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Quantitative Analysis of rRNA Populations of Bacterial Groups in Dairy Cattle Rumen Samples by Sequence-specific Small-Subunit rRNA Cleavage Method

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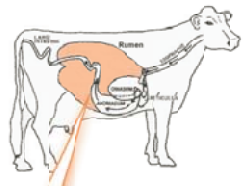
ABSTRACT

The rRNA cleavage reaction-mediated microbial quantification method was applied to the investigation of predominant bacterial groups within the rumen microbial community. A suite of oligonucleotide probes that complement the conserved sites of the 16S rRNA of phylogenetically defined groups of ruminal bacteria were designed for this cleavage method. For each probe, the target-specific SSU rRNA cleavage was achieved by optimizing the formamide concentration in the reaction mixture. The evaluated probe sets were applied to analyze the bacterial community in the rumen fluids of healthy dairy cows. The total amount of 16S rRNAs of those targeted groups accounted for 71%-78% of the total bacterial 16S rRNAs. These results indicated that quantitative detection using the probe sets coupled with the sequence-specific SSU rRNA cleavage method allows rapid and comprehensive description of active bacterial populations in rumen ecosystems.

Introduction



✓ Ruminant animals harbor a complex microbial community. Various aspects of the importance of microbiological characteristics for rumen functions have been studied (e.g., the emission of methane from livestock and the suppression of the emission). To better understand how the community effects on the rumen functions, an accurate analysis of the rumen microbiota is required.

✓ We previously developed a novel method for the quantitative detection of the small-subunit (SSU) rRNA of a specific group of microorganisms in complex ecosystems (Sequence-specific SSU rRNA cleavage method). Because of its rapid and simple procedure (☞Analytical Procedure), it seems advantageous to apply this method to the tracking of the microbial community formed in the rumen.

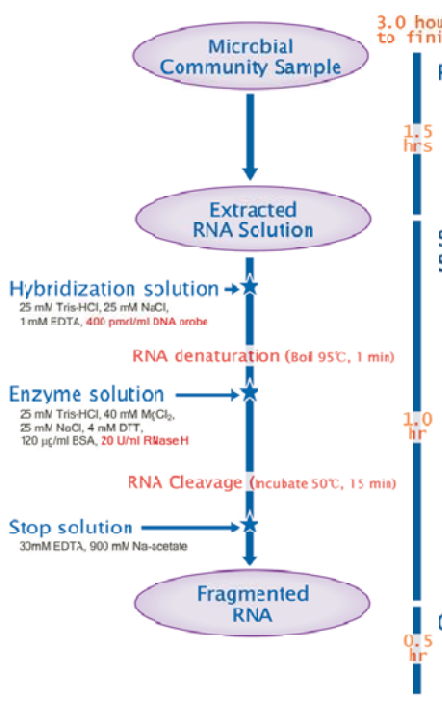
Aim of this study ✈✈✈

-- To develop and evaluate a suite of probes for the major bacterial groups of the rumen bacterial community

-- To apply the suite of probes to the quantitative detection of the SSU rRNA of specific groups in the cattle rumen fluid using the sequence-specific SSU rRNA cleavage method

Analytical Procedure

RNase H, which specifically degrades the RNA strand of RNA-DNA hybrid duplexes, could degrade the RNA at the site where DNA probe is hybridized with the RNA molecule of the target group. Therefore, if a total RNA solution retrieved from complex microbial community, only the target RNA can be fragmented.
(Uyeno et al., Appl. Environ. Microbiol. 71: 3650-3663 [2004])



RNA extraction

-- We usually apply beads-beating method to the extraction of total RNA solution.

-- Packaged reagents for the solution/purification of RNA (e.g., RNeasy kit (QIAGEN)) is helpful to eliminate oligo-deoxynucleotide from extracted RNA solution. Residual DNA sometimes causes non-specific 16S rRNA cleavage (☞Q AND A).

Sequence-specific SSU rRNA cleavage

-- Only a programmable thermalcycler is demanded as boiling and incubating device for this cleavage reaction.

-- For the correct hybridization between the scissor probes and the target 16S rRNA, denaturing reagent (e.g., formamide) could be added at a appropriate concentration in the hybridization solution (☞Result in Experiment-2).

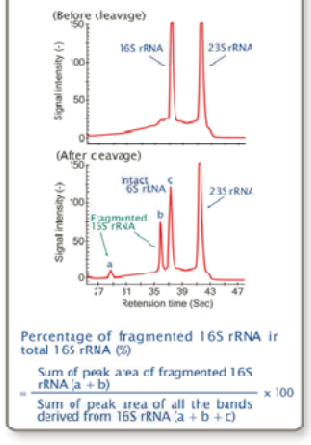
-- By adding stop solution, the cleavage reaction is terminated. Thereafter, both fragmented and non-fragmented RNA were recovered by means of EtOH precipitation or other standard protocols to collect RNA (e.g., microcentrifuge spin-column).

Quantification of RNA fragments

-- Fragmented 16S rRNA is readily distinguished from the intact one by a mean of electrophoresis (See right column).

How to visualize and quantify RNA fragments

On-chip capillary electrophoresis device enables to visualize both fragmented and intact 16S rRNA as peaks in different positions in the phenogram. The quantity of the molecule is measured by calculating the size of the peak area.



Percentage of fragmented 16S rRNA in total 16S rRNA (%)

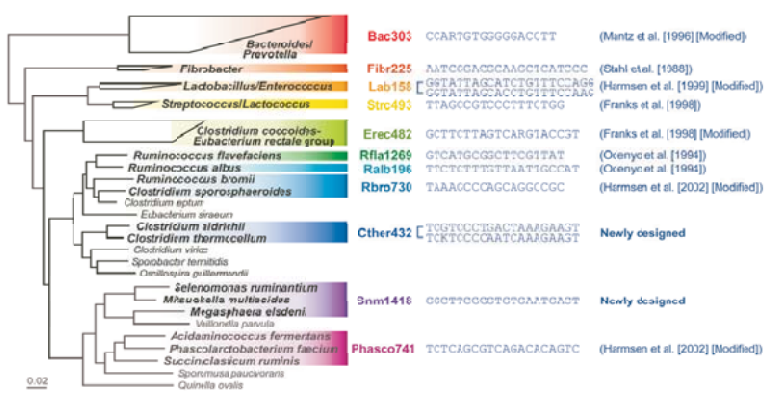
$$= \frac{\text{Sum of peak area of fragmented 16S rRNA } (a + b)}{\text{Sum of peak area of all the bands derived from 16S rRNA } (a + b + c)} \times 100$$

Results and Discussion

EXPERIMENT-1

Scissor probe design and probe set construction

Phylogenetic tree illustrating the target groups of scissor probes for rumen bacterial community

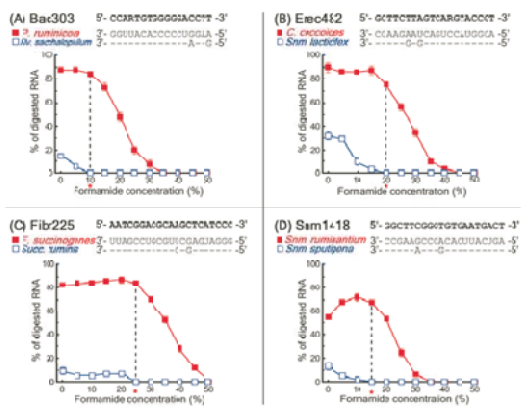


✓ To fully describe the rumen bacterial community, we have developed a systematic scissor probe set, based upon a comparative 16S rRNA sequencing approach to the rumen bacterial community. We consequently made up a set of 11 scissor probes that could detect typical groups of bacteria present in cattle rumen.

EXPERIMENT-2

Specificity of the scissor probes

Probe dissociation curves of scissor probes



✓ The specificity of the cleavage by the probes can be properly adjusted by the addition of formamide to the buffer that is used in the reaction to control the hybridization stringency between rRNA and oligonucleotides. For each scissor probe, a formamide concentration (indicated as * in the figure) was determined at which 16S rRNA of the non-target strain was not cleaved at all while 16S rRNA of the target strain was sufficiently cleaved.

EXPERIMENT-3

Quantitative detection of bacterial groups in the RNAs extracted from cattle rumen

Populations of 11 bacterial groups in cattle rumen fluid samples

		RNA population (Mean±SD) (%)			
		Cow A	Cow B	Cow C	Cow D
Eub338	(Domain *Bacteria*) (a	89.6±2.9	90.4±2.1	93.3±2.2	90.3±2.5
Arc915	(Domain *Archaea*)	7.6±1.4	6.9±0.4	7.8±1.2	6.8±0.6
Bac303	(*Bacteroides/Prevotella*)	44.8±0.5	48.2±4.1	43.9±1.4	48.9±6.2
Fib225	(*Fibrobacter*)	10.1±0.3	5.8±0.6	7.3±0.6	2.9±1.1
Lab158	(*Lactobacillus*)	ND	ND	ND	ND
Strc493	(*Streptococcus*)	ND	ND	ND	ND
Erec482	(*C. coccoides-E. rectale* group)	10.1±0.4	9.1±0.8	9.2±0.9	9.6±3.4
Rfla1269	(*R. flavefaciens*)	1.9±0.2	1.1±0.1	1.8±0.1	1.3±0.2
Ralb196	(*R. albus*)	1.4±0.1	1.7±0.4	1.6±0.3	0.8±0.4
Rbro730	(*R. bromii* group)	ND	0.4±0.1	0.7±0.1	0.5±0.3
Cther432	(*C. thermocellum* subgroup)	ND	ND	ND	ND
Snm1418	(*Selenomonas-Megasphaera* group)	1.6±0.2	0.9±0.2	1.0±0.1	ND
Phasco741	(*Phascolarctobacterium* group)	ND	ND	0.3±0.1	0.3±0.1
	Total (b	69.9	67.2	65.8	64.3
% of total bacteria 16S rRNA	(b/a x100)	71	71	71	71

✓ We applied the method to total RNA solutions extracted from the rumen fluids of four individual cattle and determined the populations of specific bacterial groups.
16S rRNA of the genera *Bacteroides/Prevotella*, the *C. coccoides-E. rectale* group, the genus *Fibrobacter*, *R. flavefaciens*, and *R. albus* were detected in all four samples. *Phascolarctobacterium* group, *Selenomonas* group, and *R. bromii* group were detected in some samples, whereas the genera *Lactobacillus/Enterococcus*, the genera *Streptococcus/Lactococcus*, and the *C. thermocellum* subgroup were not detected in all samples. In total, the group-specific probes we used detected 71-78% of the total bacterial 16S rRNAs.

Q AND A

– What is critical factor to obtain a good digestion result?

✈ The quality of RNA is the most critical factor to successfully obtain the data using this method. To avoid the degradation of RNA, which led to a higher background in the phenogram, it is recommended to extract RNA from fresh sample. Additionally, oligodeoxynucleotide should be completely removed from the extracted RNA, because it may act as "non-specific" scissor probe which hybridize to any complementary site of 16S rRNA. This causes the degradation of 16S rRNA due to the cleavage of the RNA strand of the hybrid by RNase H during the reaction.

– When designing scissor probes for the group of interest, is it possible to use the sequence information of the probes used in other probe-based methods (e.g., membrane hybridization and FISH)?

✈ Basically, the known probe sequences could be applied to the design of scissor probe in this method. However, some of scissor probes may require a few bases extension in length from their original sequences, to stabilize the hybrid formation between the scissor probe and the target 16S rRNA.

– How about the detection limit? i.e., how little 16S rRNA of target group in total 16S rRNA sample can be detected?

✈ We experimentally estimate that no less than 0.3% of the total 16S rRNA can be quantified if sample RNA is good enough to ignore the background effect caused by the degraded RNA.

– In the result of Experiment 3, only 71-78% of bacteria could be detected. Why couldn't you detect the rest?

✈ We made two hypotheses to explain why about 20-30% of the bacteria remain undetected:
(i) Compared to the vast range of genetic diversity, the coverage area we have examined with the probes may still be limited.
(ii) Due to the variations of the aligned sequence within the group, the probes may fail to detect some of the microbes that belong to the target group but have one base mismatch within the signature sequences.

– Is this method applicable to other microbial community like the microbiota formed in human intestine?

✈ Yes, it is. We have applied this method to quantitative analyses of actual microbial communities (anaerobic sludge, UASB granule and cow feces) and are now applying it to the investigation of bacterial populations in human feces.