APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2010, p. 7482–7490 Vol. 76, No. 22
0099-2240/10/$12.00 doi:10.1128/AEM.00388-10

Rumen Microbial Population Dynamics during Adaptation to a High-Grain Diet▽†

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Received 11 February 2010/Accepted 8 September 2010

High-grain adaptation programs are widely used with feedlot cattle to balance enhanced growth performance against the risk of acidosis. This adaptation to a high-grain diet from a high-forage diet is known to change the rumen microbial population structure and help establish a stable microbial population within the rumen. Therefore, to evaluate bacterial population dynamics during adaptation to a high-grain diet, 4 ruminally cannulated beef steers were adapted to a high-grain diet using a step-up diet regimen containing grain and hay at ratios of 20:80, 40:60, 60:40, and 80:20. The rumen bacterial populations were evaluated at each stage of the step-up diet after 1 week of adaptation, before the steers were transitioned to the next stage of the diet, using terminal restriction fragment length polymorphism (T-RFLP) analysis, 16S rRNA gene libraries, and quantitative real-time PCR. The T-RFLP analysis displayed a shift in the rumen microbial population structure during the final two stages of the step-up diet. The 16S rRNA gene libraries demonstrated two distinct rumen microbial populations in hay-fed and high-grain-fed animals and detected only 24 common operational taxonomic units out of 398 and 315, respectively. The 16S rRNA gene libraries of hay-fed animals contained a significantly higher number of bacteria belonging to the phylum *Fibrobacteres*, whereas the 16S rRNA gene libraries of grain-fed animals contained a significantly higher number of bacteria belonging to the phylum *Bacteroidetes*. Real-time PCR analysis detected significant fold increases in the *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* populations during adaptation to the high-concentrate (high-grain) diet, whereas the *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* populations gradually decreased as the animals were adapted to the high-concentrate diet. This study evaluates the rumen microbial population using several molecular approaches and presents a broader picture of the rumen microbial population structure during adaptation to a high-grain diet from a forage diet.

The rumen is a complex microbial ecosystem that is composed of an immense variety of bacteria, protozoa, fungi, and viruses (5). Among these microorganisms, bacteria are the most investigated population and have a significant effect on the animal's performance. However, our understanding of how rumen bacteria change and adapt to different ruminal environments is in its infancy.

In the feedlot cattle industry, when animals on a forage diet are directly put on a high-grain diet, a decrease in ruminal pH due to lactate production has been observed (23, 31, 42), which leads to the possibility of digestive disorders, which can cause a decrease in the animal's performance (23, 45). Therefore, feeding programs have been implemented to adapt feedlot cattle from a high-forage diet to a high-concentrate diet by gradually increasing the concentration of grain in the diet and decreasing the fiber content (2, 35). During this adaptation to high-grain diets, significant changes in the ruminal environment and rumen bacterial population structure have been reported (17, 46, 48). However, the microbial changes that occur during this transition phase are poorly understood (17, 21, 26, 46). Studies performed to date have utilized culture-based techniques or have looked at the fluctuation of a few indicator bacteria (48, 47) to evaluate bacterial population changes. Due to limitations in culturing rumen bacteria, the use of culture-based techniques to evaluate bacterial populations substantially underestimates the diversity of microorganisms within the rumen. In this study, we have utilized culture-independent approaches to evaluate bacterial population structure and diversity using terminal restriction fragment length polymorphisms (T-RFLPs) and sequence analysis of 16S rRNA gene libraries to compare the rumen bacterial population structure in animals on prairie hay against that in animals adapting to a high-concentrate (high-grain) diet. We have also quantified the fluctuations in the populations of previously reported indicator bacterial species using quantitative real-time PCR (qRT-PCR) to assess the role of these organisms during adaptation to a high-concentrate diet.

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† This article was approved for publication by the director of the Oklahoma Agricultural Experiment Station.

▽ Published ahead of print on 17 September 2010.

MATERIALS AND METHODS

Animals and diets. Eight ruminally cannulated beef steers (weight, 380 ± 27 kg) were fed prairie hay *ad libitum* for a period of 2 weeks. Following adaptation to prairie hay, four steers were randomly selected and were shifted to a step-up diet regimen containing incrementally increased amounts of metabolizable en-

ergy (ME) with constant incremental increases in the grain level. The step-up diets were formulated to meet the animals' nutrient requirements, as described by the National Research Council (29a), and were composed of 2.0, 2.4, 2.7, or 3.0 Mcal of ME/kg of dry matter with fiber-to-concentrate (grain) ratios of 80:20 (diet 1), 60:40 (diet 2), 40:60 (diet 3), and 20:80 (diet 4), respectively. The four steers selected were fed each diet for 7 days and were then moved to the next stage of the diet (e.g., all four animals were fed diet 1 containing 2.0 Mcal of ME/kg of dry matter with a fiber-to-grain ratio of 80:20 for 7 days and then shifted to diet 2 containing 2.4 Mcal of ME/kg of dry matter with a fiber-to-grain ratio of 60:40). The remaining four animals were maintained on prairie hay throughout the sampling period and were used as control animals to compare microbial shifts during adaptation to the high-grain diet from prairie hay. The total duration of the experiment was 6 weeks.

Sampling. Ruminal content (partially digested feed [solid] plus rumen fluid) was collected via a ruminal cannula from the dorsal sac after mixing of the contents. Sampling was done after 7 days of adaptation to each diet. The samples collected were snap-frozen in liquid nitrogen and were stored at −20°C until DNA extraction.

DNA isolation. Frozen rumen samples were snap-frozen in liquid nitrogen and homogenized (~2 to 3 min) using a domestic coffee grinder. Additional liquid nitrogen was added to the sample to maintain the cold chain during sample processing. Total DNA was extracted from 0.5 g of homogenized sample using a QIAamp DNA stool minikit (Qiagen, Valencia, CA), according to the manufacturer's protocol but with a few modifications. The modifications included (i) incubation of the sample at 95°C for 10 min after ASL buffer addition and (ii) performance of the proteinase K digestion for 30 min.

T-RFLP analysis. T-RFLP analysis (22, 27, 32) was performed using 100 ng of isolated total DNA. PCR amplification of the 16S rRNA gene was performed in a dyad thermocycler (MJ Research, Watertown, MA) using a 6-carboxyfluorescein (6-FAM)-labeled forward primer (primer FAMBacT0008F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and an unlabeled reverse primer (primer BacT0805R, 5′-GGACTACCAGGGTATCTAATCCC-3′). The 50-μl PCR mixture contained 100 ng of DNA, 1× PCR buffer (Promega, Madison, WI), 1.5 mM $MgCl_2$, 400 nM each primer, 200 μM deoxynucleoside triphosphates (dNTPs), 100 ng/μl bovine serum albumin (BSA), and 3 U *Taq* DNA polymerase (Promega). The cycling conditions were 1 cycle of 1 min at 95°C, 30 s at 52°C, and 1 min at 72°C, followed by 34 additional cycles of 30 s at 95°C, 30 s at 52°C, and 1 min at 72°C and a final extension step of 3 min at 72°C.

PCR products were ethanol precipitated (38), resuspended in 5 μl of distilled water, and independently digested with RsaI (Invitrogen, Carlsbad, CA), HaeIII (Invitrogen), and MspI (Promega). A 10-μl restriction digest mixture contained 1× buffer, 2.5 U enzyme, and 5 μl of the PCR product. The MspI digestion mixture contained an additional 250 ng/μl of BSA. All reaction mixtures were incubated at 37°C for 4 h, followed by 65°C for 20 min. Digested PCR product (2 μl) was mixed with 0.5 μl of GeneScan ROX1000 size standard (Applied Biosystems, Foster City, CA) and 3.5 μl of loading dye (Applied Biosystems) and was electrophoresed for 8 h in an ABI 377 sequence analyzer (Applied Biosystems). The resulting data were analyzed using GeneScan (version 3.1) analysis software (Applied Biosystems). The data sets were subsequently normalized by dividing the cumulative peak height of each sample by the height for the sample with the smallest cumulative peak height. The normalized T-RFLP profiles for each restriction enzyme were aligned using the T-Align program (43), and principal component analysis (PCA) was performed using the UnscramblerX program (CAMO Software Inc., Woodbridge, NJ) to identify shifts in microbial population structure. In addition, phylogenetic assignment of the T-RFLP profiles was performed using a custom database generated from 16S rRNA reads available at the Ribosomal Database Project (7) with the help of the Phylogenetic Analysis Tool (PAT) (22).

Construction and sequence analyses of 16S rRNA gene libraries. Four 16S rRNA gene libraries were constructed from the bacteria from animals on the high-concentrate diet and on prairie hay. Two libraries were constructed from the bacteria from grain-fed animals by pooling equal amounts of DNA from animals on the 80:20 (grain/hay) diet. The other two libraries were constructed from the bacteria from hay-fed animals. One library for hay-fed animals was constructed by pooling the DNA from four different animals maintained on prairie hay, while the other library was constructed using the DNA from a single animal which was later transitioned to the high-concentrate diet. A DNA fragment of ~797 bp (based on the *Escherichia coli* numbering) of the 16S rRNA gene was PCR amplified using primers BacT0008F (5′-AGAGTTTGATCCTGGCTCAG-3′) and BacT0805R (5′-GGACTACCAGGGTATCTAATCCC-3′) and a high-fidelity *Taq* DNA polymerase. The 50-μl PCR mixture contained 100 ng of DNA, 1× PCR buffer (Invitrogen) 1.5 mM $MgCl_2$, 400 nM each primer, 200 μM dNTPs, and 2 U *Taq* DNA polymerase (Invitrogen). Thermal cycling conditions were 1 cycle of 2 min at 95°C, 30 s at 52°C, and 1 min at 68°C, followed by 34 additional cycles of 20 s at 95°C, 30 s at 52°C, and 1 min at 68°C and a final extension of 3 min at 68°C. The blunt-ended PCR products generated were ligated into a PCR-Blunt (Invitrogen) plasmid vector and transformed into *E. coli* DH5α maximum-efficiency competent cells (Invitrogen). Random libraries of approximately 384 colonies were picked from each library and grown, and plasmid DNA was isolated for sequence analyses. Sequencing reactions were performed bidirectionally, as described previously (4), on an ABI 3700 capillary DNA sequencer. The resulting sequences were base called, analyzed, and assembled into contigs using the Phred, Phrap, and Consed suite of software (13, 12, 18). The resulting data were further analyzed using the NCBI BLAST program and tools available at the Ribosomal Database Project (7, 8).

Phylogenetic and comparative analyses. The assembled reads were aligned using Infernal (30) secondary structure-based alignment software, and contigs were generated at 97% similarity. Phylogenetic analysis of the contigs and singleton reads were performed using the Phyml program (19) with 100 bootstrap replications. A phylogenetic tree was constructed using the maximum-likelihood method and was visualized using the iTOL program (25). The Ribosomal Database Project classifier software tool (50) was used to classify the sequences into taxonomic units, and the library compare software tool was used to compare the libraries with each other to identify statistically significant changes in microbial population structure (7, 8). The Mothur analysis tool (40) was used for cluster analysis of sequences and for generation of diversity statistics.

Quantitative real-time PCR analyses. Fluctuations in the levels of previously isolated microbial species (*Prevotella bryantii*, *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Megasphaera elsdenii*, *Streptococcus bovis*, *and Butyrivibrio fibrisolvens*) were evaluated using qRT-PCR. The assays included samples from all four animals from each diet (biological replicates) and two technical replicates of each sample. Therefore, a single assay included 40 × 2 samples, in addition to the negative controls. Each real-time PCR assay was performed using a SYBR green I reporter assay kit (Roche Diagnostics, Indianapolis, IN). The dynamic range and PCR efficiency of each assay were evaluated using positive controls. The primers and PCR conditions are shown in Table 1. For all assays, the PCR products generated were sequenced to verify amplification of the correct bacterial species.

A 15-μl reaction mixture contained 400 nM (each) forward and reverse primer, 1× master mixture (Roche Diagnostics), and 30 ng of rumen DNA. Thermal cycling conditions were 95°C for 10 min, followed by 50 additional cycles of 95°C for 30 s, 50 to 62°C for 30 s (depending on the annealing temperature of each primer set [Table 1]), and 72°C for 40 s or 1 min, on the basis of the size of the amplicon, and finally, a melting curve was prepared. A universally conserved single-copy gene, *rpoB*, was used for normalization (10, 39). The qRT-PCRs were performed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Relative quantification of bacterial population changes was performed using the comparative threshold cycle (C_T) method, as described previously (20). Statistical analysis was performed using the PROX MIXED program in SAS software to identify statistically significant fluctuations in the population.

Nucleotide sequence accession numbers. All sequence data generated in this study have been submitted to GenBank under accession numbers HM104710 to HM105497.

RESULTS

T-RFLP analysis. T-RFLP analysis displayed distinct differences in the bacterial population structures between prairie hay-fed animals and animals adapted to a high-concentrate diet (Fig. 1). Principal component analysis displayed a shift in microbial population structure by diet 3 (60% corn, 40% hay), and the shift was more apparent by diet 4 (80% corn, 20% hay). The phylogenetic assignment of the terminal restriction fragments annotated only 30% to 50% of the terminal fragments. Thus, the phylogenetic assignments cannot be used to identify trends in the change in the microbial population structure and are therefore used only to identify what bacterial candidate species were present in the sample. On the basis of the phylogenetic assignment, the ratios of the phyla *Firmicutes*/*Bacteroidetes* were compared between hay-fed animals and animals adapted to the high-concentrate diet (Fig. 2). There was

TABLE 1. Primer sequences used in real-time PCR analyses

Primer	Sequence 5′–3′	Product size (bp)	T_m[a] (°C)	Reference
Prevotella bryantii-For	ACTGCAGCGCGAACTGTCAGA	421	58	46
Prevotella bryantii-Rev	ACCTTACGGTGGCAGTGTCTC			
Fibrobacter succinogenes-For	GGTATGGGATGAGCTTGC	445	62	46
Fibrobacter succinogenes-Rev	GCCTGCCCCTGAACTATC			
Selenomonas ruminantium-For	TGCTAATACCGAATGTTG	513	53	46
Selenomonas ruminantium-Rev	TCCTGCACTCAAGAAAGA			
Megasphaera elsdenii-For	GACCGAAACTGCGATGCTAGA	128	60	33
Megasphaera elsdenii-Rev	TCCAGAAAGCCGCTTTCGCCACT			
Streptococcus bovis-For	ATTCTTAGAGATAGGGTTTCTCTT	134	60	This study
Streptococcus bovis-Rev	ACCTTATGATGGCAACTAACAATA			
Butyrivibrio fibrisolvens-For	CGCATGATGCAGTGTGAAAAGCTC	625	56	This study
Butyrivibrio fibrisolvens-Rev	CCTCCCGACACCTATTATTCATCG			
rpoB-For	AACATCGGTTTGATCAAC	371–390	53.5	10
rpoB-Rev	CGTTGCATGTTGGTACCCAT			

[a] T_m, melting temperature.

no significant change in the ratio between hay-fed animals and high-concentrate-fed animals until diet 3, when the ratio was higher in hay-fed animals than in grain-fed animals. T-RFLP analyses identified a total of 115 different bacterial genera among the animals on prairie hay and high-concentrate diets.

16S rRNA gene library and phylogenetic analysis. The comparison of 16S rRNA gene libraries using the classifier software tool revealed significant changes in rumen bacterial population diversity and structure between animals on prairie hay and animals on a high-concentrate diet (Fig. 3). The two libraries from animals on prairie hay were not significantly different from each other. Similarly, the two libraries from animals on a high-concentrate diet were not significantly different from each other. Animals on prairie hay displayed a significantly higher number of bacteria belonging to the phylum *Fibrobacteres* than animals on a high-concentrate diet (Fig. 3A). Animals on a high-concentrate diet displayed a significantly higher number of bacteria belonging to the phylum *Bacteroidetes* than animals fed prairie hay (Fig. 3A). All the bacteria detected in the phylum *Fibrobacteres* belonged to the genus *Fibrobacter*, while a majority of the bacteria detected in the phylum *Bacteroidetes* belonged to the genus *Prevotella*. Although the fluctuations in *Firmicutes* were not statistically significant, further analysis of members within the phylum detected significantly higher numbers of organisms belonging to the families *Clostridiaceae* and *Acidaminococcaceae* in animals fed a high-concentrate diet (Fig. 3B).

Hierarchical classification of the 16S rRNA gene libraries attributed 620 clones from animals on the prairie hay diet and 677 clones from animals on the high-concentrate to the domain *Bacteria* and 5 clones to the domain *Achaea*. Among the 620 sequences generated from the 16S rRNA libraries from animals fed prairie hay, 15 clones belonged to the phylum *Spirochaetes*, 19 to the *Fibrobacteres*, 219 to the *Firmicutes*, 15 to the *Proteobacteria*, and 147 to the *Bacteroidetes*. Out of the 677 sequences from the 16S rRNA libraries from animals fed a high-concentrate diet, 24 clones belonged to the *Spirochaetes*, 2 to the *Fibrobacteres*, 271 to the *Firmicutes*, 6 to the *Proteobacteria*, and 303 to the *Bacteroidetes*. The rest of the clones (202 from prairie hay-fed animals and 67 from high-concentrate-fed animals) could not be classified into a phylum. Comparison of the ratios of *Firmicutes*/*Bacteroidetes* predicted using the classifier software tool (see Material and Methods) showed that hay-fed and high-concentrate-fed animals displayed ratios of 1.49 and 0.89, respectively. Phylogenetic analysis demonstrated significant bacterial diversity both in animals fed prairie hay and in animals fed a high-concentrate diet (Fig. 4). Libraries

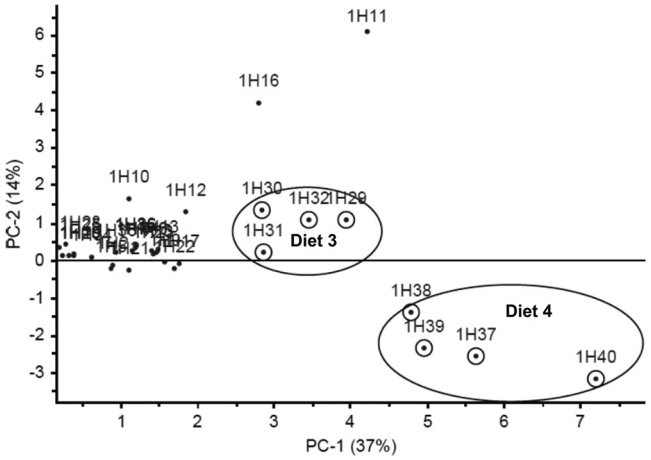

FIG. 1. PCA of T-RFLP data. A shift in bacterial population structure begins by diet 3 (60:40 grain/hay) and is more apparent by diet 4 (80:20 grain/hay).

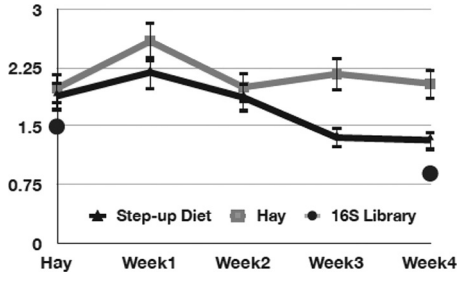

FIG. 2. *Firmicutes*/*Bacteroidetes* ratio during adaptation to high-concentrate diet based on T-RFLP analysis and 16S rRNA library analysis. Gray line, prairie hay; black line, high-concentrate diet; black filled circles, rRNA 16S libraries.

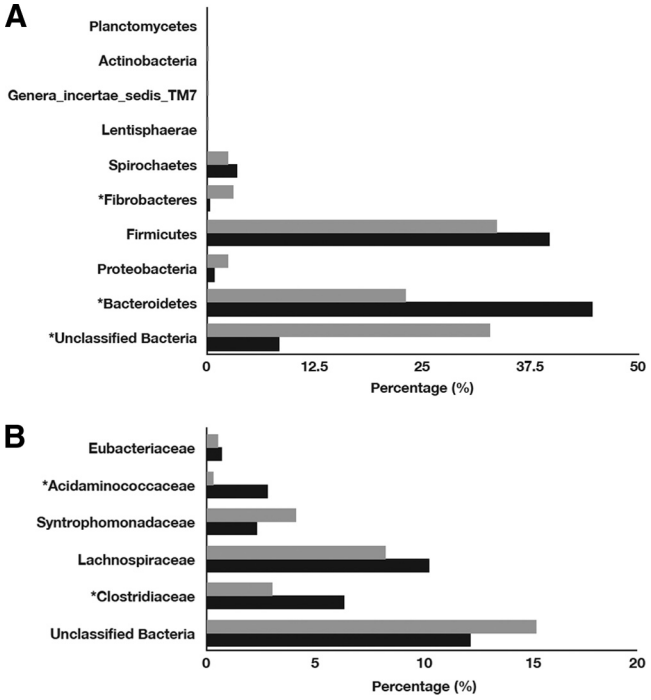

FIG. 3. Comparison of 16S rRNA gene libraries at the phylum level. Populations that are significantly different at a P value of >0.01 are indicated by an asterisk. (A) Total population (prairie hay [gray bars] versus high-concentrate diet [black bars]); (B) distribution of organisms within the phylum *Firmicutes* among animals on prairie hay (gray bars) versus a high-concentrate diet (black bars).

constructed from animals on prairie hay consisted of 398 different operational taxonomic units (OTUs), and libraries constructed from animals on a high-concentrate diet had 315 OTUs. There were only 24 OTUs that were shared between the animals on the two different diets. A significantly larger number of clones belonging to the phylum *Firmicutes* was detected in animals fed prairie hay (Fig. 4). These *Firmicutes* clones accounted for 44% of the total bacterial sequences in the libraries from animals fed prairie hay. The libraries constructed from animals on a high-concentrate diet displayed a significantly higher number of *Bacteriodetes* spp. (Fig. 4). A few *Archaea* were also detected from the libraries constructed from animals fed prairie hay. These sequences belonged to the genus *Methanobrevibacter*.

The diversity statistics (4) calculated for each diet estimated significant diversity within the rumen and had Chao1 values of 2,725 and 1,177 for hay-fed and grain-fed animals, respectively, and ACE values of 4,445 and 2,699 for hay-fed and grain-fed animals, respectively.

Quantitative real-time PCR analysis. The qRT-PCR results are summarized in Fig. 5. qRT-PCR analysis displayed fold increases in the *Megasphaera elsdenii*, *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii* populations during adaptation to the high-concentrate diet, whereas the *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* populations gradually decreased as animals were adapted to the high-concentrate diet.

DISCUSSION

Programs aimed at adaptation of animals to a high-grain diet are widely used in the U.S. feedlot cattle industry in an effort to balance enhanced growth against the risk of acidosis (2). During adaptation to high-grain diets, significant changes in the ruminal environment and microbial populations have been reported (17, 46, 47). This stepwise adaptation to a high-grain diet from a high-forage diet is known to help establish a stable microbial population within the rumen (23, 2). The change in the rumen microbial population structure due to a change in diet is of great interest, as it increases the energy density within the rumen and helps improve feed efficiency and average daily gain (ADG) (2). However, microbial population dynamics during this transition phase are poorly understood, as only a few studies have been reported (6, 17, 48). Most of these studies have utilized culture-based techniques on a few indicator species, which have limited these studies to a few rumen bacterial species (46, 48, 51). This study, based on culture-independent molecular methods, presents a broader picture of the rumen microbial population dynamics during adaptation to a high-concentrate diet from a forage diet.

In this study we detected a significant change in population structure during adaptation to a high-concentrate diet from prairie hay (Fig. 1). When the animals on prairie hay were first put on the step-up diet, no significant change in population structure was detected in the principal component analysis. However, by diets 3 and 4 the change in microbial population structure was clearly apparent. This change in microbial population structure and diversity may be due to the increased fermentable substrate present in the diet favoring the growth of amylolytic and other starch-digesting bacterial species. Goad and coworkers (17) also detected a similar change in the rumen microbial population numbers, where they observed an increase in the total amount of viable anaerobic and amylolytic bacteria in animals fed high-concentrate compared to the amount in animals fed prairie hay. Analysis of the T-RFLP data using the Ribosomal Database Project 16S rRNA database was able to assign phylogeny to only 30% to 50% of the terminal fragments generated, suggesting the presence of a large number of uncharacterized bacteria within the rumen. Therefore, the phylogenetic assignments were used only to identify the bacterial species present in animals on each diet.

A majority of the bacteria identified during diets 3 and 4 were *Proteobacteria*. *Proteobacteria* are predominantly composed of Gram-negative bacteria, which have highly diverse metabolic functions (15, 16). The increased detection of *Proteobacteria* during diets 3 and 4 suggests an increase in the numbers of bacterial species that are metabolically capable of handling the newly available fermentable carbohydrates. The increasing numbers of *Proteobacteria* detected by T-RFLP analysis was not consistent with the results obtained by use of the 16S rRNA libraries, which did not detect an increase in *Proteobacteria*. This can be explained by looking at the 16S rRNA reads available in the databases. A majority of the reads in databases and sequenced genomes are from *Proteobacteria*; thus, out of the T-RFLP fragments characterized, a majority represent *Proteobacteria* due to the availability of more proteobacterial sequence information. *Firmicutes* were abundant in the 16S rRNA libraries and also in the T-RFLP profiles for

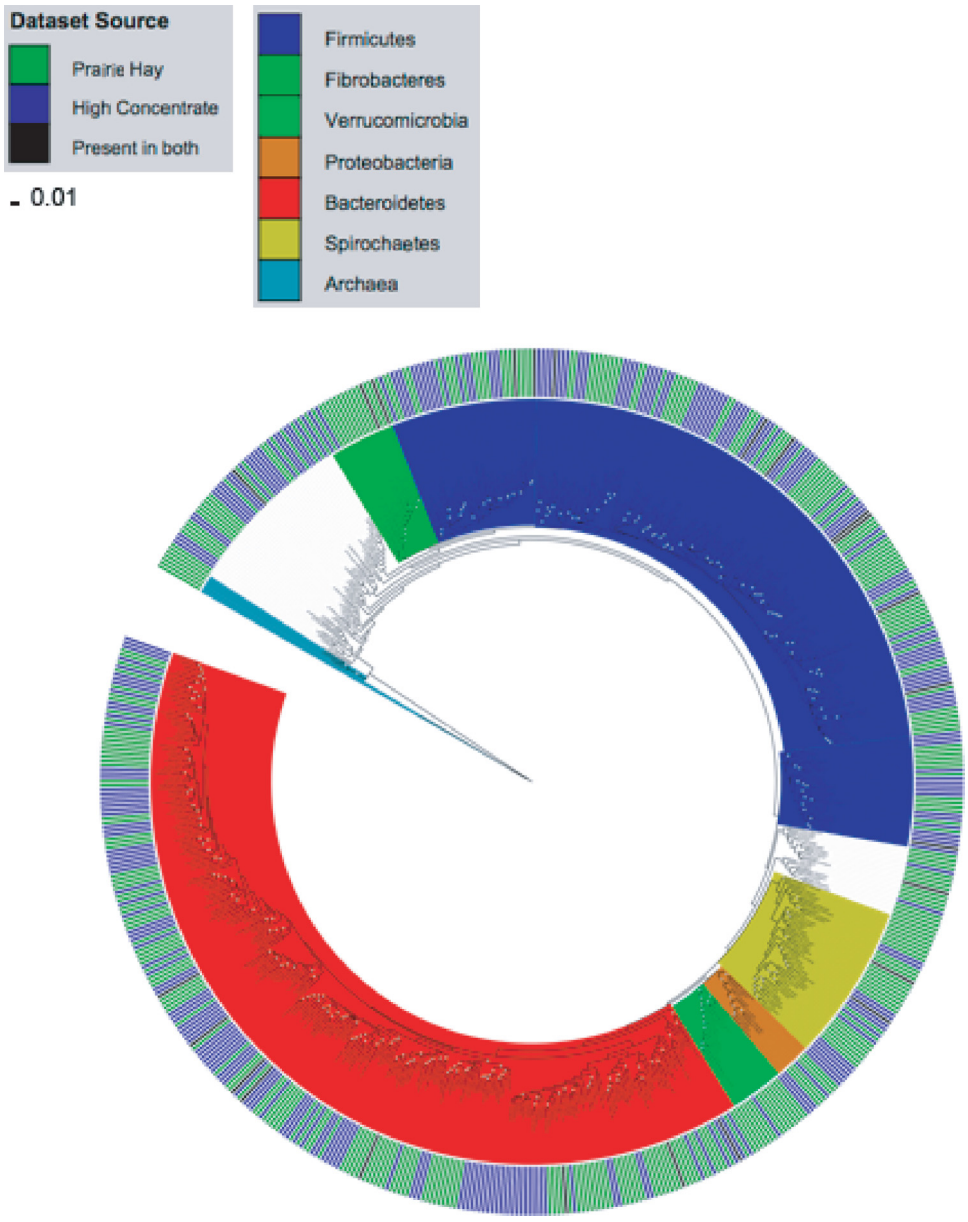

FIG. 4. Phylogenetic analysis of libraries constructed from hay-fed animals and grain-fed animals. A consensus phylogenetic tree constructed using the maximum-likelihood method is shown. The scale bars indicate the length of 1 substitution per 100 residues. Phylogenetic analysis was performed on a nonredundant set of OTUs identified from each treatment (diet). The inner ring shows the distribution of each clone at the phylum level (see the key); the white region represents unclassified sequences. The outer ring shows where each clone originated. Green, prairie hay; blue, high concentrate; black, both.

both diets. *Firmicutes* are mainly comprised of Gram-positive, low-G+C-content bacteria (3). Thus, the presence of *Firmicutes* in high numbers in both ruminal environments suggests that *Firmicutes* represent a core bacterial component within the rumen. The ratios between *Firmicutes* and *Bacteroidetes* in the T-RFLP analysis (Fig. 2) displayed a significant difference by diet 3, when the ratio was larger in hay-fed animals than in grain-fed animals. This observation was consistent with the results obtained with the 16S rRNA library sequences, where ratios of 1.49 and 0.89 were observed in hay-fed and high-concentrate-fed animals, respectively (Fig. 2). This is contrary to the ratios observed in the human gut, where an increase in weight gain was reflected by a higher *Firmicutes*/*Bacteroidetes* ratio (49). However, the gastrointestinal tracts of humans and ruminants are completely different, with humans being hindgut fermenters and cattle being foregut fermenters (5, 52). As such, it is possible that in foregut fermenters the *Firmicutes*/*Bacteroidetes* ratio is lower during weight gain.

T-RFLP analysis identified over 350 bacterial species belonging to 115 different genera. The bacterial genera identified included many previously described genera. These include *Bifidobacterium* spp., *Butyrivibrio* spp., *Eubacterium* spp., *Lactobacillus* spp., *Prevotella* spp., *Ruminococcus* spp., *Selenomonas* spp., *Streptococcus* spp., *Fusobacterium* spp., and *Pep-*

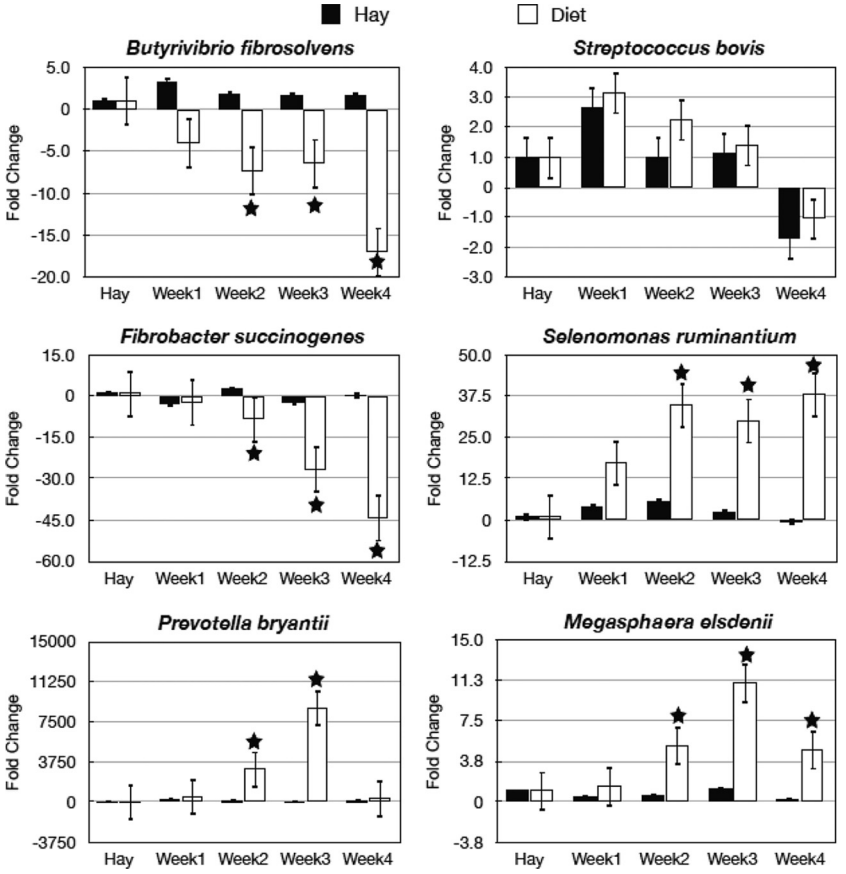

FIG. 5. qRT-PCR-based population changes of some selected rumen bacterial species during adaptation to a high-concentrate diet. Assays included four biological replicates and two technical replicates. Changes in population are shown as the fold change compared to the size of the population when the animals were fed hay during the adaptation phase. *rpoB* was used for normalization, and fold changes in bacterial populations were calculated as described in Materials and Methods.

tostreptococcus spp. T-RFLP analysis, although reliable and highly reproducible, allows identification only of previously characterized bacterial species. In addition, multiple bacterial species may share the same terminal restriction fragment. Thus, additional independent molecular approaches are needed to verify terminal restriction fragment analysis results.

For definitive identification of the bacterial species in the samples analyzed, we constructed four 16S rRNA gene libraries and sequenced 384 clones from each library (4×384). Sequence analysis of the reads from animals fed prairie hay displayed a significantly larger number of bacteria belonging to the phylum *Bacteroidetes*. This *Bacteroidetes* population accounted for a majority of the clones sequenced and was composed of bacteria belonging to the genera *Prevotella*, *Anaerophaga*, and *Tannerella*. However, 82% of the bacteria belonging to the phylum *Bacteroidetes* were unclassified *Bacteroides* spp., suggesting that a greater number of bacterial species within the rumen are yet to be characterized. The libraries from prairie hay-fed animals displayed significantly higher numbers of bacteria belonging to the phylum *Fibrobacteres*. *Fibrobacter* spp. have been identified as the major cellulolytic bacterial species present within the rumen (14, 24, 46); as such, this observation of *Fibrobacter* species within our library is consistent with previous reports. Although we did not detect significant changes in total rumen *Firmicutes* populations, the hierarchical classification of the microbial populations within the phylum *Firmicutes* revealed significant differences between the *Firmicutes* populations in animals on the two diets (Fig. 3B). The bacterial populations in animals fed high-concentrate diet contained more species belonging to the genera *Mitsuokella*, *Anaerovibrio*, and unclassified *Clostridiaceae*. *Mitsuokella* is an amylolytic organism that flourishes in the presence of fermentable sugars (44); therefore, an increase of *Mitsuokella* isolates during adaptation to a high-concentrate diet was expected. We also detected the presence of a few *Archaea* belonging to the genus *Methanobrevibacter* within our libraries from animals fed prairie hay. Previous studies using *Archaea*-specific primers have demonstrated that *Methanobrevibacter* is a common inhabitant in the bovine rumen and is present in forage diets (41).

Hierarchical classification further demonstrated that 202 and 67 sequences from libraries constructed from animals fed prairie hay and a high-concentrate diet, respectively, were uncharacterized. This validates our previous assertion that the bovine rumen microbiome is yet to be properly characterized and is confirmed by diversity estimates that predict >1,700 bacterial species based on chao1 and ACE. In addition, the significantly higher number of unclassified bacteria in prairie

hay-fed animals suggests that the ruminal bacterial diversity in animals on forage-based diets is higher than that in grain-fed animals. The diversity statistics also support this notion, as the predicted chao1 and ACE values are much higher for hay-fed animals than grain-fed animals.

Phylogenetic analysis was performed on the sequenced 16S rRNA gene libraries to identify the phylogenetic relatedness among the bacterial sequences (Fig. 4). This analysis demonstrated significant diversity among animals fed prairie hay and those on a high-concentrate diet, suggesting that the rumen microbiome is composed of a diverse bacterial population with flexible metabolic capabilities. This was apparent by the fact that only 24 OTUs were common between the animals on the two diets, showing minimum overlap and the presence of distinct microbial population structures. This also suggests that the rumen microbial environment is highly dynamic and changes constantly. The libraries constructed from animals fed prairie hay contained 398 OTUs, and the libraries constructed from animals fed a high-concentrate diet displayed 315 OTUs. Although the numbers of OTUs identified were relatively similar, the distribution and relatedness of the 16S rRNA sequences were significantly different among the two ruminal environments (Fig. 4). This suggests that when animals are shifted from a forage diet to a high-concentrate diet, the microbial diversity in terms of the number of different species remains somewhat similar but the composition or the species makeup changes significantly to adapt to the new ruminal environment. Phylogenetically distant *Firmicutes* observed in prairie hay-fed animals accounted for more than 44% of the total sequences and were composed of *Anaerobaculum*, *Butyrivibrio*, *Acetivibrio*, and other *Clostridium* spp. The few *Archaea* identified in animals on prairie hay belonged to the genus *Methanobrevibacter*. Although we were initially surprised at the observation of archaeal sequences, further analysis of these primers reveals that they do amplify a small group of *Archaea* (1). We also detected a phylogenetically diverse *Bacteroidetes* population in animals fed a high-concentrate diet. This *Bacteroidetes* population was twice the size of the population found in animals fed prairie hay. The *Bacteroidetes* population observed in grain-fed animals was mainly composed of unclassified *Bacteroidetes* spp. but also contained a high number of bacteria belonging to the genera *Anaerophaga* and *Prevotella*. The rarefaction curves for the two diets and the chao1 and ACE diversity estimates show that the rumen microbial population is highly complex and diverse.

In addition to T-RFLP analysis and 16S rRNA libraries, we also evaluated microbial population changes using real-time PCR analysis. Real-time PCR was used to quantify the population shifts of some of the better-characterized rumen microbial species. The prevalence of *Fibrobacter succinogenes* gradually decreased as animals were adapted to a high-concentrate diet, and their numbers were 40-fold lower than those in animals on prairie hay. *Fibrobacter succinogenes* is a fibrolytic bacterium that digests fiber (24, 46) and is predominantly present in diets high in fiber. Therefore, *Fibrobacter* populations were expected to drop during adaptation to a high-grain diet. The 40-fold decrease in the numbers of *Fibrobacter* isolates detected was consistent with previous observations reported by Tajima et al. (2001), who reported a 20-fold decrease in population size by day 3 and a 57-fold decrease by day 28 in animals on high-concentrate diets (46). The population of *Butyrivibrio fibrisolvens* also declined 20-fold during adaptation to a high-concentrate diet. *Butyrivibrio fibrisolvens* is also known to be a fibrolytic organism, but it also has a high affinity toward maltose and sucrose utilization (36) and produces butyrate (17). As such, *Butyrivibrio fibrisolvens* is an organism capable of utilizing both cellulose and starch. The population of *Butyrivibrio fibrisolvens* had a slight decrease during the first three stages of the step-up diet (~5-fold) and decreased 20-fold on the 4th diet. This suggests that *Butyrivibrio fibrisolvens* is able to utilize both fiber and concentrate. However, the drop in the *Butyrivibrio fibrisolvens* population during diet 4 may be because of ruminal pH changes due to the increased amount of fermentable substrates present within the rumen. This is consistent with the results of a recent study that shows *Butyrivibrio fibrisolvens* populations increasing in high-fiber diets and decreasing in high-energy diets (28). Quantitative real-time PCR analysis displayed an 11-fold increase in the *Megasphaera elsdenii* population by diet 3 of the step-up diet and a 6-fold decrease by diet 4. *Megasphaera elsdenii* is one of the most widely studied rumen organisms (37) and is known to utilize the lactic acid produced within the rumen to help prevent lactic acid accumulation and acidosis (9). The increase in the *Megasphaera elsdenii* population is a mechanism of maintaining ruminal pH by utilizing the increasing lactic acid produced within the rumen on high-energy diets. The 5-fold decrease in the *Megasphaera elsdenii* population during the 4th stage of the step-up diet may be due to the decrease in ruminal pH beyond the survival limits of the organism. The *Streptococcus bovis* population increased 2-fold by the start of the step-up diet but decreased by the end of the step-up diet regimen and did not show a significant change. *Streptococcus bovis* is a facultative anaerobe and is known to predominate during lactic acidosis (34, 11, 42). The rapid growth of *Streptococcus bovis* had not been reported in animals adapted to grain but was seen to be similar to that in animals on a forage diet (29). Therefore, our observation of no significant change in the *S. bovis* population is consistent with previous reports and suggests that by using a step-up diet to adapt animals to a high-concentrate diet, the *S. bovis* population can be controlled and will help control the ruminal pH. The *Selenomonas ruminantium* population increased 30-fold by the second stage of the step-up diet and thereafter was consistently 30-fold higher than that in the animals fed prairie hay. *Selenomonas ruminantium* is a propionate-producing species and is known to produce propionate through succinate decarboxylation (44). *Selenomonas ruminantium* also has the ability to utilize a wide range of substrates, including lactate (36). Hence, the increasing *Selenomonas ruminantium* population may help utilize the increasing amount of fermentable substrates and the lactic acid produced within the rumen during adaptation to a high-concentrate diet. Thus, the increasing *Selenomonas ruminantium* and *Megasphaera elsdenii* populations during adaptation to a high-concentrate diet may help decrease lactic acid concentrations by utilization. *Prevotella bryantii* populations increased gradually, reaching 8,000-fold by the 3rd regimen of the step-up diet, and then decreased rapidly by diet 4. A similar trend in population change was reported by Tajima et al. (2001), who reported a 263-fold increase in the *P. bryantii* population by day 3 of the step-up program and a large decrease in the *P. bryantii* popu-

lation in later stages (46). This decrease in the *P. bryantii* population on diet 4 may be due to the decreasing ruminal pH caused by the excess fermentable substrates in the diet.

This study of microbial population dynamics utilizing several molecular approaches provides valuable insight into microbial population structure and diversity in hay-fed and grain-fed animals. This approach also helps overcome the drawbacks of one or another technique and helps get a better understanding of the community. T-RFLP profiles provide a global view of the population structure and allow a low-cost approach to monitor microbial population shifts. However, T-RFLP analysis does not allow characterization of the microbial community, as classification of terminal restriction fragments is dependent on previously characterized sequences. Thus, only a fraction of the terminal fragments can be characterized. In addition, T-RFLP analysis prevents the identification of novel species. However, using 16S rRNA libraries in combination with T-RFLP profiles helps overcome several of these drawbacks. 16S rRNA gene sequence analyses provide both specific sequence identification and the relative representation of each species and offer a unique perspective into the rumen microbiomes of hay-fed and grain-fed animals. Quantitative real-time PCR analysis adds a new dimension to the community analysis, as it allows quantifying the change in selected bacterial species and helps identify how important the change in a bacterial species is for the ruminal function. This study, using three different molecular approaches, provides a broader picture of the rumen microbial population dynamics in hay-fed and grain-fed animals during adaptation to high-grain diets and shows the value of using multiple approaches to study microbial community structure and diversity. Our analyses identified numerous organisms that were not previously reported from the rumen. Although it is impossible to functionally characterize them without isolation, the hierarchical classification performed should help predict their function. As far as we are aware, this investigation of rumen microbial population structure and diversity using 16S rRNA gene libraries is among the largest sequence-based analyses of the rumen microbiome conducted thus far.

ACKNOWLEDGMENT

This research was supported under HATCH project OKL02612.

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