RF-076 Development of rapid and simple methods for quantitative detection of microbial taxa in complex microbial communities for environmental monitoring（Abstract of the Final Report）

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[Abstract]

We previously reported a rapid and simple method for the quantitative detection of microorganisms based on hybridization of oligonucleotide probes (scissor probes) with small-subunit rRNAs and subsequent digestion of RNA-DNA heteroduplexes with ribonuclease H (RNase H). Here this method is further expanded to detect important members of microorganisms in environmental monitoring. First, the method was further improved by modifying the protocol, by using thermostable RNase H from *Thermus thermophilus*, and by labeling fluorescent at the 3' terminal of virtually all SSU (small subunit) rRNA molecules in sequence-dependent manner. Second, several oligonucleotide scissor probes were developed to cover a wide range of environmental microorganisms. 70 oligonucleotides in total were developed and evaluated in RNase H sequence-specific cleavage reactions. 16S (18S) rRNA library (170 plasmids in total) was also constructed from a variety of organisms, including anaerobic and aerobic bacteria, methanogenic archaea, nitrifying bacteria, and dehalorespiring bacteria. These probes were used for actual complex microbial community samples, including anaerobic sludges, soils, and groundwater samples. The results demonstrated that the probes were useful for quantitative detection of microbial groups of interest in complex communities.

1. Introduction

Microorganisms are an essential component of the earth's biota, playing integral roles in ecosystems in terms of function and sustainability. In environmental monitoring, quantitative detection of important microorganisms in environment is often required. To precisely and rapidly evaluate the abundance and activity of selected groups of microbes in complex ecosystems, more direct, rapid, simple, quantitative, and cost-effective tools should be developed, which can also be

applied to various types of heterogeneous environments.

2. Research Objective

In order to advance rRNA-based molecular techniques for quantitative detection of selected groups of microorganisms in environment, rRNA-cleavage method (RNase H method, Fig. 1) is improved to give higher detection level and simpler procedures. In addition, to construct rRNA-targeted oligonucleotide probe library for environmental monitoring, various probes are developed for a wide range of microbial taxa at the phylum to species levels. Furthermore, rRNA reference standards are made for use in quantitative detection of rRNA molecules.

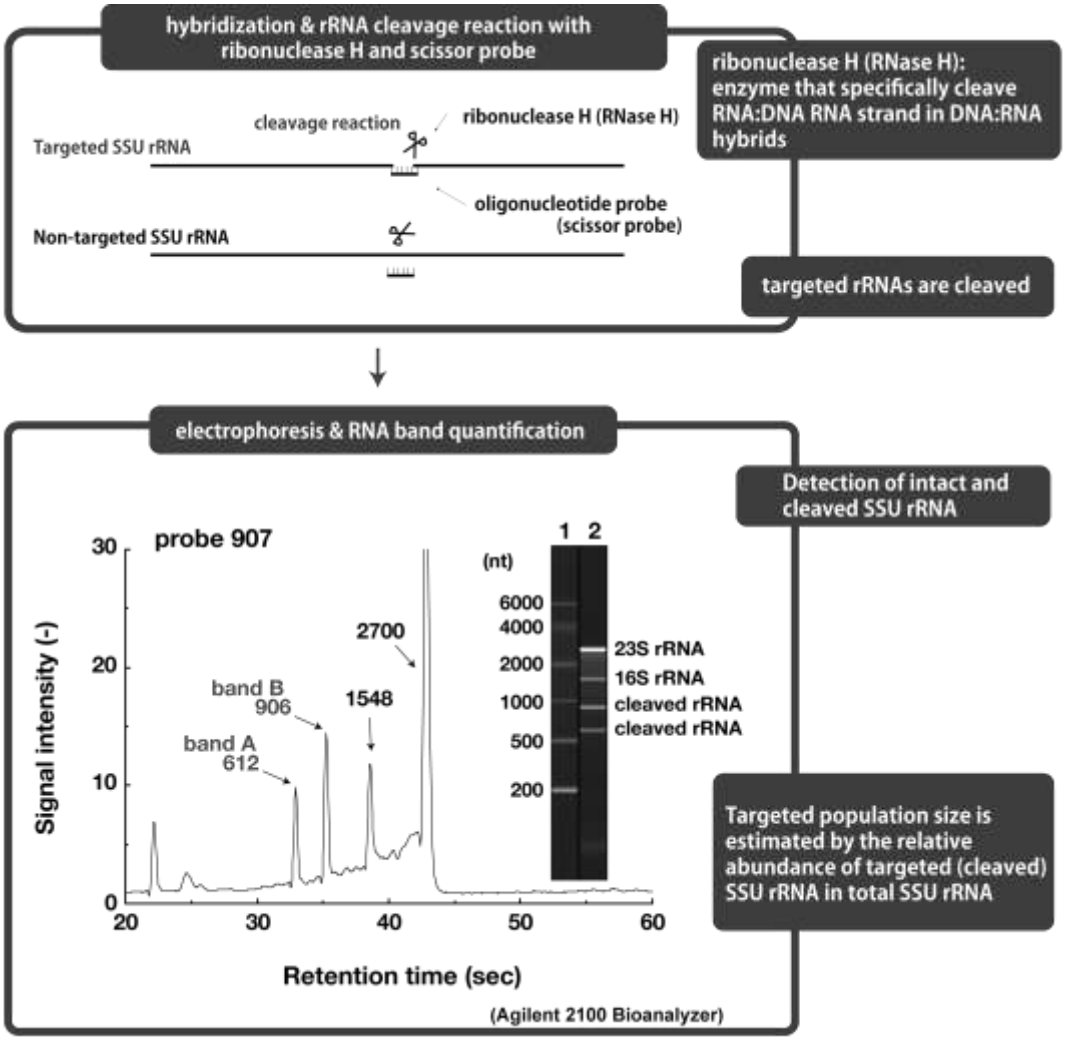

Fig. 1 Schematic representation of the sequence specific cleavage method for SSU rRNA (RNase H method).

3. Results and Discussions

We previously reported a rapid and simple method for the quantitative detection of microorganisms based on hybridization of oligonucleotide probes (scissor probes) with small-subunit rRNAs and subsequent digestion of RNA-DNA heteroduplexes with ribonuclease H (RNase H)[1]. The method was further improved by modifying the protocol, by using thermostable RNase H from *Thermus thermophilus*, and by labeling fluorescent at the 3' terminal of virtually all SSU (small subunit) rRNA molecules in sequence-dependent manner.

First, thermostable RNase H (Ribonuclease H from *T. thermophilus*) was employed to cleave rRNA in the reaction instead of *E. coli* RNase H that had been used in our previous study[1]. The use of thermostable RNase H to improve single-base mismatch discrimination was evaluated by using two *E. coli*-targeted probes with perfect-matched or single-mismatched sequences against targeted *E. coli* 16S rRNA sequence. Results showed that the single-base mismatch discrimination at 50% of cleavage efficiency was improved from a 15% disparity of formamide concentration with RNase H to a 20% disparity with thermostable RNase H, suggesting that the use of thermostable RNase H for cleavage could increase the optimum formamide concentration for each probe (i.e., probes can hybridize under a more stringent condition). Therefore, thermostable RNase H was used in this study hereafter for the determination and optimization of the specificities of all the probes used in the RNase H reaction.

Second, several rRNA-based oligonucleotide probes for virtually all organisms, for members of *Bacteria*, and for members of *Archaea* were evaluated by the newly developed protocol of the RNase H method with approximately 20 reference rRNAs, and the best probes were selected based upon the cleavage coefficient values of these oligonucleotide scissor probes.

Third, labeling fluorescent at the 3' terminal of virtually all SSU rRNA molecules was attempted. By using Klenow fragment (or Taq polymerase), Cy5-labeled dCTP, and O-methyl-RNA-DNA chimera probes, rRNA fragments were found to be labeled with Cy5 in sequence-dependent manner.

To develop molecular probes for quantitative detection of microbes in environment, several oligonucleotide scissor probes were developed to cover a wide range of environmental microorganisms. 70 oligonucleotides in total were developed and evaluated in RNase H sequence-specific cleavage reactions. Most of these probes were for the detection of important anaerobic bacteria residing in anoxic (methanogenic) environments.

To develop rRNA quantification standards, 16S (18S) rRNA library (170 plasmids in total) was also constructed from a variety of organisms, including anaerobic and aerobic bacteria, methanogenic archaea, nitrifying bacteria, and dehalorespiring bacteria. These reference standards were used for specificity evaluation of oligonucleotide scissor probes.

These probes were then used for actual complex microbial community samples, including anaerobic sludges (six samples), soils, and groundwater samples. The results demonstrated that the probes were useful for quantitative detection of microbial groups of interest in complex communities.

References

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Major Publications

1) Narihiro, T., T. Terada, A. Ohashi, J.H. Wu, W.T. Liu, N. Araki, Y. Kamagata, K. Nakamura and Y. Sekiguchi. 2009. Quantitative detection of culturable methanogenic archaea abundance in anaerobic treatment systems using the sequence-specific rRNA cleavage method. ISME J. in-press.