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Short communication

The effects of monensin on amino acid catabolizing bacteria isolated from the Boer goat rumen☆

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ABSTRACT

When ruminants consume feed, as much as half of the amino acid nitrogen can be lost due to microbial degradation in the rumen. Hyper ammonia-producing bacteria (HAB) are primarily responsible for amino acid loss in sheep and cattle, and ionophores (e.g. monensin) are used to inhibit the HAB. Ionophores are sometimes included in the diets of meat goats, but the effects on caprine HAB have not been investigated. Five amino acid catabolizing bacteria were isolated from the rumina of Boer goats, which were consuming a supplement that contained monensin. Two of the isolates were most closely related to the bovine HAB, *Peptostreptococcus anaerobius*, but the other three were more closely related to *Peptoniphilus indolicus*. When the isolates were exposed to monensin, intracellular potassium was lost, and ammonia production was inhibited. However, the *P. indolicus*-like isolates demonstrated a greater capacity to overcome this inhibition in growth experiments. These results indicate that the monensin sensitivity of these bacteria can be variable, even when previous exposure to monensin occurred in the rumen environment.

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1. Introduction

The rumina of cattle and sheep contain bacteria that catabolize amino acids and produce ammonia (Russell et al., 1988; Chen and Russell, 1989; Atwood et al., 1998; Wallace et al., 2003). This ecological guild is termed hyper ammonia-producing bacteria (HAB), and previously described species include *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, *Clostridium aminophilum*, and *Eubacterium pyruvativorans*. Some of the ammonia that is produced by the HAB is assimilated into microbial protein, which is nutritionally available to the animal. However, as much as half of the ammonia can enter the blood and be lost in the urine. Ionophores (e.g. monensin) are often added to the diet to promote growth (Callaway et al., 2003). These membrane-active antimicrobials inhibit the HAB and permit more amino acids to reach the intestines.

Some protein supplements fed to meat goats in the United States contain monensin, and the current study utilized such a supplement. The effects of monensin on caprine HAB have not been described, and it should be noted that the bovine and ovine HAB were isolated from ionophore-naïve animals (Chen and Russell, 1989; Wallace et al., 2003). A thorough comparison of the ruminal flora of goats to other ruminants has not been performed. However, a recent study has shown that differences in rumen microbial communities can even be found between breeds of goats (Shi et al., 2007). Given these facts, it was reasonable to suspect that the caprine bacteria described here would be phylogenetically and physiologically distinct from the bovine and ovine HAB.

☆ 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, nor exclusion of others that may be suitable.

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2. Materials and methods

2.1. Media composition

Basal medium contained (per liter) 240 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 64 mg $CaCl_2 \cdot 2H_2O$, 600 mg cysteine hydrochloride, 4.0 g Na_2CO_3, 0.5 g yeast extract and 1.0 g Trypticase (Fisher BioReagents, Fair Lawn, NJ). The initial pH was adjusted to 6.5 by adding NaOH, and the medium was prepared as previously described (Russell et al., 1988). Casamino acids (Fisher BioReagents, Fair Lawn, NJ) were added aseptically (15 mg ml^{-1} final concentration).

2.2. Bacterial isolation

The Kentucky State University Institutional Animal Care and Use Committee approved animal sampling. The herd (grade Boer goats) was maintained on pasture (primarily Kentucky 31 tall fescue), and a 15% crude protein commercial supplement (wheat middlings, soybean hulls, dried distillers grains, corn screenings, soybean meal, ammonium chloride, and 0.25 g kg^{-1} Rumensin™) was given at 1.15 kg $head^{-1}$ day^{-1} for 30 days prior to sampling. The rumen liquor ($n = 5$ mature bucks) was obtained by gastric intubation. The samples (1 ml) were subjected to 10-fold serial dilution in basal medium to a final dilution of 10^{-12}, and the tubes were incubated (39 °C, 48 h). Tubes with any increase in optical density were plated onto basal medium with Casamino acids (15 mg ml^{-1}) and 1.5% agar in an anaerobic chamber (Coy, Grass Lake, MI; 39 °C, 95% CO_2, 5% H_2). *P. anaerobius* C was previously described (Russell et al., 1988).

2.3. Growth and washed cell experiments

All experiments were performed at 39 °C in basal medium. Optical density was monitored in a Biowave II spectrophotometer (Biochrom, Cambridge, UK). Five percent inocula were used in growth experiments. Sodium monensin (Acros Organics, Geel, Belgium) was added when indicated. Monensin concentrations resulting in a 90% reduction in final optical density are reported. Washed cell experiments were performed with 16 h cultures as previously described (Flythe and Russell, 2005).

2.4. Fermentation products and biochemical analysis

Volatile fatty acids were quantified by HPLC (Dionex, Sunnyvale, CA) with a refractive index detector (Shodex/Showa Denko, Kanagawa, Japan). The column (Aminex HP-87H, Bio-Rad, Hercules, CA) was operated at 50 °C, 0.4 ml min^{-1} flow rate, H_2SO_4 (0.17N) mobile phase. Cell protein was determined using the method of Lowry et al. (1951). Ammonia was quantified using the method of Chaney and Marbach (1962). Urease activity was determined by ammonia production from urea. Indole production was determined in 24 and 48 h cultures with Kovak's reagent. Differential stains were performed with standard methods (Seeley et al., 1998).

2.5. Intracellular potassium

Cells were separated from the culture supernatant by centrifugation through silicone oil as described by Barker and Kashket (1977). The cell pellets were digested in 3N nitric acid, and potassium concentrations were determined by flame photometry (Cole-Parmer 2655-00 Digital Flame Analyzer, Cole-Parmer Instruments, Vernot Hills, Illinois). Control treatments received valinomycin and nigericin (5 mM).

2.6. Phylogenetic analysis

Overnight cells were suspended in water, and incubated (37 °C, 30 min) with lysozyme (Sigma–Aldrich, St. Louis, MO). The cells were subjected to centrifugation and the DNA was precipitated from the supernatant with cold ethanol, dried, and resuspended in water. Amplification of the 16S gene (Lane, 1991) was performed using 1 μl template DNA, PuReTaq™ Ready-to-Go™ PCR beads (GE Healthcare, Buckinghamshire, UK), and 10 pmol primers (IDT, Coralville, IA; 16S rRNA for: AGAGTTTGATCCTGGCTCAG, 16S rRNA rev: ACGGCTACCTTGTTAC GACTT). PCR cycles were: 94 °C for 5 min, 35 cycles of 94 °C for 0.5 min, 55 °C for 1 min, 72 °C for 1 min, final extension step at 72 °C for 5 min. The products were transformed into *E. coli* using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Plasmids were purified (QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA) from four clones per isolate, and the insertions were sequenced using the BigDye™ system and an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Percent identity was determined by pairwise alignment with the Geneious© software package (BioMatters, Aukland, New Zealand). The sequences were deposited in GenBank (Benson et al., 2007), and the accession numbers are: BG1 (EU011743), BG2 (EU011744), BG3 (EU011745), BG4 (EU011746), and BG5 (EU011747).

3. Results and discussion

3.1. Identification and characterization

When rumen fluid samples from five male Boer goats were subjected to serial dilution in basal medium with casamino acids, growth was observed up to 10^{-6} in each case. The enrichments were plated, and a single colony type was observed from each 10^{-6} dilution. These results are consistent with an earlier study that employed oligonucleotide probes to detect bovine HAB (Krause and Russell, 1996). In that case, HAB were detected in the 10^{-6}, but not in the 10^{-8} dilution.

One isolate was selected per animal, and designated BG1 to BG5. All of the isolates grew rapidly in basal medium, and produced ammonia and volatile fatty acids (Table 1). Oxygen was inhibitory to all of the isolates. Microscopic examination revealed coccal cell morphologies, and four of the five were Gram positive. Isolate BG4 did not reproducibly stain Gram positive. No spores were observed (>100 microscopic fields), and heating cultures (80 °C, 30 min) eliminated viability. Glucose stimulated the growth of isolates BG1 and BG2, and some sugars were metabolized to acids even when growth did not occur.

Alignment of the16S sequences revealed that BG1 and BG2 were most closely related to *P. anaerobius* (>95% identity each). Therefore, the bovine HAB, *P. anaerobius* C, was included in this study to provide points of comparison. The other isolates also had close relatives in the family Peptostreptococcaceae. However, BG3, BG4 and BG5 were most closely related to *Peptoniphilus indolicus* (>97% identity each), and this result was supported by the observation that these latter isolates produced indole (Table 1). We believe this is the first report of *Peptoniphilus* species isolated from the gastrointestinal tracts of goats or other ruminant species. Members of the genus are typically associated with anaerobic tissue infections, and *P. indolicus* was first isolated from bovine mastitis (Ezaki et al., 2001).

3.2. Effects of monensin on the isolates

In the first HAB studies, Russell and co-workers described the effects of monensin on the metabolism and bioenergetics of *P. anaerobius*, *C. sticklandii*, and *C. aminophilum* (Russell et al., 1988; Chen and Russell, 1989). These bacteria have rapid rates of ammonia production when incubated in the presence of amino acids. Likewise, washed cell suspensions of the caprine isolates had rapid rates of ammonia production that were linear for at least 60 min. Under our experimental conditions, *P. anaerobius* C produced 750 nmol NH_3 mg cell $protein^{-1}$ min^{-1}. BG1 and BG2 produced about 600 nmol NH_3 mg cell $protein^{-1}$ min^{-1}. BG3, BG4 and BG5 produced approximately 300 nmol NH_3 mg cell $protein^{-1}$ min^{-1}. These rates are similar to previously described HAB, which indicates