The Effect of Protozoa on the Composition of Rumen Bacteria in Cattle Using 16S rRNA Gene Clone Libraries

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The effect of the presence of protozoa on the composition of rumen bacteria was investigated in cattle. Seven castrated Holstein cattle were divided into two groups: four faunated and three unfaunated, and 16S ribosomal RNA gene (rDNA) clonal libraries were constructed. A total of 312 clones were sequenced across 1,500 bp. The 151 sequences of the faunated cattle were classified into 98 operational taxonomic units (OTUs) having at least 97% similarity. The sequences derived from the faunated cattle were classified into Firmicutes (59.7%), Bacteroidetes (34.4%), Spirochaetes (2.6%), Actinobacteria (2.0%), and Proteobacteria (1.3%). Bac-
teroides and Prevotella (34.4%) were the major groups in the faunated cattle. The 161 sequences in the unfaunated cattle were classified into 72 OTUs. The sequences derived from the unfaunated libraries were classified into Firmicutes (65.7%), Bacteroidetes (31.1%), Proteobacteria (1.9%), and Spirochaetes (1.2%). The Clostridium botulinum group and its relatives (36.0%) were the major groups in the unfaunated cattle.

An analysis by the computer program LIBSHUFF clarified that the presence of ruminal protozoa markedly affected the composition of rumen bacteria.

Key words: rumen; protozoa; cattle; 16S rRNA gene; phylogenetic analysis

The rumen microbial ecosystem comprises a diverse symbiotic population of obligatory anaerobic bacteria, ciliate protozoa, and fungi. These microorganisms appear to account for most of the fermentative activity in the rumen.1) They change feedstuff into volatile fatty acids (VFA), microbial cells, and vitamins, providing the host ruminant with most of its nutritional requirements.1) Rumen fermentation proceeds under very complex interaction among microorganisms.2,3)

Several studies have examined the influence of protozoa on rumen microorganisms. For example, it has been reported that total elimination of ruminal protozoa brings about an increase in the bacterial population and a decrease in methanogenic bacteria in the rumen.4–6) The number of amylolytic bacteria and cellulolytic bacteria decreased markedly after inoculation of protozoa into the rumen of unfaunated cattle.7)

In those reports, however, the bacteria were counted using cultural methods. Considering that only a small fraction of the total microbial diversity of a natural ecosystem can be recovered by such methods, it is not clear whether these reports accurately reflect the composition of bacteria in the rumen. Most molecular techniques for the detection, identification, and classification of bacteria at the species level are based on the nucleotide sequences of the 16S ribosomal RNA gene (rDNA).8) The composition of rumen bacteria obtained from faunated cattle has been well studied9–11) by analysis of 16S rDNA, but 16S rDNA genes from unfaunated cattle have not been investigated.

In this study, to analyze the influence of protozoa, we measured the concentrations of VFA and ammonia-N in the rumen and compared the bacterial compositions of faunated and unfaunated cattle using 16S rDNA clone libraries.

Materials and Methods

Animals and sampling. Seven castrated Holstein cattle (half-siblings, mean body weight 144 kg) reared at the Field Science Center of Tokyo University of Agriculture and Technology were divided into two groups: four faunated and three unfaunated. The cattle were isolated from their dams within a few days of birth, and were penned individually in an isolated building. After weaning at 6 weeks of age, four cattle were inoculated with a mixed protozoa population obtained from other adult cattle. The three other cattle were maintained protozoa-free.

All seven cattle were fed a diet consisting of 66% (dry matter basis) chopped Sudangrass hay and 34% concentrate mixture at a maintenance level for energy and crude protein, twice daily at 09:00 h and 17:00 h in equal amounts. The concentrate mixture contained 41% corn,
Chemical assay of ruminal fluid. Ruminal fluid samples were acidified with 3 N sulfuric acid solution containing 12% metaphosphoric acid, and then the concentrations of VFA were determined by gas chromatography (model GC-14B, Shimadzu, Kyoto, Japan). The concentration of ammonia-N was determined by the microdiffusion

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DNA extraction. Each 0 h rumen sample (2 ml) was centrifuged at 13,000 x g for 10 min. The supernatant was transferred to a fresh 15 ml tube. The pellet was mixed with 650 μl PBS in a 2-ml tube with 0.5 g of glass beads. After adding 20 μl of 20% SDS and 650 μl TE-buffered phenol (pH 8.0), the samples were bead-beaten 3 times for 2 min on a Mini-Beater (BioSpec Products, Bartlesville, OK). The tubes were put onto ice at intervals of 1 min between each step. Then they were centrifuged at 13,000 x g for 15 min. The supernatant was mixed each time with a fresh 15 ml tube. The mixture was then extracted with buffered phenol and was isopropanol-precipitated. RNase was added to a mixture was then extracted with buffered phenol and stored at -20°C until analysis. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Tokyo University of Agriculture and Technology.

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PCR amplification of protozoa. Protozoa-specific primer P-SSU-342f (5’-CTTTCCAGTGGTAGTGTAT-GACTAC-3’)(13) and reverse primer Medlin B (5’-TGATCCTTCTGAGGTTACCTAC-3’)(14) were used in PCR amplification of 1,360 bp of 18S rDNA. PCR amplification was performed in a total volume of 100 μl containing 100 ng of DNA extracted from a rumen sample and TaKaRa Ex Taq Hot Start Version (Takara Shuzo, Kyoto, Japan). The PCR was conducted with an iCycler thermal cycler (Bio-Rad, Hercules, CA). The reaction mixtures used the following program: 95°C for 3 min, followed by 30 cycles consisting of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and a final extension period of 72°C for 7 min. The PCR products were analyzed using gel electrophoresis.

PCR amplification of bacterial 16S rDNA and cloning. Two universal primers, 27F (5’-AGAGTTTGATCTGGCTCAG-3’) and 1544R (5’-AAGGAGGTGTGACGGCACCG-3’), were used in PCR amplification of the 16S rDNA. PCR amplification was performed in a total volume of 100 μl containing 100 ng of DNA extracted from a rumen sample and Takara Ex Taq Hot Start Version (Takara Shuzo). The reaction mixtures used the following program: 95°C for 3 min, followed by 12 cycles consisting of 95°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, and a final extension period of 72°C for 7 min. PCR products from each rumen sample were transformed into One Shot TOP 10 competent cells by use of the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Colonies were randomly selected, and the insert DNAs (about 1,500 bp long) were amplified with M13 primers P7 and P8.

DNA sequencing. The DNA products were purified using the PCR Product Pre-Sequencing kit (USB, Cleveland, OH) and were sequenced using the BigDye version 3.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequences were read on a model ABI PRISM 3730 DNA analyzer system (Applied Biosystems).

Phylogenetic analysis. All sequences were compared with similar sequences of the reference organisms by a BLAST search,(17) and chimeric sequences were removed on the basis of the results of the CHECK CHIMERA program of the Ribosomal Database Project (RDP). Sequence data were aligned with the ClustalX version 1.83 package. Phylogenetic trees were constructed using the neighbor-joining method.(20) The sequences were assigned to individual operational taxonomic units (OTUs) based on a sequence similarity of at least 97%. Similarities in this range allow discrimination between bacterial species on the basis of DNA–DNA reassociation values.(21)

Statistical comparison of coverage. 16S rDNA clone libraries from faunated and unfaunated cattle were compared using the LIBSHUFF computer program.(22) LIBSHUFF was written in perl. Detailed instructions on its use are to be found at http://www.arches.uga.edu/~whitman/libshuff.html. The DNADIST program of PHYLIP(23) using the Jukes–Cantor model was used to generate the distance matrix analyzed by LIBSHUFF.

Nucleotide sequence accession numbers. The sequence data determined in this study have been assigned DDBJ, EMBL, and GenBank accession numbers AB185506-AB185817.
Results

Characteristics of ruminal fluid

Protozoa in the ruminal fluid were counted under an optical microscope. In addition, protozoa-specific primers were used for detection of protozoa at the molecular level. The numbers of ciliate protozoa in the ruminal fluid are shown in Table 1. The protozoal population was composed mainly of Entodinium spp., Dasytricha sp., and Polyplastron sp. The PCR products were analyzed using gel electrophoresis (Fig. 1). No PCR products were detected in unfaunated cattle. The concentration of ruminal ammonia-N was significantly higher in the faunated than in the unfaunated group at all sampling times (Table 1). Although the concentration of total ruminal VFA did not differ between the faunated and unfaunated groups, the proportion of butyric acid was higher in the faunated group at 0 and 5 h after feeding (*p < 0.05) (Table 1).

Sequence similarity

The distributions of the numbers of clones between the faunated and unfaunated cattle are shown in Table 2. In this study, 312 clones were subjected to sequence analysis. One hundred ten clones (35.2%) showed high levels of sequence relatedness (>97%) with 12 known species. The remaining 202 clones (64.7%) had a sequence similarity of less than 97%. The 151 sequences of the unfaunated group were classified into 98 OTUs with sequence similarity of less than 97%. The 151 sequences of the faunated group were classified into 98 OTUs with at least 97% similarity. The sequences derived from the faunated group were classified into Firmicutes (59.7%), Bacteroidetes (34.4%), Spirochaetes (2.6%), Actinobacteria (2.0%), and Proteobacteria (1.3%). The 161 sequences of the unfaunated group were classified into 72 OTUs. The sequences derived from the unfaunated libraries were classified into Firmicutes (65.7%), Bacteroidetes (31.1%), Proteobacteria (1.9%), and Spirochaetes (1.2%).

In the Clostridium botulinum group and among its relatives, as well as in the Clostridium coccoides group,
the detection rates of clones and OTUs differed between the faunated and unfaunated libraries. The results of phylogenetic analysis are shown in Fig. 2. In the *C. botulinum* group and its relatives, CLUSTER-B and CLUSTER-E, two deep-branching clusters, were found according to the definitions in Leser et al. 24)

In the faunated group, 52 (34.4%) sequences, classified into 31 OTUs representing the largest group, belonged to the *Bacteroides* and *Prevotella* groups. Thirty-three (21.9%) sequences classified into 23 OTUs belonged to the *C. botulinum* group and relatives. Twenty-seven (17.9%) sequences classified into 14 OTUs belonged to the *C. coccoides* group. Ten (6.6%) sequences classified into 6 OTUs belonged to the *Sporomusa* and relatives group. Four (2.6%) sequences classified into 4 OTUs belonged to the *Treponema* group. Three (1.9%) sequences classified into 3 OTUs were minor groups in the clones analyzed, and 2 of those clones were identified as *Ruminococcus flavefaciens*.

In the unfaunated group, 58 (36.0%) sequences, classified into 14 OTUs representing the largest group, belonged to the *C. botulinum* group and relatives. Fifty (31.1%) sequences classified into 27 OTUs belonged to the *Bacteroides* and *Prevotella* groups. Two (1.2%) sequences classified into 2 OTUs belonged to an unclassified group of *Proteobacteria*. Five (3.1%) sequences classified into 3 OTUs were minor groups in the clone analyzed. No sequences exhibited a level of similarity higher than 97% with any known species in these 5 classified groups. Fifteen (9.3%) sequences classified into 10 OTUs belonged to the *C. coccoides* group. Two of those clones were identified as *Pseudobutyrivibrio ruminis*, and 3 of the clones were identified as *Butyrivibrio fibrisolvens*. Nineteen (11.8%) sequences classified into 9 OTUs belonged to the *Clostridium leptum* subgroup, and 1 clone was identified as *R. flavefaciens*. Seven (4.3%) sequences were classified into 3 OTUs belonging to the *Sporomusa* and relatives group, and 1 clone was identified as *Selenomonas ruminantium*. Two (1.2%) sequences classified into 2 OTUs belonged to the *Treponema* group, and 1 clone was identified as *Treponema bryantii*.

### Table 2. Number of Clones, Diversity of Species, and Known Ruminal Bacteria of Faunated and Unfaunated Cattle

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Group</th>
<th>Faunated</th>
<th>Unfaunated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clones (%)</td>
<td>No. of OTUs</td>
<td>No. of known species (%)</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td><em>Clostridium botulinum</em> group and relatives</td>
<td>33 (21.9%)</td>
<td>23</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td><em>Clostridium leptum</em> subgroup</td>
<td>11 (7.3%)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td><em>Clostridium coccoides</em> group</td>
<td>27 (17.9%)</td>
<td>14</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td><em>Sporomusa</em> and relatives group</td>
<td>10 (6.6%)</td>
<td>6</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>Mycoplasma and relatives group</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>Other clostridia</td>
<td>9 (6.0%)</td>
<td>7</td>
</tr>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td><em>Bacteroides/Prevotella</em></td>
<td>52 (34.4%)</td>
<td>31</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>Unclassified</td>
<td>3 (2.0%)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Spirochaetes</strong></td>
<td><em>Treponema</em></td>
<td>4 (2.6%)</td>
<td>4</td>
</tr>
<tr>
<td><strong>Proteobacteria</strong></td>
<td>Unclassified</td>
<td>2 (1.3%)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>151</td>
<td>98</td>
</tr>
</tbody>
</table>

Significance of the difference between coverage curves

The results of LIBSHUFF included homologous and heterologous coverage curves indicating that 16S rDNA
Fig. 2. Phylogenetic Tree Showing the Relationship between 16S rRNA Genes Sequences in Faunated (beginning with F) and Unfaunated (beginning with U) Samples within the *Firmicutes* (the *Clostridium botulinum* group and two deep branching clusters, and the *C. coccoides* group). 16S rDNA gene sequences (about 1,500-bp long) were used to draw the tree. The tree was constructed using neighbor-joining analysis based on 16S rDNA sequences. Scale bar = 0.01 substitutions per nucleotide position. Clones obtained from samples appear as bold letters. An accession number is given for each published sequence. Numbers in parentheses are the numbers of clones detected in the same group. *Escherichia coli* is used as the outgroup for rooting the tree.
The predator–prey relationship between ruminal protozoa and bacteria is well known. The number and composition of bacteria are thus influenced by protozoa. For example, total viable counts and amylolytic and cellulolytic bacteria are higher in unfaunated than in faunated rumen. But since these studies were analyzed by cultural methods, it was unclear whether they accurately reflect changes in the bacterial population.

In the present study, we applied molecular biology techniques to determine the effect of protozoa on bacterial composition. Since there were fewer OTUs in unfaunated cattle than in faunated ones (Table 2), it appeared that bacterial diversity was lower in the unfaunated cattle. From the results of phylogenetic analysis, it was shown that the clones of CLUSTER-B and CLUSTER-E were detected at a high rate in unfaunated cattle (Fig. 2 and Table 2). LIBSHUFF results indicated that the cloned clone library was significantly different from that of the unfaunated (\( P = 0.001 \)) (Fig. 3 and Table 3). This means that the faunated library differs greatly from the unfaunated at high levels of genetic distance but shares all deep taxa (\( D < 0.1 \)). This suggests that the presence of protozoa clearly affects bacterial diversity in the rumen. Especially, the clones of U29-G05, U29-C09, and U28-E05 were detected only in the unfaunated cattle. Thus, these clones may play an important role in the unfaunated rumen. The two large clusters that had no cultured representatives were found to be close to the \( C. leptum \) subgroup. These clusters branched off deeply in the tree, suggesting that they were high-order taxonomic structures. CLUSTER-B was positioned between the \( C. leptum \) subgroup and the \( Clostridium thermolacticum \) subgroup. CLUSTER-E was located near the STR.16SX subgroup. Although CLUSTER-B and CLUSTER-E were first described using clones obtained from the pig intestine, the sequence similarity between the rumen bacteria of cattle and the intestinal bacteria of pig was low.

Using cultural methods, Arakaki et al. also reported that the number of \( Ruminobacter amylophilus \) was reduced, while those of \( Butyrivibrio \) spp., \( Selenomonas \) spp. and \( Clostridium \) spp. increased after inoculation of protozoa into unfaunated cattle. In the present study, no clone of \( Ruminobacter amylophilus \) was detected, and there was no difference in the detection rate of \( Butyrivibrio \) spp. and \( Selenomonas \) spp. between faunated and unfaunated cattle.

There has been some work on the role of ciliate protozoa in ruminal nutrition. It has been found that digestibility of plant cell walls is improved by 25–30% when protozoa are present. Takenaka et al. showed by molecular techniques that ruminal protozoa have fibrolytic enzymes. Recently, the specific functions of protozoal species and their relation to animal production have been studied. For example, entodinimorph protozoa actively metabolize lactic acid and prevent a decrease in ruminal pH, which is very
important, especially for cellulolytic bacteria. In contrast, holotrich protozoa produce lactic acid and can have a negative effect on pH value. The nutritional advantages of entodiniomorphid over holotrich protozoa, then, have been discussed. Overall effects must be taken into account to assess the actual advantages for ruminants of controlling the protozoal population in the rumen. The impact of protozoa on ruminant digestion should be studied in detail using culture-independent molecular biology techniques. We are now trying to develop a methodology to isolate rumen bacteria including characterization of their functions using specific primers designed on the basis of clone libraries.

In conclusion, we are the first to find that the bacterial compositions of faunated and unfaunated cattle are significantly different. The major groups in unfaunated cattle were *C. botulinum* and its relatives (CLUSTER-B and CLUSTER-E), although the major groups in faunated cattle were *Bacteroides* and *Prevotella*. This study provides an outline of the effect of ciliate protozoa on bacterial composition in the rumen.

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References


