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Evaluations of Different Hypervariable Regions of Archaeal 16S rRNA Genes in Profiling of Methanogens by *Archaea*-Specific PCR and Denaturing Gradient Gel Electrophoresis[▽]

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Different hypervariable (V) regions of the archaeal 16S rRNA gene (*rrs*) were compared systematically to establish a preferred V region(s) for use in *Archaea*-specific PCR-denaturing gradient gel electrophoresis (DGGE). The PCR products of the V3 region produced the most informative DGGE profiles and permitted identification of common methanogens from rumen samples from sheep. This study also showed that different methanogens might be detected when different V regions are targeted by PCR-DGGE. Dietary fat appeared to transiently stimulate *Methanosphaera stadtmanae* but inhibit *Methanobrevibacter* sp. strain AbM4 in rumen samples.

Archaea can thrive in various natural and engineered environments, and many of them can grow in habitats at the extreme limits (in terms of temperature, pH, salinity, anaerobiosis, etc.) of conditions that allow life on earth (7a). From a pragmatic perspective, archaeal extremophiles can be exploited for biotechnological applications (9a, 25a), while methanogens mediate the terminal step of the carbon cycle (through methanogenesis) in anaerobic environments, including rumens and anaerobic digestors (2b, 20a). The methanogens present in the latter two habitats have recently received renewed interest because it has been recognized that ruminal methanogens contribute significantly to emission of methane (a potent greenhouse gas) and to loss of feed energy (22a), while the methanogens present in anaerobic digestors produce methane biogas as a valuable bioenergy source from a variety of types of biomass (2a). Since *Archaea* are typically fastidious organisms that are difficult to culture in the laboratory, they are often analyzed by molecular biology techniques (1, 28).

Community profiling by PCR-denaturing gradient gel electrophoresis (DGGE) has been widely used to examine both temporal succession in microbial communities and spatial variations in microbial diversity in the same type of microbial communities (21, 24). It is especially useful for identifying samples of interest for in-depth analyses in a large number of samples (9, 13, 16, 20). However, even using universal primers, *Archaea* were probably not accounted for in such studies because sequencing of excised DGGE bands rarely yielded *Archaea*-like sequences (13, 14). This is probably attributable to the low abundance of *Archaea* in most microbial communities and/or primer mismatch. Thus, *Archaea*-targeting PCR-DGGE has been used increasingly for profiling *Archaea* in various environmental samples (1, 17, 27, 33). A recent study also reported profiling of ruminal methanogens by PCR-DGGE, but no primer was reported (8). In all these studies different primer sets were used and different hypervariable (V) regions were targeted, but no justification or rationale was presented. In a previous study we noted that different V regions of the bacterial 16S rRNA gene (*rrs*) can produce different DGGE patterns (number and separation of DGGE bands) (31). We hypothesized that this is also true for *Archaea*-specific PCR-DGGE. In this study we first evaluated a battery of *Archaea*-specific primer sets targeting different V regions of archaeal *rrs* in PCR-DGGE by using the same approach (31). The primer sets that produced the most informative DGGE profiles were used to assess the impact of dietary fat on the methanogenic subcommunities in the sheep rumen.

In silico analysis of V regions of archaeal *rrs* genes. Among the 218 representative phylogenetic *rrs* genes (RDP II, release 8.1; release 9, the most recent release, does not contain archaeal *rrs* genes), 21 are of archaeal origin (8a). The archaeal representatives containing these genes belong to 21 different genera representing all nine recognized orders of *Euryarchaeota* (15 genera), all four recognized orders of *Crenarchaeota* (five genera), and one uncultured marine *Crenarchaeota* clone. These representative *rrs* genes were used to calculate, exactly as described previously (31), the average sequence identities, the average melting temperatures of the lowest melting domain [$T_{m(L)}$], and the predicted denaturant gradients needed to resolve 95% of the representative amplicons delineated by the corresponding primer sets. The 5′ half of the archaeal *rrs* gene appeared to be more divergent than the 3′ half (Table 1). This trend toward sequence heterogeneity is similar to that observed for the bacterial representative phylogenetic *rrs* genes (31). The V1 to V5 regions also form more $T_{m(L)}$ groups (≥0.1°C difference) and thus more hypothetical DGGE bands than the other V regions. Compared to their bacterial counterparts (31), the archaeal V regions are less divergent, resulting in more similar average $T_{m(L)}$ and narrower predicted

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TABLE 1. Sequence identities of the PCR amplicons calculated from the representative phylogenetic archaeal *rrs* genes in RDP II and their $T_{m(L)}$

Hypervariable region(s)	% Sequence identity[a]	No. of $T_{m(L)}$ groups[b]	No. of sequence sharing the same $T_{m(L)}$	Avg $T_{m(L)}$ (°C)[a]	Predicted denaturant gradient (%)[c]
V1-V2	70.3 (6.2)	19	1.11	75.8 (3.7)[c]	28–77
V3	71.2 (6.8)	15	1.40	79.2 (2.9)	45–83
V3-V4	71.5 (6.4)	16	1.31	76.0 (3.9)	27–79
V3-V5	75.4 (5.6)	16	1.31	75.6 (3.7)	27–76
V6-V8	80.3 (4.6)	11	1.91	76.1 (1.8)	41–66
V7	77.4 (5.7)	11	1.91	79.0 (2.1)	49–77

[a] The values in parentheses are standard deviations.
[b] Difference, ≥0.1°C.
[c] For DGGE gels run at 60°C.

denaturant gradients needed to resolve various archaeal *rrs* amplicons by DGGE. It should be noted that since these *rrs* sequences represent all the recognized orders of both *Euryarchaeota* and *Crenarchaeota*, the predicted denaturant gradients should be wide enough to accommodate most *Archaea* encountered in most habitats. As demonstrated in this study and our previous study (31), these predicted gradients serve as a starting point from which optimal denaturant gradients can be determined empirically for specific samples. In fact, the gradients used in previous studies (1, 17, 28, 33) all fell within the gradient ranges predicted in this study.

Comparison of different V regions by PCR and DGGE. A set of gastrointestinal samples (collected from sheep rumens, cow rumens, and cow feces) and anaerobic digestor samples (collected from five laboratory anaerobic digestors that were inoculated with the same anaerobic digestor content but were operated under different conditions) were used to compare different V regions by PCR-DGGE. The genomic DNA was extracted using the repeated bead beating and column purification (RBB+C) method (32), which results in efficient recovery of PCR-quality community DNA. The quality of the DNA was assessed using agarose gel electrophoresis, and DNA concentrations were determined using a Quanti-it kit (Invitrogen, Carlsbad, CA) (30). Table 2 shows all the primers, targeted V regions, annealing temperatures, and $MgCl_2$ concentrations used in this study. Primers A329r and A693r were designed in this study by aligning the consensus sequence of the archaeal phylogenetic representatives with the consensus sequence of bacterial representatives. The specificity of these two primers was confirmed using the Probe Match program of RDP. The PCRs were performed as described previously (31), except that the final extension time at 72°C was extended to 30 min to eliminate artifactual double DGGE bands resulting from a possible heteroduplex (10). To eliminate the potential impact of different amounts of DNA template on DGGE profiles, 100 ng was used for each PCR. Positive controls containing *Methanobacterium thermoautotrophicum* genomic DNA as the template and no-template controls were included in parallel. Aliquots (15 μl) of PCR products were resolved on 7.5% polyacrylamide gels (37.5:1) containing denaturant gradients (see below) that were optimized empirically using the predicted denaturant gradients (Table 1) for each V region. DGGE, gel staining, image capturing, and gel analysis were done as described previously (31). For each primer set, PCR and DGGE were repeated once, and identical DGGE profiles were obtained (data not shown).

Primer sets A2Fa/GC-A348r and A24f/GC-A348r yielded much smaller, and variable, amounts of PCR products and very few DGGE bands (data not shown). Primer set GC-ARC344f/ARC915r yielded amounts of PCR products comparable to the

TABLE 2. Primers used for *Archaea*-specific PCR-DGGE targeting *rrs* genes

Primer	Sequence (5′→3′)	Annealing positions[a]	Targeted V region(s)	Amplicon length (bp)[b]	Annealing temp (°C)	$MgCl_2$ concn (mM)	Reference(s)
A2Fa	TTC CGG TTG ATC CYG CCR GA	7–27	V1-V2	343	56	3.0	5, 11, 19
GC-A348r[c]	CCC CRT AGG GCC YGG	335–349					
GC-A24f[c]	TCY GKT TGA TCC YGS CRG A	8–26	V1-V2	306	56	3.0	3
A329r	TGT CTC AGG TTC CAT CTC CG	310–329					This study
A24f	TCY GKT TGA TCC YGS CRG A	8–26	V1-V2	342	56	3.0	3
GC-A348r[c]	CCC CRT AGG GCC YGG	335–249					
GC-ARC344f[c]	ACG GGG YGC AGC AGG CGC GA	344–363	V3	191	56	1.75	4, 15
519r	GWA TTA CCG CGG CKG CTG	519–534					
GC-A357f[c]	CCC TAC GGG GCG CAG CAG	340–357	V3-V4	372	53	3.0	
A693r	GGA TTA CAR GAT TTC	693–707					This study
GC-ARC344f[c]	ACG GGG YGC AGC AGG CGC GA	344–363	V3-V5	591	56	1.75	4, 7, 23
ARC915r	GTG CTC CCC CGC CAA TTC CT	915–934					
GC-ARCH915[c]	AGG AAT TGG CGG GGG AGC AC	915–934	V6-V8	492	65	1.75	12
UNI-b-rev	GAC GGG CGG TGT GTR CAA	1390–1407					
A1040f	GAG AGG WGG TGC ATG GCC	1043–1060	V7	171	56	1.75	3, 25
GC-UA1204r[c]	TTM GGG GCA TRC IKA CCT	1196–1213					

[a] *Escherichia coli rrs* gene numbering.
[b] Calculated using the *rrs* genes of *E. coli*.
[c] Primer with a 40-bp GC clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) attached to the 5′ end.

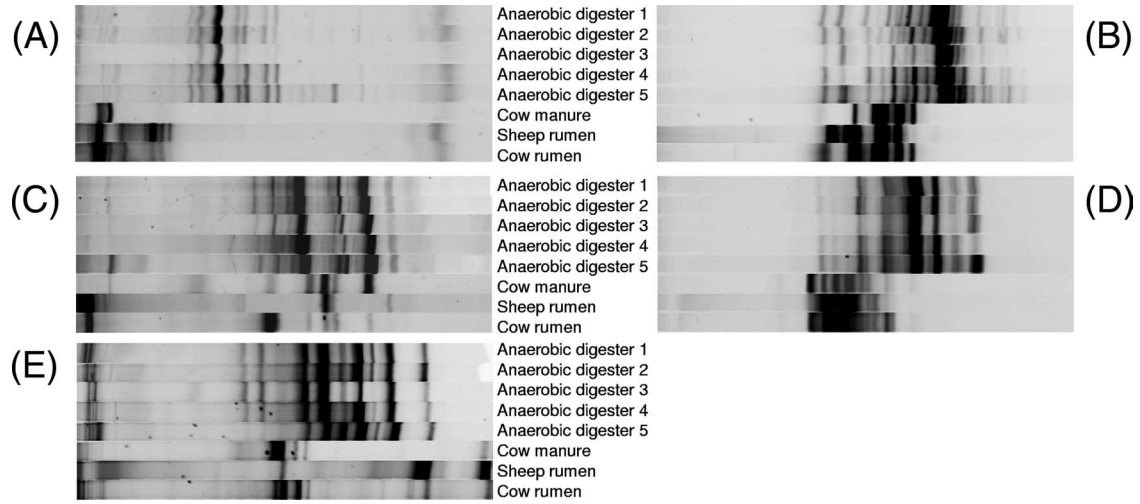

FIG. 1. Methanogenic DGGE banding profiles based on different V regions produced using the community DNA from three gastrointestinal samples and five anaerobic digestors. (A) V1-V2 (delineated by primers A24f/A329r), with a 40 to 60% denaturant gradient. (B) V3, with a 40 to 60% denaturant gradient. (C) V3-V4, with a 40 to 55% denaturant gradient. (D) V7, with a 50 to 70% denaturant gradient. (E) V6-V8, with a 40 to 55% denaturant gradient.

amounts yielded by the rest of the primer sets but only several diffuse DGGE bands with several denaturant gradients (data not shown). These three primer sets were not used in further analyses. The remaining five primer sets all generated informative DGGE profiles (Fig. 1). As expected, with all these primer sets the digestor samples produced significantly more DGGE bands than the gastrointestinal samples. In agreement with the PCR-DGGE employing universal bacterial primer set GC-357f/518r, the V3 region of *Archaea*, as targeted by primer set GC-ARC344f/519r, generated the highest number of well-separated bands, and the V1-V2, V3-V4, V7, and V6-V8 regions generated fewer but similar numbers of bands. The dual hypervariable regions V1-V2 and V3-V4 did not yield any better DGGE profiles than the single V region (Fig. 1 and 2), even though there was high sequence divergence (Table 2). The greater lengths of these amplicons may have complicated their denaturing behavior and thus reduced the DGGE resolution.

Assessment of the impact of dietary fat on ruminal methanogens in sheep. The utility of the five promising primers sets (GC-A24f/A329r, GC-ARC344f/519r, GC-357f/A693r, GC-ARCH915/UNI-b-rev, and A1040f/GC-UA1204r) was tested by analyzing a set of rumen samples collected from four sheep; two sheep (C-1 and C-2) were fed a mixture of corn and hay (40:60 on a dry weight basis), and the other two sheep (F-3 and F-4) were fed a mixture of corn, hay, and tallow (animal fat) (20:60:20). The rumen samples were collected via rumen cannula 14 and 23 days after the sheep were first fed the diets. DNA extraction, PCR, and DGGE were performed as described above, except that the different gradients were optimized for this sample set (Fig. 2). The PCR and DGGE were also repeated, and identical DGGE profiles were obtained (data not shown). The V6-V8 and V7 regions generated very few DGGE bands (data not shown) so they were not used in further analyses. For the remaining three V regions (V1-V2, V3, and V3-V4) and their corresponding primers sets, the two rumen samples collected on day 14 from the two sheep fed

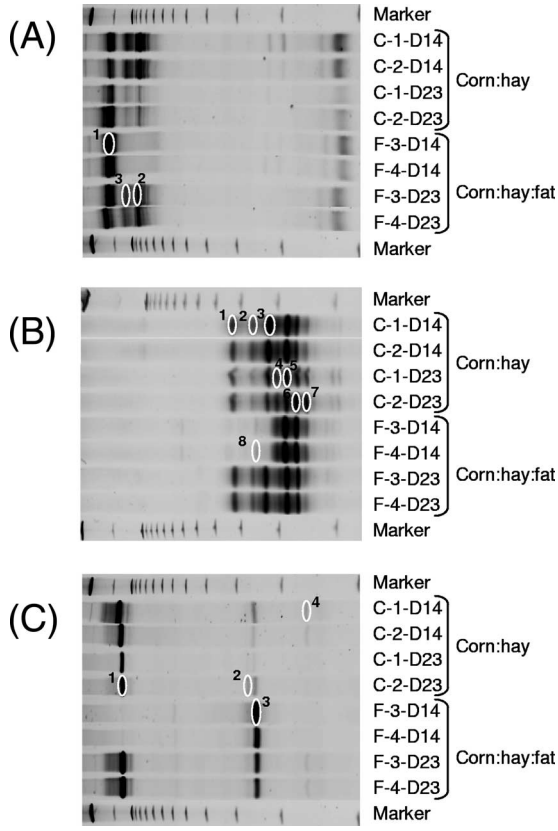

FIG. 2. Methanogenic DGGE profiles of the rumen contents of four sheep. The marker was a 100-bp DNA ladder (Invitrogen); C indicates the controls fed corn and hay without fat; F indicates animals fed corn, hay, and fat; the numbers 1 to 4 indicate the four sheep; and D-14 and D23 indicate 14 and 23 days, respectively, after the animals were first fed the diets before sampling. (A) V1-V2-targeted DGGE profiles (40 to 55% denaturant gradient). (B) V3-targeted DGGE profiles (50 to 55% denaturant gradient). (C) V3-V4-targeted DGGE profiles (40 to 55% denaturant gradient). The methanogens identified by sequencing the circled bands are shown in Table 3.

TABLE 3. Sequence matches for the bands excised from the DGGE gels containing sheep rumen samples[a]

Amplicon	Band	Accession no.	Most similar sequence (accession no.)	% Identity[b]	Occurrence
V1-V2	1	EF442682	*Methanobrevibacter* sp. strain NT7 (AJ009959)	100	All samples
	2	EF442683	*Methanobrevibacter* sp. strain AbM4 (AJ550156)	98	All samples except F-3-D14 and F-4-D14
	3	EF442684	*Methanobrevibacter* sp. strain AbM4 (AJ550156)	97	All samples except F-3-D14 and F-4-D14
V3	2	EF442689	*Methanobrevibacter* sp. strain AbM4 (AJ550156)	99–100[c]	All samples except F-3-D14 and F-4-D14
	4	EF442690	*Methanobrevibacter smithii* PS (AY196669)	100	All samples
	5	EF442691	*Methanobrevibacter runimantum* M1 (AY196666)	99	All samples
	6	EF442692	*Methanobrevibacter woesei* GS (U55237)	100	All samples
	7	EF442693	*Methanobrevibacter smithii* PS (AY196669)	100	All samples
	8	EF442694	*Methanosphaera stadtmanae* MCB-3 (AY196684)	96	F-3-D14 and F-4-D14
V3-V4	1	EF442685	*Methanobrevibacter* sp. strain AbM4 (AJ550156)	98	All samples except F-3-D14 and F-4-D14
	2	EF442686	*Methanobrevibacter thaueri* CW (U55236)	100	C-2-D23
	3	EF442687	*Methanobrevibacter runimantum* NY-1 (AB034183)	99	All samples
	4	EF442688	*Methanobrevibacter smithii* PS (AY196669)	96	C-1-D14, C-2-D14, F-3-D23, F-4-D23

[a] See Fig. 2.
[b] Roundup values and ambiguous bases were considered the same.
[c] The sequences of bands 1 and 3 are very similar to the sequence of band 2.

dietary fat (samples F-3-D14 and F-4-D14) exhibited different DGGE profiles than the rumen samples collected from the two control sheep (samples C-1-D14 and C-2-D14) (Fig. 2). However, this effect diminished by day 23. Despite some temporal changes in the intensity of several bands for the control sheep, these preliminary results suggest that there was a transient impact on the ruminal methanogens that was probably attributable to the dietary fat. Further studies involving a larger number of ruminants are needed to confirm this observation. It is also evident that the V3 region produced the largest numbers of well-separated DGGE bands from these samples (Fig. 2B).

Intense DGGE bands (including one or two bands at the same position in neighboring lanes of the same gel) were excised, reamplified, and sequenced to identify the major methanogens. Briefly, immediately following image capturing, select DGGE bands were excised and placed into 30 μl of Tris-EDTA buffer. Freezing (−80°C for 10 min) and thawing (65°C for10 min) were performed three times to facilitate diffusion of the DNA fragments into the buffer. After centrifugation (4°C, 5 min), 1 μl of the supernatant was used as the template in a PCR to reamplify the DNA fragment as described above, with two modifications: no GC clamp was attached to either primer, and only 35 regular cycles were performed. The PCR products were confirmed by agarose (1.5%) gel electrophoresis, purified using a QIAquick PCR product purification kit (Qiagen, Inc., Valencia, CA), and sequenced at the Genotyping-Sequencing Core Facility at The Ohio State University. The base calling was visually examined using the chromatogram. All the sequences appeared to be good quality with little ambiguous base calling. These sequences were compared to the GenBank sequences using BLASTn (2). The most similar sequence derived from a known member of the *Archaea* was recorded as the most similar match. All the recovered sequences are closely related to sequences of known methanogens (Table 3), confirming the specificity of the primers used in this study. It was noticed that in bacterial PCR-DGGE analysis employing universal primers, ambiguous bases sometimes result from direct sequencing of DNA reamplified from excised DGGE bands (13). The success of direct sequencing of the DNA reamplified from the DGGE bands may be attributed to the limited diversity of methanogens in the samples.

It is especially interesting that different subsets of methanogens were detected in the samples by targeting the three different V regions (Table 3). This can probably be attributed to the inherent bias of different primers sets. This explanation is in accordance with a quantitative study in which different PCR efficiencies were observed for different *rrs* sequences (22). Thus, DGGE data should be interpreted with caution. More than one primer set may be needed to detect most archaeal members in a given sample. The sequenced DGGE bands also suggest that there was high methanogen diversity at low taxonomic levels (species and subspecies), at least for *Methanobrevibacter* spp. Additionally, *Methanobrevibacter* spp. appeared to be the most common methanogens in all the rumen samples analyzed (Table 2). This is consistent with the high prevalence of this genus determined by two other approaches: clone libraries (29) and rRNA probing (26). Furthermore, the V3-targeted PCR-DGGE permitted recovery of *rrs* sequences of common rumen methanogens, including *Methanobrevibacter smithii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter woesei*, and *Methanosphaera* sp. These results suggest that the V3 region should be the preferred V region to target when the archaeal communities in the rumen are profiled by PCR-DGGE analysis.

Dietary fat supplementation has been shown to inhibit methane production in the rumen (18, 34) and in in vitro cultures (6). However, which methanogen species was affected was not determined. This study provided some preliminary results suggesting that dietary tallow may stimulate *Methanosphaera stadtmanae*, which grows only by using H_2 to reduce methanol to methane, while it inhibits *Methanobrevibacter* sp. strain AbM4 (Table 3). However, such effects probably diminish gradually after adaptation. These results also suggest that the methanogenic subcommunity in the rumen is resilient. More studies are required to confirm such effects. Additionally, since not all the methanogens in the rumen are equally sensitive to an inhibitor, a combination of several inhibitors may have to be used to effectively mitigate methanogenesis in the rumen.

Taken together, the results showed that V3 appeared to be

the best choice for community profiling of *Archaea* by specific PCR-DGGE. Other V regions, such as V1-V2 and V3-V4, may also need to be targeted by *Archaea*-specific primers to detect most archaeal members in a microbial community. Preliminary results also indicate that dietary fat has only a transient impact on some ruminal methanogens. We expect this study to be useful for the standardization of *Archaea*-specific PCR-DGGE needed to produce the most informative profiles of methanogenic subcommunities.

Nucleotide sequence accession numbers. The *rrs* sequences determined in this study have been deposited in the GenBank database, and the accession numbers are shown in Table 3.

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