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Diversity of fumarate reducing bacteria in the bovine rumen revealed by culture dependent and independent approaches

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Abstract

Diversity of fumarate reducing (dissimilating) bacteria in the bovine rumen was analyzed by both culture dependent and independent methodologies. A total of 39 strains were isolated by using three different media and belonged to three different phyla (*Proteobacteria*, *Fusobacteria*, and *Firmicutes*). A primer set that amplified the fumarate reductase gene (*frdA*) from *Proteobacteria* was developed and two *frdA* clone libraries were constructed. Identities of deduced amino acid sequences of cloned *frdA* amplicons against known sequences ranged from 58% to 85% suggesting the presence of unknown fumarate reducing bacteria. This is the first report on the diversity of fumarate reducing bacteria in the rumen.

Keywords: Fumarate reducing bacteria; Fumarate reductase; *frdA*; Rumen; Molecular ecology

1. Introduction

Methanogenesis is a final step of carbohydrate metabolism in the rumen of ruminants. Methane emission from ruminants is estimated to constitute 15% of total global methane emission [1]. Methanogenesis represents a 2–12% loss of dietary energy ingested by host animal [2]. Therefore, reduction of methanogenesis in the rumen is one of important issue for global ecology and animal nutrition. Supplementation of fumarate is a possible way to reduce methane production. Fumarate is an intermediate compound of succinate–propionate pathway in the rumen and is reduced to succinate by fumarate reductase (EC. 1.3.99.1) [3]. Since hydrogen is utilized to reduce fumarate in the pathway, fumarate reducing (dissimilating) bacteria and methanogenic bacteria will compete for hydrogen in the presence of fumarate. Thus, methane production can be reduced by supplementation of fumarate. In fact, reduction of methane production by fumarate supplementation was observed both in vitro and in vivo [4,5]. Several rumen bacteria were assessed for their fumarate reducing activity [6]. However, diversity of fumarate reducing bacteria in the rumen is still unclear.

Microbial community structure analysis by 16S rRNA gene (16S rDNA) demonstrated complexity of microbial ecology and presence of many novel uncultured bacteria in the rumen [7]. However, 16S rDNA based analysis has one limitation. This gene does not target nucleic acid sequence directly related to the physiology and metabolic capacity of microbes. Thus, use of a functional gene encoding a key enzyme involved in a characteristic metabolic pathway is advantageous if a specific function within the microbial community needs to be studied. Molecular ecological studies based on functional genes have been successfully applied to analyze the diversity of methanogenic [8], acetogenic [9], and sulfate reducing bacterial [10] populations in the intestinal tract. The approach contributes not only diversity analysis but also to analysis of functional enzymes in the intestinal ecosystem.

In this study, we isolated fumarate reducing bacteria from the bovine rumen and, in addition, developed a new primer set that amplifies genes encoding the α-subunit of fumarate reductase (*frdA*) from *Proteobacteria*. Using this primer set, we constructed clone libraries of *frdA* recovered from the bovine rumen enabling analysis of *frdA* gene diversity.

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2. Materials and methods

2.1. Animal and sampling

Two Holstein cow fitted with ruminal cannulas were used in this study. Animal 1 (body weight: 650 kg) was housed at the Mie Prefectural Science and Technology Promotion Center. The animal was offered 1.5 kg of oat hay, 1.5 kg of italian ryegrass straw, 1.5 kg of beat pulp, 1.25 kg of dairy concentrate diet twice daily. Animal 2 (body weight: 560 kg) was kept at the National Institute of Livestock and Grassland Science (Tsukuba, Japan). The animal was offered 2.65 kg of timothy hay, 0.79 kg of steam-flaked corn, 0.55 kg of soybean meal twice daily. Whole rumen content was obtained from both animals by using a sampling tube via cannula just before morning feed. Samples were strained through 4-layers of surgical gauze. The sample from animal 1 was immediately stored at −80 °C. The sample from animal 2 was divided into two parts. One was used for isolation and another one was used for DNA extraction. The sample for DNA extraction was immediately stored at −80 °C.

2.2. Strains

Selenomonas ruminantium subsp. *lactilytica* JCM6582 was purchased from Japan Collection of Microorganisms, RIKEN BioResource Center (Tsukuba, Japan). *Fibrobacter succinogenes* S85 was kindly provided from Dr. Kiyoshi Tajima, National Institute for Livestock and Grassland Science. These strains were maintained as described in Matsui et al. [11].

2.3. Preparation of media

Three types of medium were used in this study based on the report of Joblin [12] with some modification. Sugars were omitted from the basal medium. Hydrogen or formate was used as electron donors, while fumarate was used as electron acceptor. Fumarate and either of the two electron donors were supplemented to the basal medium as described in Table 1. Hydrogen was added to headspace of the tube at 1.92×10^5 Pa after inoculation. For enrichment incubation, 54 ml of medium was dispensed into 120-ml serum bottle and sealed with a butyl-rubber septum and an aluminum crimp cap. Monensin sodium salt was added to the FHM medium immediately prior to inoculation to suppress bacteria that produce acetic acid and hydrogen [13]. For roll-tubes, 6 ml of medium containing 1.5% of Difco Bacto-agar (Becton Dickinson, Sparks, MD) was dispensed into Balch tube. For broth medium, 10 ml of medium was dispensed into Hungate tube. All media were prepared under O_2-free N_2 gas and autoclaved for 15 min at 121 °C. Anaerobic dilution solution [14] was prepared under O_2-free CO_2 gas and autoclaved.

2.4. Isolation of fumarate reducing bacteria

Enrichment was performed prior to roll-tube isolation using 6 ml of strained rumen content inoculated into the enrichment medium and incubated for 24 h with shaking horizontally (80 rpm). A portion (6 ml) of culture fluid was then inoculated into the same medium and incubated in same manner. After enrichment, the culture was serially diluted from 10^{-8} to 10^{-11} with anaerobic dilution solution and inoculated into roll-tubes and then incubated for 24–48 h. Visible colonies in the agar film were inoculated into broth medium and incubated for 24 h with horizontal shaking at 120 rpm. The isolation step was repeated until purified. All incubations were carried out anaerobically at 37 °C [13].

2.5. DNA extraction

DNA from bacterial isolates was extracted using UltraClean Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., Solana Beach, CA) according to manufacturer's protocol. DNA from rumen contents of two animals was separately extracted by bead beating method with the FastPrep instrument (Bio 101, Vista, CA) as described by Godon et al. [15]. The crude DNA was purified with Genomic-tip 100/G (QIAGEN, Hilden, Germany) and dissolved in TE buffer. The DNA concentration was adjusted to 45 ng μl^{-1} for animal 1 and 25 ng μl^{-1} for animal 2, respectively.

2.6. Development of PCR primers

To design the *frdA* specific primer set, nucleotide sequences of a wide variety of fumarate reductase gene were retrieved from the GenBank database. *Campylobacter jejuni* (GenBank accession no. AL139075), *Escherichia coli* (NZ_AAJU01000026), *Helicobacter pylori* (NC_000915), *Pasteurella multocida* (AE006054), *Proteus vulgaris*

Table 1
Combination of electron donors and electron acceptor, and monensin in three different media for the enrichment and isolation of fumarate reducing bacteria (mmol per L medium)

Components	Fumarate + H_2 medium (FH)	Fumarate + formate medium (FF)	Fumarate + H_2 + monensin medium (FHM)
Disodium fumarate	40	40	40
Hydrogen	40	–	40
Sodium formate	–	40	–
Monensin	–	–	0.025