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The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production in vitro[1]

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Abstract

Methane represents a loss of feed energy to ruminant animals, and nutritionists have sought methods of inhibiting ruminal methane production. When mixed ruminal bacteria (approximately 400 mg protein ml^{-1}) from a cow fed timothy hay were incubated in vitro with carbon dioxide and hydrogen (0.5 atm) for less than 8 h, the first-order rate of methane production was 17 μmol ml^{-1}. Semi-purified bacteriocin from *Streptococcus bovis* HC5 (bovicin HC5) inhibited methane production, by as much as 50%, and even a low concentration of bovicin HC5 (128 activity units (AU) ml^{-1}) caused a significant decrease. Mixed ruminal bacteria that were transferred successively retained their ability to produce methane from carbon dioxide and hydrogen, and the first-order rate of methane production did not decrease. Cultures that were treated with bovicin HC5 (128 AU ml^{-1}) gradually lost their ability to produce methane, and methane was not detected after four transfers. These latter results indicated that ruminal methanogens could not adapt and become resistant to bovicin HC5. When the chromosomal DNA was amplified with 16S rDNA primers specific to archaea, digested with restriction enzymes (*Hha*I and *Hae*III) and separated on agarose gels, approximately 12 fragments were observed. DNA from control and treated cultures (third transfer) had the same fragment pattern indicating bovicin HC5 was not selective. Given the perception that the routine use of antibiotics in animal feeds should be avoided, bacteriocins may provide an alternative strategy for decreasing ruminal methane production.
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Keywords: Ruminal fermentation; Bacteriocin; Methane

1. Introduction

Ruminal fermentation produces methane as a by-product, and the methane production of a single cow can be as great as 17 l h^{-1} [1]. Because methane represents a loss of feed energy to the animal and a significant source of 'greenhouse' gas, ruminant nutritionists have sought methods of inhibiting ruminal methane production [2]. In the 1970s, researchers in Belgium demonstrated that methanogens could be inhibited by chlorinated hydrocarbons (e.g. chloroform and carbon tetrachloride) [3]. However, these effects did not persist, and a similar adaptation was noted with pyromellitic diimide [4].

The ionophore, monensin, decreases the methane production of cattle, but it does not seem to have a primary effect on methanogens [5]. When mixed ruminal bacteria were treated with monensin, methane production from hydrogen and carbon dioxide did not decrease [6], and later work showed that monensin was inhibiting carbohydrate fermenting bacteria that produced hydrogen [7]. Monensin decreased methane production from formate [8], but Hungate demonstrated that ruminal formate was converted to hydrogen and carbon dioxide before it was consumed by methanogens [9].

Monensin is widely used as a feed additive for beef cattle in the United States [2,5], but many groups have opposed the routine use of antibiotics in animal feed [10]. In vitro experiments indicated that nisin, a bacteriocin produced by *Lactococcus lactis*, could decrease the methane production of mixed ruminal bacteria that were incubated with hay, and this inhibition was correlated with

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[1] Proprietary or brand names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product, and exclusion of others that may be suitable.

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an increase in the ratio of propionate to acetate [11]. However, later work indicated that some ruminal bacteria become nisin-resistant [12], and an in vivo feeding trial indicated that nisin could not increase the acetate to propionate ratio [13].

Recent work indicated that *Streptococcus bovis* HC5 produced a very potent bacteriocin against a variety of Gram-positive bacteria, and bacterial adaptation to it has not been demonstrated [14,15]. The following experiments examined the effect of bovicin HC5 on ruminal methane production in vitro.

2. Materials and methods

2.1. Animals

Three non-lactating dairy cows were fitted with ruminal cannulae (10 cm i.d.) according to surgical procedures approved by the Cornell Institutional Animal Care and Use Committee. The cows were fed medium quality timothy hay (14% crude protein, 40% neutral detergent fiber) ad libitum.

2.2. Ruminal bacteria

Ruminal contents (2 l) were squeezed through four layers of cheesecloth, transported to the laboratory and placed in a water bath (39°C). Once gas production had buoyed the feed particles to the top of the flask and protozoa had sedimented to the bottom, fluid from the middle was collected. The mixed ruminal bacteria (5 ml) were then dispensed anaerobically into tubes (18 mm×150 mm) that were sealed with butyl rubber stoppers and aluminum caps. The tubes had a head space of 21 ml, and 10 ml of hydrogen was added. The tubes were incubated at 39°C on a Labquake shaker (Barnstead/Thermolyne, Dubuque, IA, USA) for 24 h. The mixed ruminal bacteria (2.5 ml) were transferred successively into basal medium (2.5 ml). The basal medium contained (per liter): 292 mg K_2HPO_4, 292 mg $KHPO_4$, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 500 mg cysteine hydrochloride, 25 mg $Na_2S \cdot 9H_2O$, 1 g Trypticase (BBL Microbiology Systems, Cockeysville, MD, USA), 4 g Na_2CO_3, and 0.5 g yeast extract.

2.3. Specific activity of methane production

Head space samples were removed from the Bellco tubes after 0–6 h of incubation with gas-tight syringes. Methane and hydrogen were measured with a Gow Mac (Bound Brook, NJ, USA) gas chromatograph (Carboseive S 8100 mesh column; Supelco Inc., Bellafonte, PA, USA). Bacteria were harvested by centrifugation (10 000×*g*, 10 min, 5°C), washed with 0.9% NaCl (w/v) and treated with 0.2 *N* NaOH (100°C, 15 min) prior to protein determination. Bacterial protein was measured by the method of Lowry et al. [16].

2.4. Preparation and activity of bovicin HC5

Stationary phase *S. bovis* HC5 cultures (1 l, approximately 400 μg ml^{-1}) were heated to 70°C for 30 min, and the cells were harvested by centrifugation (8000×*g*, 15 min, 5°C). The cell pellets were washed in sodium phosphate buffer (50 ml, 5 mM, pH 6.7) and re-suspended in acidic sodium chloride (100 mM, pH 2.0, 4°C, 2 h). The cell suspensions were then re-centrifuged (8000×*g*, 15 min, 5°C), and the cell-free supernatant was lyophilized. The lyophilized material was re-suspended in sterile distilled water (2 ml, 2500 activity U ml^{-1}). The preparation was assayed for antibacterial activity by serially diluting the extract in distilled water (two-fold increments), and placing each dilution (10 μl) on a lawn of *S. bovis* JB1 (approximately 10^5 cfu ml^{-1}). Activity units (expressed per ml) were calculated from the reciprocal of the highest serial dilution showing a visible zone of clearing.

2.5. PCR of archaeal 16S rDNA

Mixed ruminal bacteria were harvested by centrifugation (8000×*g*, 15 min, 5°C). Total DNA was isolated using a Q-BIOgene Fast DNA SPIN kit (Carlsbad, CA, USA) and mini-bead-beater (Biospec Products, Bartlesville, OK, USA). Archaeal 16S rDNA genes were amplified using the archaeal primers, Ar109f (5′-ACG/T GCT CAG TAA CAC GT-3′) and Ar912r (5′-CTC CCC CGC CAA TTC CTT TA-3′) [17]. The protocol had an initial denaturation step (5 min, 94°C) followed by 30 cycles of denaturation (60 s, 94°C), annealing (60 s, 52°C), and extension (90 s, 72°C) in a Sprint Thermocycler (Hybaid Ltd., Middlesex, UK) using PureTaq Ready-To-Go PCR Beads (Amersham, Piscataway, NJ, USA). After terminal extension (6 min, 72°C), samples were kept at 4°C. The PCR products were digested overnight with *Hha*I and *Hae*III (37°C). Restriction fragments were separated on Metaphor agarose gels (3.0%, w/v) and stained with ethidium bromide.

2.6. Statistical methods

All incubations were performed in triplicate with ruminal fluid from three different cows and the standard deviations are reported [18].

3. Results

Mixed ruminal bacteria that were incubated with hydrogen and carbon dioxide produced methane stoichiometrically, and the production rate was first order for approximately 8 h (Fig. 1). Once the hydrogen was depleted, no

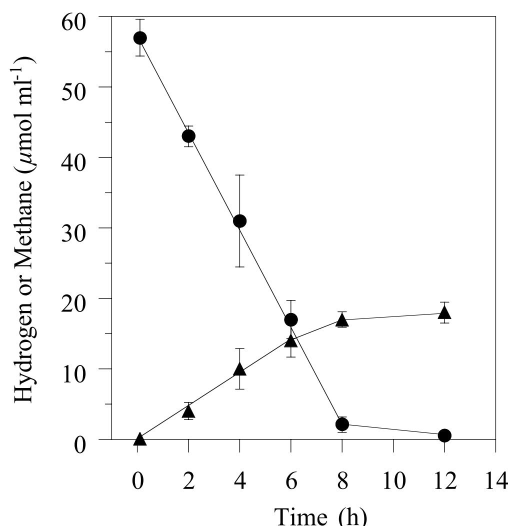

Fig. 1. The methane (▲) production of mixed ruminal bacteria that were incubated in vitro with hydrogen (●) and carbon dioxide.

further increase in methane was detected. If ruminal bacteria were treated with bovicin HC5 for 6 h, the initial rate of methane production decreased by as much as 53%, and large decreases in methane production were observed even if less than 100 AU ml^{-1} were added (Fig. 2). Mixed ruminal bacteria that were transferred successively (50% v/v) in basal medium retained their ability to produce methane from hydrogen and carbon dioxide for a least five transfers, but those treated with 128 AU ml^{-1} of bovicin HC5 lost their ability to produce methane after only four transfers (Fig. 3). When the chromosomal DNA was isolated from the third transfer (Fig. 3), it was possible to amplify archaeal 16S rDNA and separate the PCR products on an agarose gel. Mixed ruminal bacteria from all three cows that were treated with bovicin HC5 had the same dominant band (approximately 800 bp) as those that were not treated. When *Hha*I and *Hae*III were added, at least 12 fragments were detected. The restriction fragment profile of archaeal 16S rDNA from treated and untreated cells was similar (Fig. 4).

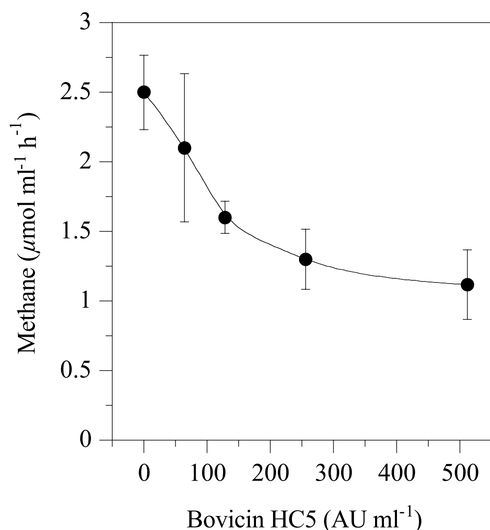

Fig. 2. The effect of bovicin HC5 on the methane production of mixed ruminal bacteria that were incubated in vitro with hydrogen and carbon dioxide.

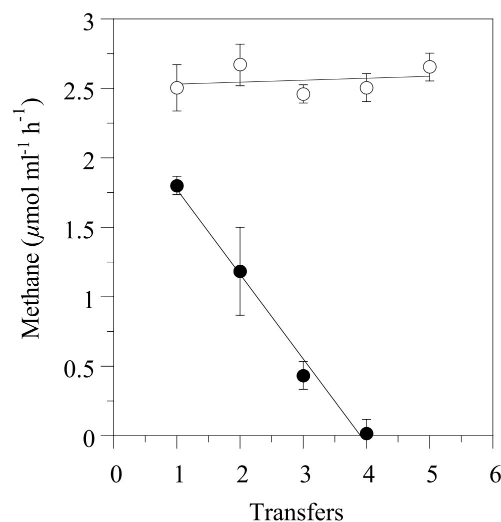

Fig. 3. The methane production of mixed ruminal bacteria that were transferred successively (50% v/v in basal medium) every 24 h. Open symbols show untreated controls. Closed symbols show those that were treated with 128 AU ml^{-1} bovicin HC5.

4. Discussion

Many Gram-positive bacteria produce small peptides that inhibit other Gram-positive species, but bacteriocin activity can be highly species- or even strain-specific [19].

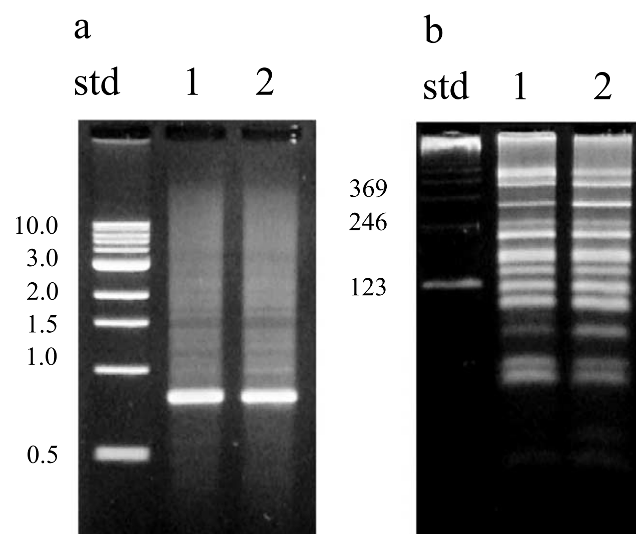

Fig. 4. 16S archaeal rDNA (a) and 16S archaeal rDNA that was digested with *Hha*I and *Hae*III (b). Lanes 1 and 2 show untreated controls and those that were treated with 128 AU ml^{-1} bovicin HC5, respectively. DNA was obtained from the third transfer shown in Fig. 3. The DNA ladder (std) for part (a) was 1 kb. The DNA ladder (std) for part (b) was 123 bp.

The specificity of some bacteriocins is thought to be mediated by specific membrane receptors, but direct evidence is lacking [20]. Most Gram-negative bacteria (e.g. *Escherichia coli*) are resistant to the bacteriocins of Gram-positive bacteria, but they, too, can be inhibited by peptides that disrupt membrane function [21]. These latter peptides (e.g. colicins) are typically larger than the bacteriocins of Gram-positive bacteria, and specific receptors have been identified.

Nisin is the best understood Gram-positive bacteriocin, and it has a broad spectrum of activity [19]. Nisin recognizes the lipid II of the cell membrane, and most eubacteria have this cell wall precursor [22]. Archaea do not have peptidoglycan and many utilize a protein layer to maintain cellular integrity [23]. Whether archaea have lipid II or an analogous anchor has, to our knowledge, not yet been determined. Nisin decreased the methane production of mixed ruminal bacteria, but this effect was explained by its ability to inhibit carbohydrate fermenting bacteria that produced hydrogen [11].

Previous work indicated that the inhibitory activity of *S. bovis* HC5 was mediated by a peptide of approximately 2500 Da [15]. Purified and crude preparations have the same spectrum of activity, and they both inhibited a variety of Gram-positive ruminal bacteria, several lactobacilli, *Bacillus subtilis* and *Listeria monocytogenes* [15]. Bovicin HC5 catalyzed potassium efflux from *S. bovis* JB1 [15], a non-bacteriocin producing strain, and *L. monocytogenes* [24]. *S. bovis* [12] and *L. monocytogenes* [25] can become resistant to nisin after a short period of exposure, but these bacteria did not become highly resistant to bovicin HC5 [14,15].

Some bacteria do not produce bacteriocin until they reach stationary phase [20,26], but continuous cultures of *S. bovis* HC5 produced bovicin HC5 when the rate of glucose consumption was slow enough to relieve the catabolite repression [26]. *S. bovis* HC5 produced more bovicin HC5 at acidic pH values, but even continuous cultures that were maintained at pH 6.7 had significant activity [26]. Based on these results, it appeared the *S. bovis* could produce bovicin HC5 under conditions that would simulate the rumen.

Previous work indicated that approximately 100 AU ml^{-1} of bovicin HC5 would inhibit *S. bovis* JB1 or *L. monocytogenes* [24], and a similar amount of bovicin HC5 inhibited methane production of mixed ruminal bacteria. Even high concentrations of bovicin HC5 did not completely inhibit methane production, but in vivo studies indicate that monensin (the most widely used feed additive) only decreases methane production by 33%. Based on these results, bovicin HC5 has potential as a ruminal methane inhibitor.

Cattle fed forages typically produce more methane and have a higher ruminal pH than those fed grain [27]. The activity of many bacteriocins is highly pH-dependent, and some bacteriocins have little activity at neutral pH [20]. However, our results indicated bovicin HC5 could inhibit ruminal methane production even if the pH was 6.7. Because recent work with *L. monocytogenes* indicated that bovicin HC5 was more active at pH 5.5 than 6.5 [24], it is conceivable that bovicin HC5 would be even more effective in cattle fed grain than those fed forage.

Because chemical inhibitors of ruminal methane production have only been effective for short periods of time and methanogens can adapt [28], we tested the ability of bovicin HC5 to inhibit methane production in a successive fashion. Untreated enrichments retained their ability to produce methane from hydrogen and carbon dioxide at a rapid rate, but those treated with 128 AU ml^{-1} gradually lost activity. By the fourth transfer, methane was no longer being detected. These results indicated that the methanogens did not adapt to bovicin HC5, and there was a gradual decrease in the methanogenic population.

Early work indicated that most ruminal methanogens could be classified as *Methanobrevibacterium ruminantium* [29], but Whitford et al. [30] indicated that the rumen has a highly diverse population of methanogens. When the 16S archeal rDNA from our methanogenic enrichments was amplified, only a single dominant product of approximately 800 bp was observed, but more than 12 fragments were observed if the PCR produce was digested with *Hha*I and *Hae*III. Based on this observation that treated and untreated cultures had a similar fragment pattern, it appears that the effect of bovicin HC5 on ruminal methanogens is not selective.

The contribution of ruminants to greenhouse gas emission and global warming is difficult to estimate [2], but the most recent studies indicate that domestic cattle and sheep could account for as much as 25% of the world's methane production [31]. Given the perception that the routine use of antibiotics in animal feeds should be avoided, on the basis that this use has in at least some cases led to an increase in antibiotic resistance [10], the use of bacteriocins as an alternative strategy for modifying ruminal fermentation is feasible, and our results indicate that bovicin HC5 is effective in decreasing methane.

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