Diversity of the Formyltetrahydrofolate Synthetase Gene (fhs), a Key Enzyme for Reductive Acetogenesis, in the Bovine Rumen

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A clone library of the partial formyltetrahydrofolate synthetase gene (fhs), a key enzyme in reductive acetogenesis, was constructed from the DNA of bovine rumen contents. Diverse sequences were recovered, the majority of which were clustered with the fhs of authentic acetogens. Low similarity values to known fhs were observed in all sequences, suggesting the presence of unknown acetogens.

Key words: formyltetrahydrofolate synthetase gene (fhs); reductive acetogenesis; acetogenic bacteria; methanogenesis; rumen

Reductive acetogenesis is one of the metabolic pathways for utilizing hydrogen during fermentation in anaerobic environments. It is known to occur in the rumen.1) Enhancement of reductive acetogenesis is an attractive technique for reducing methanogenesis in the rumen.2) The addition of different acetogenic bacteria to mixed rumen microorganisms in vitro reduces methanogenesis to varying extents.3,4) To establish a technique to control and enhance acetogenesis and suppress methanogenesis in the rumen, it is important to understand the ecology of acetogenic bacteria, but little is known about the community structure or the diversity of acetogenic bacteria in the rumen. Molecular ecological analysis using functional genes has illustrated the community structure and microbial diversity of various functional groups of microorganisms, responsible for nitrogen fixation,5) methanogenesis,6) and sulfate reduction.7) Drake et al.8) pointed out that it is impossible to develop 16S rRNA oligonucleotide probes and primers that exclusively target all known acetogens, since their 16S rRNA sequences are not monophyletic and are often very closely related to non-acetogenic species.

Formyltetrahydrofolate synthetase is a key enzyme of the reductive acetogenesis pathway. Degenerated oligonucleotide primers that amplify partial gene sequence encoding the enzyme have been developed and applied in diversity analysis of acetogenic bacteria.9) In this study, a clone library of partial formyltetrahydrofolate synthetase gene (fhs) sequences was constructed from genomic DNA extracted from a microbial community in the contents of the bovine rumen, and the diversity of the genes was analyzed.

A Holstein cow (body weight, 560 kg) fitted with a ruminal cannula was used. The animal was housed at the National Institute of Animal and Grassland Science. It was offered 2.65 kg of timothy hay, 0.79 kg of steam-flaked corn, and 0.55 kg of soybean meal twice daily. Rumen contents were obtained through a sampling tube via the cannula before morning feeding, and were strained through four layers of surgical gauze. The sample was immediately stored at -80°C. Microbial cells in the rumen contents were physically disrupted with a FastPrep instrument (Bio 101, Vista, CA), and DNA was extracted as described by Godon et al.10) The crude DNA was purified with Genomic-tip 100/G (QIAGEN, Hilden, Germany) and dissolved in TE buffer. The purified DNA (22 ng μl-1) was used as the PCR template. The PCR reaction was performed using TaKaRa Ex Taq (TaKaRa, Otsu, Japan) with an iCycler thermal cycler (Bio Rad, Hercules, CA). A total of 20 μl of PCR reaction mixture contained 1.0 μl of template DNA, 1X Ex Taq reaction buffer, 200 μM of each deoxynucleoside triphosphate (dNTP mixture), 0.5 unit of Ex Taq DNA polymerase, and 0.5 g l-1 of bovine serum albumin. fhs forward primer and reverse primer10) were used at a concentration of 2.5 μM. A modified touchdown PCR protocol as described by Lovell and Leaphart9) was employed to amplify the fhs fragment. Initial denaturation was performed at 94°C for 2 min. Nine touchdown cycles of 94°C for 30 s, 63°C for 30 s (decreased by 1°C per cycle to 55°C), and 72°C for 30 s were performed. After the touchdown cycles, 15 additional cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s were performed, followed by a final step at 72°C for 10 min. Amplification of the PCR products was confirmed by 1.0% agarose gel electrophoresis. The PCR products were cloned with a TA Cloning Kit.

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Abbreviations: fhs, formyltetrahydrofolate synthetase gene; 16S rDNA, 16S ribosomal RNA gene
(Invitrogen, Carlsbad, CA), as described in the manufacturer’s protocol. Positive clones were randomly selected from the clone library. The cloned fragments were amplified from the recombinant plasmid by PCR. The PCR products were purified with a Pre-Sequencing kit (USB, Cleveland, OH), and then sequenced with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ). The DNA sequence of the cloned fragment was read in both strands with an M13 P8 primer (5'-AGATAACAATTTGACACGAGAAAC-3') and an M13 P7 primer (5'-CGCCAGGTTTTCCAGTCACGAC-3'). All sequences were analyzed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The deduced amino acid sequences from the DNA sequences were searched for homology by BLASTX program.\textsuperscript{11)} The amino acid sequences were aligned and phylogenetic trees were constructed as described elsewhere.\textsuperscript{6)}

The GenBank accession numbers of the reference sequences are shown in parentheses. Thermoplasma acidophilum was used as the outgroup. Bootstrap values of 70% or above of 1,000 replicates are shown at the node of the tree.

**Fig. 1.** Phylogenetic Analysis of Deduced Amino Acid Sequence of the fhs Gene Fragment Recovered from the Bovine Rumen. The GenBank accession numbers of the reference sequences are shown in parentheses. Thermoplasma acidophilum was used as the outgroup. Bootstrap values of 70% or above of 1,000 replicates are shown at the node of the tree.
technique.\textsuperscript{1,12} Surprisingly, the diversity of this functional group in the rumen had not been analyzed previously. This is the first report on diversity analysis of them using a functional gene. A total of 53 clones were randomly selected, and cloned DNA fragments were sequenced. The nucleotide sequences of the fragments after removal of the primer regions ranged from 1,054 to 1,069 bp, and the deduced amino acid residues ranged from 351 to 356. No identical sequence for known acetogenic bacteria was found in our library. The similarity of the clone to \textit{fhs} of known acetogenic bacteria was less than 80\% (data not shown). A phylogenetic tree was constructed from the amino acid sequences of the \textit{fhs} recovered from the bovine rumen and the \textit{fhs} sequences deposited in the GenBank database (Fig. 1), and the clones were classified into clusters A, B, and C, as defined by Lovell and Leaphart.\textsuperscript{13} Forty-one clones were classified into cluster A, and no clone was affiliated with cluster B or C. Within cluster A, six clones were grouped with \textit{Ruminococcus productus}. Four clones were grouped with horse manure clone H2. Eight clones were related to horse manure clone H4. Although three clones (R018, R041, and R049) out of eight were closely related to H4, the others were only distantly related to it. Cluster A includes \textit{fhs} sequences of \textit{Treponema primitia} or \textit{Treponema azotonutricium}, spirochetes isolated from termite guts.\textsuperscript{14} No sequence was closely related to these sequences. A tight grouping of 10 clones (R001, R003, R020, R009, R015, R017, R020, R025, R042, R046, R050) clustered with \textit{Clostridium magnum} was found. Twelve clones were grouped with \textit{Sporomusa ovata} and sulfate-reducing bacteria BG9. \textit{Moorella thermoacetica} was also grouped with these sequences. A group of R010, R036, and R039 was deeply branched with cluster A. Although these sequences showed about 67\% similarity to \textit{M. thermoacetica}, no closely related reference sequence was found in the database. Nine diverse clones were not associated with cluster A, B, or C, but were grouped with horse manure clones H1 and H3, \textit{Proteus vulgaris} and \textit{Clostridium acetobutylicum}. Since \textit{P. vulgaris} and \textit{C. acetobutylicum} are non-acetogenic bacteria,\textsuperscript{15} these nine sequences should be excluded from the acetogenic bacterial community in the rumen. Because the phylogenetic placements of these sequences were obviously separated, they were distinguishable from the \textit{fhs} of authentic acetogenic bacteria. The deduced amino acid sequences of the \textit{fhs} fragment recovered from the bovine rumen contents were searched for the signature sequence published in Prorule\textsuperscript{15} in PROSITE (http://au.expasy.org/prosite/prorule.html). The amino acid sequence of \textit{fhs} (PROSITE accession no., PDOC00595) included two signature sequences. The amplified fragment contained signature 2 of \textit{fhs} (V-[ASV]-[TS]-[IVLA]-[RIQ]-[AGS]-[LIM]-[KER]-x-[HN]-[GAS]-[GLKD]). We found 14 different signature sequences within the cloned sequences in this study (Table 1). Among these sequences, five exceptional sequences were found. The most abundant was \textit{VATVRALKMHGG}. This sequence was found in the clones grouped with \textit{S. ovata} except for R023 and R027 (Fig. 1). The \textit{fhs} sequences from \textit{S. ovata}, sulfate-reducing bacteria BG9 and \textit{M. thermoacetica}, included this signature sequence. The second most abundant sequence was \textit{VATIKALKYNGG} and 10 clones were assigned to the sequence. It contained exceptional residue. The fifth residue was [K] instead of [RQ] in the published signature sequence. The clones that included this signature sequence were grouped with \textit{C. magnum}. Both \textit{VATIRALKYNGG} and \textit{VATVRALKYNGG} included five clones. The former sequence was found in cloned sequences that were grouped with horse manure clones H1 and H3 (Fig. 1). The latter sequence was found in R004, R011, and R032, which were also grouped with H1 and H3. This sequence was also found in R012 and R016. These clones were found in cluster A, and were distantly related to H1 and H3. The signature sequence was found in \textit{fhs} of authentic acetogens such as \textit{C. magnum}, \textit{Acetobacterium woodii}, \textit{Acetobacterium psammophilicum}, \textit{Eubacterium limosum}, \textit{Thermoanaerobacter kurvii}, and \textit{R. productus}.

A wide variety of \textit{fhs} gene fragments was recovered from the bovine rumen in the present study. Most of the cloned sequences were involved in cluster A. Because this cluster included many authentic acetogenic bacteria, the cloned sequences found in it were derived from acetogenic bacteria in the bovine rumen, but about one-third of the clones within the cluster had an exceptional signature sequence of \textit{fhs} (Fig. 1 and Table 1). Functional analysis of these sequences is required. The cloned sequences had relatively low similarity to the \textit{fhs} sequences of known acetogenic bacteria. Furthermore,
phylogenetic placements of these sequences were
distantly related to known acetogenic bacteria. These
results suggest that unknown acetogenic bacteria are
colonizing the bovine rumen. Efforts should be made
to isolate acetogenic bacteria from the rumen. fhs se-
quencies from isolates and environmental samples should
be deposited to enrich the database for future research.

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