S2-5

Phylogenetic Diversity Analysis of Rumen Microbes and Screening of Novel Gene from Rumen Microbial Metagenomic Library

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The rumen is a complex, open ecosystem which is inhabited by a dense, diverse, and competitive microbial population such as fungi, protozoa, bacteria and archaea. This is the study to examine the phylogenetic diversity of the microbial community in the rumen fluid, rumen solid, and rumen epithelium of the cow by direct retrieval and analysis of rDNA sequences in a culture-independent manner. The rumen is a site of remarkably efficient microbial breakdown of plant structural polysaccharides. The plant cell degradative enzyme genes were picked out from the cosmid library and characterized.

The animal was rumen-fistulated Korean cow (Hanwoo) fed with a mixed ration twice a day. Representative samples of total rumen contents were collected from the animal via the ruminal fistula before the morning feeding. The samples were separated into the fluid, the feed particle, and the epithelium fractions of the rumen for the library construction. The products of PCR with the specific primers for bacteria, archaea, protozoa, and yeast were cloned, and sequenced. All reference sequences were obtained from the GenBank and RDP databases. Sequences were analyzed by the CHECK_CHIMERA program to exclude sequences from chimerical rDNA clones. The fractionated 40 kb fragment of the metagenomic DNA was ligated and cloned into pCC1FOS^{TM} fosmid vector. We obtained about 200,000 cosmid clones from the library and selected the active clones from it. For the subcloning of the active cosmid clones, we partially digested the active cosmid DNA to size of 2-5 kb with *Sau3A*I and subcloned into SK+ vector.

Diversity analysis of rumen microbes in rumen contents

Bacteria: A total of 113 clones, containing almost full size 16S rDNA sequences (1.5 kb), were completely sequenced and subjected to an on line similarity search (Table 1). Only 19 sequences from the 113 clones in our libraries (16.8%) can be identified as belonging to *C. algidicarnis*, etc. About 47.9% of the sequences have a similarity level with database sequences in the range of 90-97% and for the remaining 6.1%, the similarity was less than 90%. In the library from the rumen fluid, the sequences were affiliated with the following major phyla: *Cytophaga-Flexibacter-Bacteroides* (CFB) (67.5%), low G+C Gram-positive (LGCGP) bacteria (30%) and *Proteobacteria* (2.5%). The vast majority of sequences from the rumen epithelium were found to relate to CFB (94.4%) and the remaining sequences were LGCGP bacteria (5.5%) phyla. In the library from the

rumen solid, the sequences were affiliated with the following major phyla: LGCGP bacteria (75.7%), CFB (10.8%), *Proteobacteria* (5.4%), high G+C Gram-positive (HGCGP) bacteria (5.4%) and *Spirochaetes* (2.7%). It has been assumed that the rumen fluid, the rumen solid, and the rumen epithelium host different populations of bacteria.

Archaea: The 45 clones (0.9 kb) of the rumen fluid could be divided into three groups and the largest group was affiliated with the *Methanomicrobiaceae* family (96% of clones) (Table 1). The clones of the rumen fluid contained a high proportion of unidentifiable clones (67%). The 39 clones of the rumen epithelium could be divided into two groups and the largest group was also affiliated with the *Methanomicrobiaceae* family (95% of clones). The clones of the rumen epithelium contained a low proportion of unidentifiable clones (5%). The 20 clones of the rumen solid could be divided into two groups that were affiliated with either the *Methanobacteriaceae* family (55%) or the *Methanomicrobiaceae* family (45%). The clones of the rumen solid contained a moderate proportion of unidentifiable clones (40%). The predominant family of whole rumen archaea was found to belong to the *Methanomicrobiaceae* (85%). *Methanomicrobiaceae* were predominant in the rumen epithelium and the rumen fluid while *Methanobacteriaceae* were predominant in the rumen solid.

Protozoa: The 23 clones (1.3 kb) of the rumen fluid could be divided into two genera, the largest of which was affiliated with the *Entodinium* (69.6% of clones and the smaller with the *Epidinium* (31.4% of clones). The rumen fluid clones included a moderate number of unidentifiable clones (30.4%). All 14 clones of the rumen solid library were affiliated with the *Entodinium* genera (no *Epidinium*-related sequence was obtained). The predominant genus of whole rumen protozoa was found to belong to the *Entodinium* group (81.1%). Protozoa was not detected from the rumen epithelium.

Yeast: A total of 97 clones, containing a partial 26S rDNA sequence of 0.6 kb length, were sequenced. The 41 clones of the rumen fluid could be divided into five classes. The largest class was affiliated with *Pezizomycotina* class (85.4% of clones), and the remaining classes were related with the *Urediniomycotina* (2.4%), *Hymenomycetes* (4.9%), *Ustilaginomycetes* (4.9%), and *Saccharomycotina* (2.4%) classes. The 26 clones of the rumen epithelium could be divided into three classes and the *Saccharomycetes* class (92.4% of clones) was the largest group. The remaining classes were related with either *Pezizomycotina* (3.8%) or *Ustilaginomycetes* (3.8%). The 30 clones of rumen solid were all affiliated with *Saccharomycotina*. *Saccharomycotina* were predominant in rumen epithelium and rumen solid while *Pezizomycotina* were predominant in rumen fluid. Yeast belonging to the *Saccharomycotina* class was predominant in the rumen as a whole (57.0%).

Screening of genes for plant cell wall degradative enzymes from the rumen microbial metagenomic library

We inoculated cosmid clones on plates containing the cellulase, xylanase, and amylase active selection mediums and selected the active clones. Three kinds of the active clones were subcloned and characterized, respectively. Cellulase gene (*cel*5A) was 1,191 bp in size and encodes a protein of

396 amino acids with a predicted molecular mass of 44,600 Da. The conserved region of glycosyl hydrolase family 5 was confirmed. Xylanase gene (*xyn*10A) is 1,581 bp in size and encodes a protein of 526 amino acids with a predicted molecular mass of 58,750 Da. The conserved region of glycosyl hydrolase family 10 was confirmed. Amylase gene (*amy*A) of partial sequences was very close to *Vibrio parahaemolyticus*. The novel gene isolated was confirmed by Blast search. Furthermore, we extracted genomic DNA from 50 strains of the cultivable rumen bacteria and conducted the PCR with the internal primers within the ORF of the active gene to know whether the cloned genes come from the cultivable rumen bacteria or uncultivable. We confirmed that each clone was originated from uncultivable rumen bacteria.

Table 1. Number of the microbial rDNA clone in the cow rumen

Rumen content	Bacteria	Yeast	Archaea	Protozoa
Rumen fluid	40	38	45	23
Rumen epithelium	36	26	39	-
Rumen solid	37	30	20	14
Total	113	94	104	37

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Center Program (Grant MG02-0101-003-1-0-1), Ministry of Science & Technology, Korea

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