (CSIRO-WA02, CSIRO-WA03, and CSIRO-WA12) or undetectable (CSIRO-WA04, CSIRO-WA05, CSIRO-WA06, CSIRO-WA07, CSIRO-WA08, CSIRO-WA09, CSIRO-WA10, and CSIRO-WA11) in the control animals (Table 2). CSIRO-WA01 increased in number by 61% and was the predominant clone recovered from the control animals. Six additional clones (CSIRO-WA13, CSIRO-WA14, CSIRO-WA15, CSIRO-WA16, CSIRO-WA17, and CSIRO-WA18), which were not detected during prevaccination, were recovered from the control animals (Table 2).

Feed intake, methane output, and LW. There was no effect ($P > 0.05$) of vaccination treatment on feed intake, which averaged 1.39 kg DM day^{-1} over the experimental period (Table 4). There was no effect ($P > 0.05$) of vaccination treatment on total methane output by sheep (data not shown) or on methane output corrected for DMI (Table 4). Methane outputs measured after the second and third vaccinations were 20% and 18% higher, respectively, than those for controls, but the difference was not significant (Table 4). Furthermore, there was no significant effect ($P > 0.05$) of vaccination on LW at the end of the experiment (72.0 ± 0.70 kg [mean ± standard error]) or LW gain between the primary vaccination and the end of the experiment (81 ± 4.2 g day^{-1}).

DISCUSSION

We used a variation of a vaccination protocol aimed at inducing a humoral response against methanogens (30) that had previously resulted in small but significant reductions in methane emissions from vaccinated sheep where only 20% of the different species/strains of methanogens in the sheep were targeted by the vaccine formulation (28). Our vaccine formulation targeted approximately 52% of the different species/strains of methanogens present in the sheep and also contained three methanogens which were used in the previous successful formulation. We therefore expected a greater impact of the vaccination procedure on methane output in vaccinated sheep. However, we did not observe a decrease in methane output in sheep that received the anti-methanogen vaccine in comparison to the level in the controls. In fact, methane output was nearly 18% higher in treatment sheep vaccinated three times than in the controls ($P > 0.05$).

Vaccination did induce a humoral immune response, as indicated by the specific IgG titers in plasma and saliva, and specific anti-methanogen IgG was also delivered to the rumen, as indicated by the titers in the rumen fluid. However, the specific IgG titer levels observed in sheep plasma after the second vaccination were lower than those recorded in our previous study (30), whereas after the third vaccination, plasma IgG-specific titers increased to levels similar to those observed in our previous study (30). However, saliva and rumen IgG-specific antibodies increased only moderately, and no methane abatement was observed. This strongly highlights the variable outcomes of vaccination for decreasing enteric methane and suggests that methane abatement is obtained only when certain undefined conditions are met.

The methanogen populations in these sheep were dominated by *Methanobrevibacter* phylotypes, which is consistent with other studies of rumen methanogen populations (6, 10, 11, 15–17, 21, 24, 27, 29). Although the total numbers of methanogens were not significantly different between control and anti-methanogen sheep after vaccinations, our results suggest that some of the targeted methanogens were affected by the vaccine. The majority of clones recovered from the control animals were markedly decreased or undetectable in the treatment animals and had at least 98.7% sequence identity to *Methanobrevibacter millerae* ZA-10^T or *Methanobrevibacter* sp. strain 1Y, two of the key methanogens in the vaccine formulation. Another indication of changed populations was the difference in the Shannon indices of the clone libraries for the control and anti-methanogen-vaccinated sheep. We believe that the presence of six clones (CSIRO-WA10, CSIRO-WA19,

CSIRO-WA20, CSIRO-WA21, CSIRO-WA22, and CSIRO-WA23) in the treatment animals that were not detectable in the control animals was due to the niche created by the decrease in numbers or elimination of the dominant strains targeted with the vaccine. The increase in population of these other clones has masked the effect of the vaccine regimen.

Our results also suggest that the vaccine formulation is very specific. The predicted percents cross-reactivity between the most closely related methanogen strain in the vaccine formulation and the clones whose numbers decreased in the treatment animals ranged from 89.9 to 97.3%. The clones in treatment animals that increased in number or appeared as additional clones had lower predicted percents cross-reactivity, which ranged from <0.1 to 84.4%. There was a small exception to this observation, however, with two clones (CSIRO-WA19 and CSIRO-WA20) that appeared for the first time in treatment animals, despite having predicted cross-reactivities of 93.2% and 93.6%, respectively, to *Methanobrevibacter millerae* ZA-10^T. Even with these anomalies, the data still demonstrate that a highly specific vaccine can be made to target specific strains of methanogens and also confirm that a more broad-spectrum approach is needed for success in the rumen.

The clone library results show a difference in composition of clones between sheep prior to the experiment and control group animals. A comparison of the diversity indexes of these sheep prior to vaccination, 4 weeks after the start of the pelletized diet, with another group of grazing sheep from the same farm (29) showed they were nearly identical (Table 1). Furthermore, the Shannon indices (14) for our control sheep after 18 weeks on the experimental diet were very similar to those for the sheep used by Wright et al. (29) after more than 3 months on a similar oaten hay-based animal house diet (Table 1). We suggest that the rumen methanogen population had not fully adapted from the grazing diet to the pelletized experimental diet after only 4 weeks. If the clone library constructed postvaccination from the control sheep represents the true acclimatized methanogen population on the experimental diet, then acclimatization occurred somewhere between 4 and 18 weeks and therefore our vaccine was formulated against methanogens in grazing sheep and not those in sheep on the experimental diet.

The construction of clone libraries before diet adaptation resulted in lower target rates for the vaccine than we originally calculated. Three of the five methanogens (*Methanobrevibacter* sp. strain AK-87, *Methanomicrobium mobile* BP^T, and *Methanosphaera stadtmanae* MCB-3^T) used in the vaccine formulation, due to their presence in clone libraries during prevaccination, were not detected in the clone library from the acclimatized control sheep. However, the remaining two methanogens (*Methanobrevibacter millerae* ZA-10^T and *Methanobrevibacter* sp. strain 1Y) accounted for two-thirds of the methanogenic archaea in the acclimatized control sheep. If the vaccine had been formulated on the basis of the acclimatized data, then it would have contained approximately 50% more protein of ZA-10^T and 1Y than the current vaccine and may have been more effective. In light of this, it is questionable whether the administered load was sufficient to induce an effective response, which could explain why the two clones with predicted cross-reactivities of 93.2 to 93.6% with *Methanobrevibacter millerae* ZA-10^T appeared in the treatment animals.

The trend toward greater methane output by treatment vaccinated sheep, even though they had lower numbers of methanogens, is contrary to the general belief that methanogenesis is coupled to growth (18). It could be suggested that the other methanogens that were detected in the treatment vaccinated sheep may be more-potent producers of methane, therefore increasing methane output. This highlights the need to measure both cell numbers and methane output in this type of work.

In conclusion, we describe a vaccination regimen which induced a substantial serum antibody response against methanogens in sheep but failed to significantly affect the methane emission by these sheep and the density of methanogens. However, upon closer inspection, it would appear that the vaccine may have affected the diversity and composition of the methanogen population. Furthermore, compared to what was found for the control sheep, some *Methanobrevibacter* strains were either missing or decreased in the treatment sheep and replaced by other methanogens not detected previously before vaccination (Table 2). In the treatment sheep, the appearance of these other methanogens accounted for 44% of the different species/strains of methanogens detected and may explain why methanogen numbers were not significantly different between the control and treatment sheep but methanogen populations were significantly different between the two groups. Another complicating factor is that there does not appear to be a definite correlation between methanogen numbers and methane production, even though methanogenesis is an energetic reaction coupled to growth. Our data indicate that there is a correlation between 16S rRNA gene sequence relatedness and cross-reactivity for the methanogens and also suggest that adaptation of the rumen methanogen populations to differing diets may be much slower than expected (i.e., more than 4 weeks). This now calls into question the validity of experimental results based upon a 2- to 4-week acclimatization period normally observed for bacteria. Further studies are warranted to properly assess the acclimatization period for the methanogenic archaea.

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