論　文　の 要 約

Isolation of Previously Uncultured Rumen Bacteria by Using Modified Media
（改良した培地による未培養ルーメン細菌の分離）

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内容

Contents

Abbreviations

Chapter 1

General Introduction

Chapter 2

Effects of gelling agents on isolation of previously uncultured rumen bacteria

Chapter 3

Effects of glycerophosphate, magnesium, and gelling reagents on isolation of rumen bacteria

Chapter 4

Improved culturability of cellulolytic rumen bacteria, and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen

Chapter 5

General Discussion and Conclusion

Acknowledgements

References

要約

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Rumen fermentation is conducted by the rumen microbial ecosystem, in which many kinds of microorganism, such as bacteria, protozoa, archaea and fungi, anaerobically convert ingested feed into fermentation end products. It is known that bacteria are the dominant domain in the rumen and make the greatest contribution to rumen fermentation. To improve feed efficiency in the ruminants by controlling rumen fermentation, bacteria, therefore, have been a focus of microbiological studies of the rumen microbiome. The diversity and functional properties of rumen bacteria were investigated by using both culture-dependent and culture-independent methods. To date, more than 200 bacterial species have been isolated and characterized physiologically by the culture-based studies. In the other hand, culture-independent studies such as analyses of 16S rRNA gene sequences from rumen showed that the bacteria community is more diverse than the number of cultivated species. Although the number of phylotypes determined by 16S rRNA gene sequences has increased, understanding of the biochemical characteristics and functions in rumen fermentation of those phylotypes, which are fully defined by pure-culture studies, are still poor. To provide better understanding of the rumen functions, more bacteria need to be isolated from the rumen, and their physiological and functional properties can be defined in the pure culture. This study attempted to isolate the previously uncultured bacteria from the rumen by improvement of a roll-tube (RT) method, which is a typical method for isolation of rumen bacteria, and to classify the isolates by 16S rRNA gene sequences analysis.

Firstly, new media for the RT method were developed by using gellan gum, a gelling agent, for the isolation of previously uncultured rumen bacteria. As a control, a basal liquid medium (BM) with agar (A-BM) was used. A modified BM (mBM) was made by removing KH_2PO_4 from and adding $MgCl_2$ to BM. Media, mBM with agar (A-mBM), mBM with phytagel (P-mBM) and mBM with gelrite (G-mBM), were prepared. Of the 207 isolates cultured with the media including the A-BM, 47 isolates were identified as the strains of novel species. Most of the new species were obtained from A-mBM, G-mBM and P-mBM. But the predominant cultural members, isolated from each medium, differed. Numbers of OTUs derived from isolates obtained by A-mBM and G-mBM was significantly

higher than those by A-BM ($P < 0.05$). The Shannon-Wiener diversity index (H') of the isolates from A-mBM showed the highest diversity ($H' = 3.16$) compared with those from G-mBM, P-mBM and A-BM ($H' = 2.86$, 2.49 and 2.41, respectively).

The modified media developed in previous study that contained magnesium salt and no additional phosphate have enabled cultivation of some novel rumen bacteria. When phosphate is coexistence with magnesium in a medium, insoluble precipitation of magnesium-phosphate appears after autoclaving. Therefore, this study applied disodium glycerophosphate as a substitute for phosphate in the media to investigate the effects of phosphate, magnesium and gelling reagents on isolation of previously uncultured rumen bacteria. Four media, A-BM with disodium glycerophosphate (PA-BM), A-mBM with disodium glycerophosphate (PA-mBM), P-mBM with disodium glycerophosphate (PP-mBM) and G-mBM with disodium glycerophosphate (PG-mBM) were prepared for the RT method. Moreover, to evaluate the effect of inoculation temperature on growth of rumen bacteria, an inoculum was inoculated to PA-mBM at 56°C (PA-mBM-LT), and at 60°C (PA-mBM-HT). From the media, 265 isolates were obtained, of which 47 isolates were the strains of novel species. Most of the novel species were obtained from PA-BM and PA-mBM-LT. The predominant cultural members isolated from each medium were different. The Shannon-Wiener diversity indexes indicated that the isolates from PA-mBM-LT were more diverse than those from PA-BM, PA-mBM-HT, PP-mBM and PG-mBM. Comparison of the community compositions between any two media using Ribosomal Database Project Library Compare (http://rdp.cme.msu.edu/) indicated significant differences ($P < 0.01$), except in the community compositions between PA-BM and PA-mBM-HT, and those between PA-mBM-LT and PG-mBM.

In order to improve the culturability of cellulolytic rumen bacteria, two new media, A-mBM with azo-carboxymethylcellulose (CA-mBM) and G-mBM with azo- carboxymethylcellulose

(CG-mBM), were developed. From the media, 129 isolates were collected, of which the numbers of isolates showing filter-paperase (FPase), carboxymethylcellulase (CMCase) and xylanase activities were 51, 108 and 116, respectively. The isolates were classified into 6 phyla. Phylum *Firmicutes* was the most dominant group in the isolates (81.4% of the total). The unclassified taxa at the genus level accounted for 19.4% of the 129 isolates and showed low sequence similarity (< 97%) with the known species. The numbers of *Streptococcus* were significantly different ($P < 0.01$) between CA-mBM and CG-mBM. Isolates showing FPase activity were obtained from the both media. CG-mBM significantly supported growth of more isolates showing higher CMCase activity than CA-mBM ($P < 0.05$).

総括

In conclusion, this study developed 5 agar-based, 2 phytagel-based and 3 gelrite- based media for the RT method to isolate the previously uncultured bacteria from the rumen. The media supported growth of 601 isolates that consisted of 172 OTUs at species level, which were classified into six phyla and into 24 known genera and unclassified genera. Sixty-six of 172 OTUs were identified as novel species. These results indicated that the novel species were successfully isolated from all media used in this study. This study has concluded that the culturable rumen bacteria can be increased by using improved culture media.