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Comparisons of Different Hypervariable Regions of *rrs* Genes for Use in Fingerprinting of Microbial Communities by PCR-Denaturing Gradient Gel Electrophoresis

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Denaturing gradient gel electrophoresis (DGGE) has become a widely used tool to examine microbial diversity and community structure, but no systematic comparison has been made of the DGGE profiles obtained when different hypervariable (V) regions are amplified from the same community DNA samples. We report here a study to make such comparisons and establish a preferred choice of V region(s) to examine by DGGE, when community DNA extracted from samples of digesta is used. When the members of the phylogenetically representative set of 218 *rrs* genes archived in the RDP II database were compared, the V1 region was found to be the most variable, followed by the V9 and V3 regions. The temperature of the lowest-melting-temperature ($T_{m(L)}$) domain for each V region was also calculated for these *rrs* genes, and the V1 to V4 region was found to be most heterogeneous with respect to $T_{m(L)}$. The average $T_{m(L)}$ values and their standard deviations for each V region were then used to devise the denaturing gradients suitable for separating 95% of all the sequences, and the PCR-DGGE profiles produced from the same community DNA samples with these conditions were compared. The resulting DGGE profiles were substantially different in terms of the number, resolution, and relative intensity of the amplification products. The DGGE profiles of the V3 region were best, and the V3 to V5 and V6 to V8 regions produced better DGGE profiles than did other multiple V-region amplicons. Introduction of degenerate bases in the primers used to amplify the V1 or V3 region alone did not improve DGGE banding profiles. Our results show that DGGE analysis of gastrointestinal microbiomes is best accomplished by the amplification of either the V3 or V1 region of *rrs* genes, but if a longer amplification product is desired, then the V3 to V5 or V6 to V8 region should be targeted.

The inherent limitations associated with cultivation-based approaches to characterizing microbial communities are widely recognized, and a number of cultivation-independent, molecular methods have emerged in recent years to improve our understanding of this aspect of microbial ecology. Techniques such as denaturing gradient gel electrophoresis (DGGE) (8, 20, 21, 27), terminal restriction fragment length polymorphism (15, 22, 28, 38), length heterogeneity PCR (29, 34), automated rRNA intergenic spacer analysis (13), and ribosomal intergenic spacer length polymorphism (9, 40) are now widely used and reported in the literature. In the in silico analyses of 41 completely sequenced bacterial genomes, DGGE appeared to be one of the best molecular community fingerprinting techniques in terms of predicting the actual Shannon-Wiener diversity index, richness, and evenness (3). Additionally, DGGE supports the identification of community members because the amplification products can be recovered and sequenced (4, 6, 20, 31). This may explain why DGGE has become the most frequently used method of molecular community fingerprinting.

In PCR-DGGE, either a single hypervariable (V) region or a combination of two or three V regions in *rrs* genes is amplified (1, 2, 7, 12, 14, 16, 17, 19, 31–33, 35–37). In studies of rumen and gastrointestinal microbiomes, the V1, V3, V1 to V3, and V6 to V8 regions have all been examined (5, 11, 24, 25, 31). Although it is well recognized that the quality of information produced by PCR-DGGE is dependent on both the number and resolution of the amplicons in denaturing gradient gels, few authors have explained their justification of primer choice and DGGE conditions. Indeed, there appears to have been little or no systematic evaluation of how the choice of primers or denaturing conditions influence data quality and veracity of the analysis.

In this context, we have compared the PCR-DGGE profiles arising from a common set of community DNA samples, using primer sets that have been previously reported in the literature. We have also used the phylogenetically representative set of *rrs* genes archived in the RDP II database to calculate melting temperature (T_m) values of the lowest-melting-temperature ($T_{m(L)}$) domains for each V region and to formulate DGGE conditions for each set of amplification products. We expect that our results can be used to standardize the primer sets and DGGE conditions needed to produce the most comprehensive information about the microbial diversity present in rumen and gastrointestinal microbiomes.

MATERIALS AND METHODS

Microbial community samples and DNA extraction. Microbial community samples were collected from four sheep. Two were fed a mixture of hay and grain (60:40 on a dry-matter basis; sheep are referred to as C1 and C2), and the other two were fed a mixture of hay, grain, and tallow (60:20:20 on a dry-matter basis; sheep are referred to as F1 and F2). Samples of rumen digesta were collected via rumen cannulae 14 and 23 days after the sheep were started on those diets. The digesta were strained through two layers of cheesecloth, and community DNA from these eight rumen fluid samples was extracted using the RBB + C method

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TABLE 1. PCR primers, targeted hypervariable regions, PCR, and DGGE conditions used in this study

Primer	Sequence (5′→3′)	Annealing positions[b]	Target	Annealing temp	Amplicon length (bp)[c]	DGGE conditions	Reference(s)
−63f[a]	GCC TAA CAC ATG CAA GTC	46–63	V1	58→53°C, −0.5°C/cycle	80	8%, 30–50%, 620 V · h	2, 31
109r	ACG TGT TAC TCA CCC GT	109–125					
−63f[a]	GCC TAA CAC ATG CAA GTC	46–63	V1–V3	58→53°C, −0.5°C/cycle	489	6.5%, 20–70%, 1,230 V · h	5
518r	ATT ACC GCG GCT GCT GG	518–534					
−357f[a]	CCT ACG GGA GGC AGC AG	341–357	V3	61→56°C, −0.5°C/cycle	194	6.5%, 40–60%, 1,230 V · h	7, 35
518r	ATT ACC GCG GCT GCT GG	518–534					
533f	GTG CCA GCA GCC GCG GTA A	515–533	V4–V5	56→51°C, −0.5°C/cycle	412	6.5%, 20–70%, 1,230 V · h	16
907r[a]	CCG TCA ATT CCT TTG AGT TT	907–926					
−357f[a]	CCT ACG GGA GGC AGC AG	341–357	V3–V5	56→51°C, −0.5°C/cycle	586	6.5%, 30–60%, 1,230 V · h	1, 37
907r	CCG TCA ATT CCT TTG AGT TT	907–926					
−954f[a]	GCA CAA GCG GTG GAG CAT GTG G	933–954	V6–V8	61→56°C, −0.5°C/cycle	456	6.5%, 35–60%, 1,230 V · h	18, 19
1369r	GCC CGG GAA CGT ATT CAC CG	1369–1388					
F-968[a]	AAC GCG AAG AAC CTT AC	968–984	V6–V8	61→56°C, −0.5°C/cycle	434	6.5%, 35–60%, 1,230 V · h	25, 42
R-1401	CGG TGT GTA CAA GAC CC	1385–1401					
1070f	ATG GCT GTC GTC AGC T	1055–1070	V8	53→48°C, −0.5°C/cycle	352	6.5%, 40–60%, 1,230 V · h	12, 14
−1392r[a]	ACG GGC GGT GTG TAC	1392–1406					

[a] Primers with a 40-bp GC clamp at the 5′ end.
[b] Numbering according to the *rrs* gene of *Escherichia coli.*
[c] Calculated from the *rrs* gene of *E. coli*; the primers were included.

(41). The DNA recovered from each sample was quantified using the PicoGreen double-stranded DNA quantitation kit (Molecular Probes, Inc., Eugene, Oreg.) and diluted to a final concentration of 50 ng/μl with Tris-EDTA.

In silico analysis of *rrs* gene V regions. The phylogenetically representative set of 218 *rrs* genes was downloaded from the RDP II database (release 8.1). The V regions were delimited by the primer sequences flanking them, and the respective sequence identities were calculated using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The average (± standard deviation) of the percent sequence identities for each individual V region, or combination thereof, was calculated using Microsoft Excel.

The $T_{m(L)}$ value of each V region was also calculated for the same sequences with Primo Melt 3.4 software (http://www.changbioscience.com/primo/primomel.html), and the frequencies of $T_{m(L)}$ values for the V1, V3, and V5 regions were graphed using Microsoft Excel. It was assumed that a 40-bp GC clamp was present at one end of each amplicon. The average $T_{m(L)}$ for each V region and its standard deviation were calculated, and these values were used to predict the denaturing gradients needed to effectively resolve 95% of all the amplicons produced by a specific primer set, with the following equations:

$$d_L(\%) = (T_{m(L)} - T_b) - 2\sigma/C \quad (1)$$

$$d_H(\%) = (T_{m(L)} - T_b) + 2\sigma/C \quad (2)$$

where d_L and d_H are the low and high denaturant concentrations, respectively; $T_{m(L)}$ is the average T_m value of the lowest-melting-temperature domains of each amplicon; T_b is the temperature of the running buffer (typically 60°C); σ is the standard deviation corresponding to the average $T_{m(L)}$ of that amplicon; and C is a constant, relating chemical denaturant concentration to melting temperature of double-stranded DNA (in DGGE, C = 0.3°C/1% denaturant as described in reference 10).

PCR and DGGE. The primers, annealing temperatures, and DGGE conditions used in this study are listed in Table 1. The following degenerate primer sets were used to evaluate the effects of primer degeneracy on DGGE profiles: Deg-63f (5′-GCY TAA BVC ATG CRA GTC-3′), Deg-109r (5′-AYG YRT TAC TSA SCC KT-3′); Deg-357f (5′-ACW CCT ACG GGD SGC WGC A-3′), and Deg-518r (5′-GTA TTA CCG CGG CKG CTG-3′). Degenerate bases (equimolar ratios) were introduced at positions where two or more nucleotides occur at relatively high frequency within the phylogenetically representative set of *rrs* genes. Inosine-containing primers with the underlined bases replaced with inosine were also tested. All PCR amplifications were performed using a PTC-100 thermocycler (MJ Research, Waltham, Mass.) in 50-μl volumes containing 1× PCR buffer (20 mM Tris-HCl [pH 8.4] and 50 mM KCl), 200 μM deoxynucleoside triphosphates, 500 nM (each) primer, 1.75 mM $MgCl_2$, 670 ng of bovine serum albumin/μl, and 1.25 U of Platinum *Taq* DNA polymerase (Invitrogen

TABLE 2. Sequence identities of the PCR amplicons calculated from the phylogenetically representative set of 218 *rrs* genes in RDP II and the melting temperatures of the lowest-melting-temperature domains ($T_{m(L)}$)

Hypervariable region	% Sequence identity[a]	No. of $T_{m(L)}$ groups[b]	No. of sequences sharing the same $T_{m(L)}$	Avg $T_{m(L)}$ (°C)[a]	Denaturant gradient (%)[c]
V1	34.4 (12.1)	120	1.83	77.6 (4.9)	26–91
V2	62.9 (8.0)	108	2.03	74.1 (3.3)	25–69
V3	60.9 (15.4)	89	2.46	76.3 (2.6)	37–72
V4	67.4 (8.4)	93	2.35	76.3 (2.7)	36–72
V5	73.5 (7.1)	77	2.84	76.3 (2.1)	40–68
V6	69.3 (15.3)	64	3.42	77.1 (1.6)	46–68
V8	76.5 (7.6)	73	3.00	75.7 (1.9)	40–65
V9	48.3 (16.2)	84	2.61	77.6 (2.5)	42–75
V1–V3	52.7 (20.7)	101	2.17	74.5 (3.2)	27–70
V3–V5	69.2 (9.0)	91	2.41	74.7 (2.7)	31–67
V4–V5	71.7 (7.2)	89	2.46	74.8 (2.5)	33–66
V6–V8[d]	70.6 (10.5)	80	2.74	73.6 (2.2)	31–60
V6–V8[e]	71.8 (10.3)	78	2.80	75.1 (2.2)	36–65
V7–V8	74.2 (7.5)	75	2.92	75.3 (2.2)	36–66

[a] Values in parentheses are standard deviations.
[b] Difference of >0.1°C.
[c] Assuming that DGGE gels are run at 60°C.
[d] Amplicons delimited with GC-954f and 1368r.
[e] Amplicons delimited with F-968-GC and R-1401.

Corporation, Carlsbad, Calif.), which allows for hot-start PCR. After an initial denaturation at 94°C for 5 min, 10 cycles of touchdown PCR were performed (denaturation at 94°C for 30 s, annealing for 30 s with an 0.5°C/cycle decrement at a temperature 5°C above the respective annealing temperatures indicated in Table 1, and extension at 72°C for 1 min), followed by 25 cycles of regular PCR (94°C for 30 s, 30 s at the respective annealing temperature, and 72°C for 1 min [0.5 min for PCR amplification of the V1 region]) and a final extension step for 7 min at 72°C. Negative controls, containing all the components except DNA templates, were included in parallel.

After PCR, 5-μl aliquots were subjected to agarose gel electrophoresis with 1.5% (wt/vol) agarose gels (2% [wt/vol] for the V1-region amplicons). Then, 15-μl aliquots were resolved on polyacrylamide gels (37.5:1) containing a gradient of denaturants (100% denaturants consisting of 40% [vol/vol] formamide and 7 M urea) as indicated in Table 1. All the DGGE gels were run at 60°C and 82 V to reach the volts-hours indicated in Table 1, with a Dcode Universal Mutation detection system (Bio-Rad Laboratories, Hercules, Calif.). The DGGE gels were stained with GelStar (BioWhittaker Molecular Applications, Rockland, Maine) according to the manufacturer's specifications, and the images were captured using a FluorChem Imager (Alpha Innotech Corp., San Leandro, Calif.).

DGGE gel analysis. The DGGE bands were detected using the band-searching algorithm of BioNumerics software (BioSystematica, Tavistock, Devon, United Kingdom). After normalization of the gels, only those bands with a peak height intensity exceeding 2.0% of the strongest band in each lane were included in further analyses. Diversity indices were also calculated: richness (S) was determined from the number of bands in each lane, and the Shannon-Wiener index (H') was calculated from $H' = -\Sigma P_i \ln P_i$ (30), where P_i is the importance probability of the bands in a lane, calculated from n_i/N, where n_i is the peak height of a band and N is the sum of all peak heights in the densitometric curve. Evenness (E) was calculated as $E = H'/H'_{max}$, where $H'_{max} = \ln S$ (26).

RESULTS

Sequence divergence within various V regions and their $T_{m(L)}$ heterogeneity. With the use of the phylogenetically representative set of *rrs* genes archived at RDP II, the V1 region was found to be the most divergent, as indicated by the low average sequence identities and the high standard deviation (Table 2). The V3 and V9 regions are also more divergent than the remaining V regions examined. Accordingly, the V1, V2, and V3 regions combined were found to possess the highest sequence divergence compared to the other multiple V regions.

The average $T_{m(L)}$ values for the V regions ranged from 73.6 to 77.6°C (Table 2), and the V1, V2, and V1 to V3 regions possess the greatest $T_{m(L)}$ heterogeneity, while the V5, V6, V8, and V7-V8 regions have the lowest $T_{m(L)}$ heterogeneity. Moreover, the frequency of calculated $T_{m(L)}$ values for the V1, V3, and V5 regions all appeared to be normally distributed about the average value (Fig. 1). Equations 1 and 2 described in Materials and Methods were therefore devised to predict the denaturing gradient necessary to resolve 95% of the amplification products arising for each V region. Table 2 shows that the V1 region requires the largest denaturing gradient (26 to 91%), while the V6 region requires the smallest (46 to 68%). With the use of these equations, the denaturing gradients listed for the V1, V3, and V5 regions should effectively resolve 95.0, 95.4, and 92.7% of the 218 phylogenetically representative *rrs* sequences, respectively.

DGGE profiles of rumen microbial community DNA samples. The DGGE profiles produced using the different primer sets and conditions listed in Table 1 are shown in Fig. 2, and the diversity indices calculated from the PCR-DGGE banding profiles are shown in Table 3. Amplification of either the V1 or V3 region alone yielded more intense bands, and the V3 region produced the largest number of bands (and richness score),

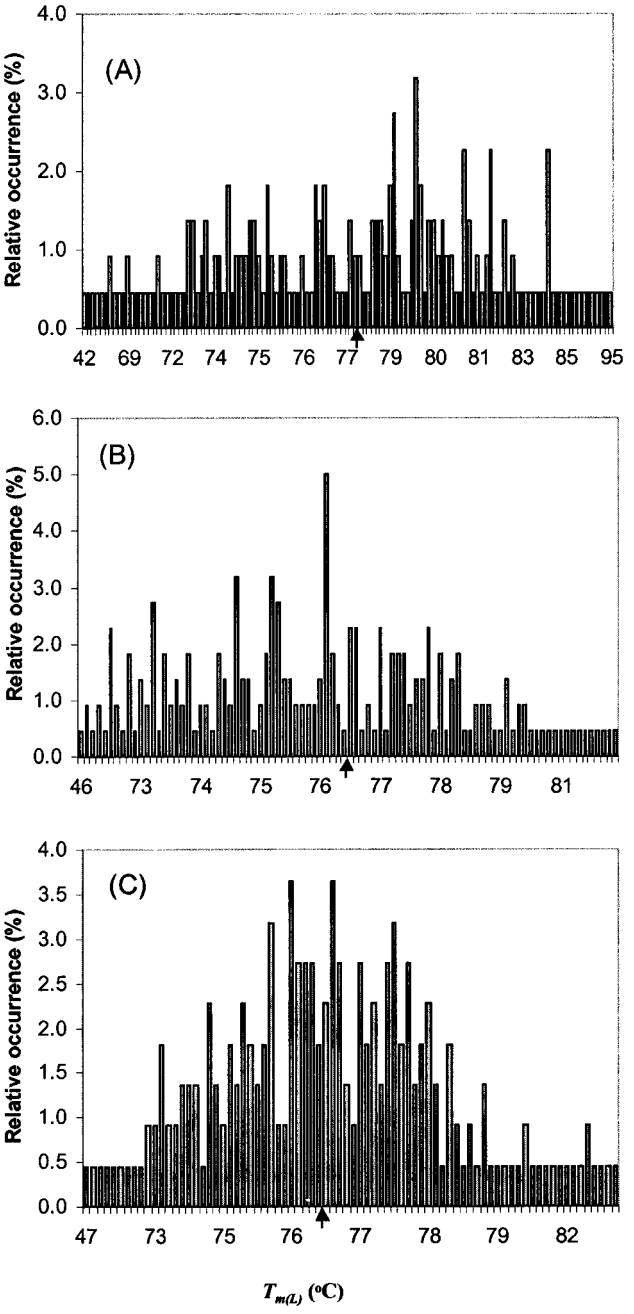

FIG. 1. Frequency distribution of $T_{m(L)}$ values for the V1 region (A), V3 region (B), and V5 region (C), derived from the phylogenetically representative set of 218 *rrs* gene sequences archived in RDP II (release 8.1). The arrows indicate the average $T_{m(L)}$ value calculated for each V region, and the $T_{m(L)}$ values appear to be normally distributed.

followed by the V1 and V8 regions. However the V1, V3, and V8 DGGE profiles all produced relatively low evenness scores, apparently due to the existence of a relatively small number of intense bands. When multiple V regions were amplified, the richness scores were all lower than that for the V3 region alone, because of the reduced number of discernible bands, and the V1 to V3 region was not amplified as efficiently as were other V regions, as judged by the intensity of the bands on the

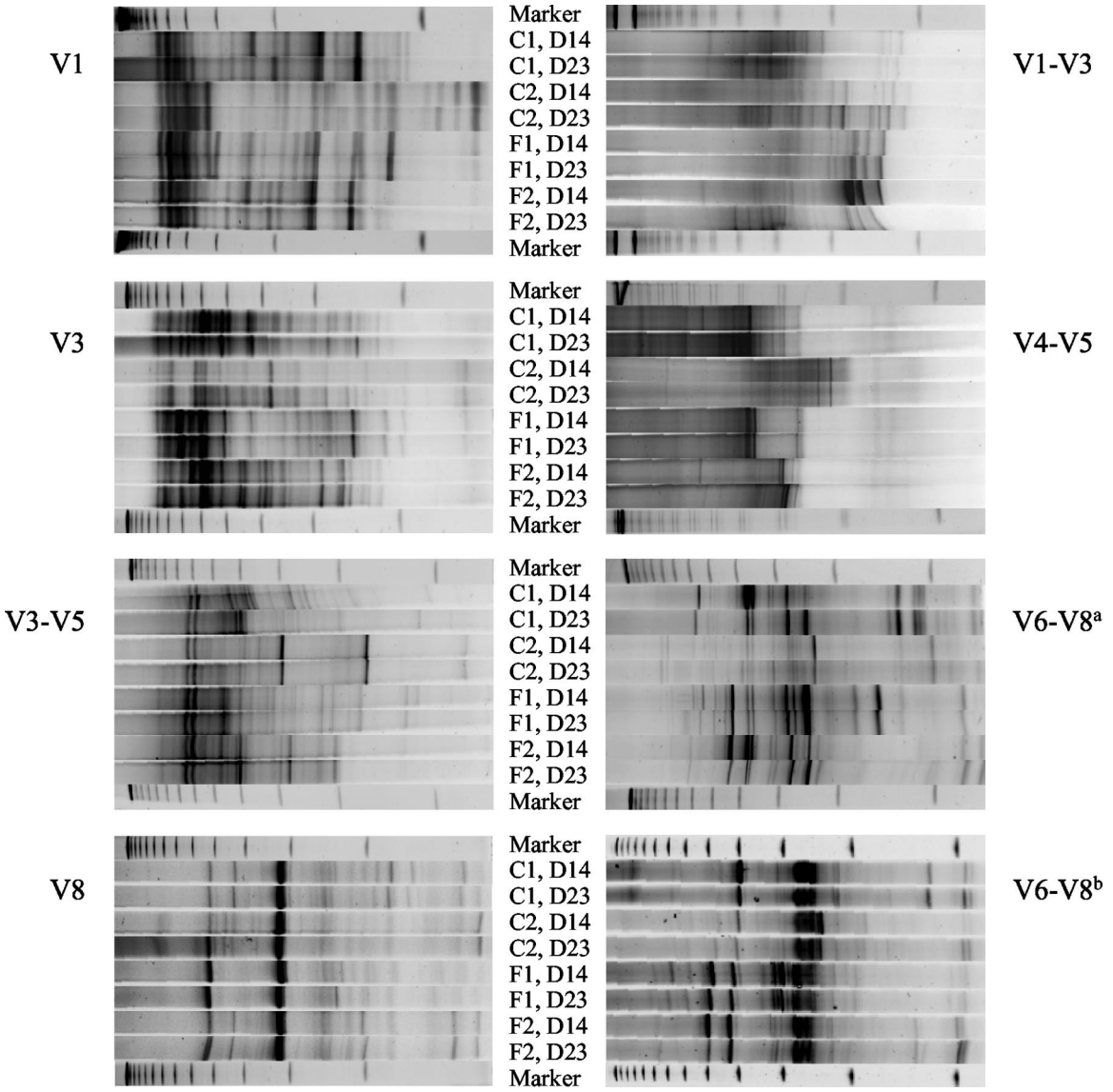

FIG. 2. DGGE banding profiles of V regions produced from the community DNA extracted from eight different samples of ruminal digesta. The nondegenerate primers and DGGE conditions described in Table 1 were used in the PCR and DGGE. The lanes labeled C1 and C2 represent samples of digesta collected from animals fed a mixture of hay and grain, and the lanes labeled F1 and F2 represent samples of digesta collected from animals fed a mixture of hay, grain, and beef tallow. D14 and D23, samples collected 14 and 23 days, respectively, after the animals were started on the diet; Marker, electrophoresis marker; V6 to V8[a], DGGE profiles generated using primers GC-954f and 1369r; V6 to V8[b], DGGE profiles generated using primers F-968-GC and R-1401.

DGGE gel (Fig. 2). In contrast, the evenness scores were all higher for the multiple-V-region profiles, suggesting that band intensity was more uniform in these DGGE profiles. Another interesting finding was the differences arising in DGGE profiles and diversity indices when the different V6 to V8 primer sets were used (Fig. 2 and Table 3). Overall, the primer set consisting of GC-954f and 1369r resulted in higher values than those obtained with F-968-GC and R-1401, although both primer sets were found to produce lower richness, Shannon-Wiener, and evenness values than those for the V3 to V5 region (Table 3). Based on these results, it appears that amplification of the V3 region alone produced the most informative DGGE profiles, and if a longer amplification product is required, then either the V3 to V5 region or the V6 to V8

TABLE 3. Diversity indices calculated from the DGGE banding profiles generated from various hypervariable regions

V region(s)	Richness (*S*)	H'^{a}	$H'_{max}{}^{b}$	Evenness (*E*)
V1	24.6	2.67	3.20	0.83
V3	31.3	3.04	3.44	0.88
V8	15.0	1.64	2.71	0.61
V1–V3	15.3	2.58	2.73	0.95
V3–V5	21.8	3.03	3.08	0.98
V4–V5	14.3	2.37	2.66	0.89
V6–V8[c]	20.8	2.95	3.05	0.97
V6–V8[d]	17.3	2.46	2.85	0.86

[a] H', Shannon-Wiener index.
[b] H'_{max}, maximum Shannon-Wiener index.
[c] Primers used were GC-954f and 1369r, as described by Iwamoto et al. (18).
[d] Primers used were F-968-GC and R-1401 as described by Nübel et al. (25).

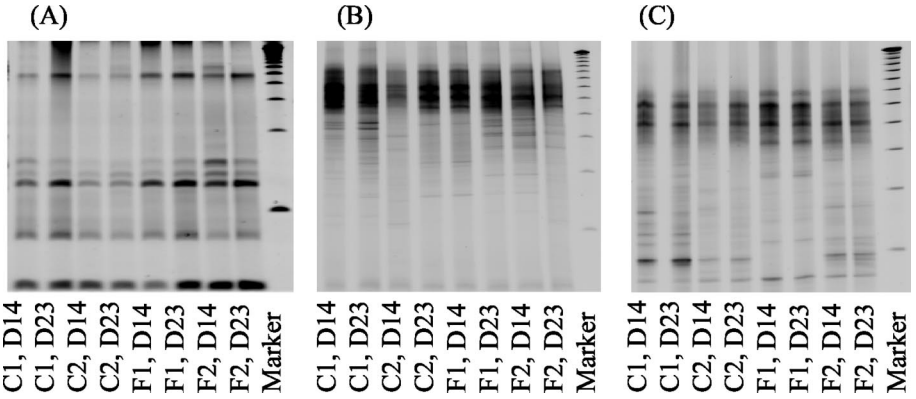

FIG. 3. DGGE gel banding profiles of the V1 and V3 regions produced with V1-specific inosine-containing primers (A), V3-specific degenerate primers (B), or V3-specific inosine-containing primers (C). The labeling of lanes follows the same protocol as described for Fig. 2.

region amplified with the GC-954f–1369r primer set should be selected.

Impact of degenerate PCR primers on DGGE profile. The introduction of degenerate bases into the V1-specific primer set substantially decreased the number of bands (Fig. 3A), and the replacement with inosine resulted in unsuccessful PCR amplification at the various annealing temperatures (42 to 64°C) and $MgCl_2$ concentrations (1.5 to 2.75 mM) tested. The degenerate primers specific for the V3 region increased the number of bands at the upper portion of the DGGE gel, but there was a decrease in the number of bands appearing at the lower portion of the gel (Fig. 3B). Although the use of inosine-containing primers resulted in a more even band distribution in the DGGE gel (Fig. 3C), there was little influence on the diversity indices (data not shown). Based on these results, it appears that the introduction of degenerate bases and/or inosine in the V1 and V3 primer sequences does not improve the PCR-DGGE profiles.

DISCUSSION

Although there are numerous published reports of using PCR-DGGE to examine microbial diversity (e.g., references 1, 12, 14, 16, 18, and 19), how the quality of the information obtained is impacted by the choice of V region(s) amplified has not been previously evaluated. The results presented here with eight different community DNA samples clearly show that the V region(s) chosen for amplification can greatly influence the PCR-DGGE profiles and diversity indices produced from community DNA samples, and even subtle differences in primer sequences can result in substantially different profiles and assessment of microbial diversity. The denaturing gradient used will also affect the results obtained, but this, too, has received little attention. The gradient estimation approach described here was very effective in producing well-resolved banding profiles, with all the primer sets used here. By using the set of 218 *rrs* gene sequences archived in RDP II to calculate average $T_{m(L)}$ values (and standard deviations), we avoided bias towards those taxa that have more sequence representations in RDP II. However, our approach to choosing denaturing gradients should also be applicable to guild-, genus-, or species-specific PCR-DGGE analyses, where closely related sequences require a narrow denaturing gradient to maximize the resolving power of DGGE.

Theoretically the V1 region, with the highest sequence divergence and $T_{m(L)}$ heterogeneity, should have produced DGGE profiles with the greatest number of resolved bands. However, the DGGE profiles of the V1 region alone were inferior to those obtained with V3-specific primers (Table 3 and Fig. 2). This may be attributed to the limited length of the V1 region, rather than the limited universality of the primer set used. This explanation is supported by the observation that degenerate primers did not improve DGGE profiles of the V1 region (Fig. 3A). For the V3 region, although inosine replacements resulted in an increase in the number of bands at the lower portion of the DGGE gel (Fig. 3C), the use of degenerate primers did not improve the assessment of microbial diversity in the samples. For these reasons, we propose that PCR-DGGE targeting the V1 region be avoided in future studies of gut microbiomes and that degenerate primers be used with caution in PCR-DGGE analyses.

A necessary requirement of all DGGE-based studies of microbial ecology is the reamplification and sequencing of excised amplicons, to provide a more detailed characterization of these microbial communities. For such purposes, longer amplicons would be preferable to facilitate identification to species level with a higher degree of probability. Most automated DNA sequencers now produce in excess of 500 bp of unambiguous sequence, which can span at least two V regions. Based on the results obtained here, amplification of the V3 to V5 region produced superior DGGE profiles. Given the results obtained when either the V1 or V3 region was amplified alone, we were also surprised by the relatively poor DGGE profiles when the V1 to V3 region was amplified. An in silico analysis of the primer set consisting of GC-63f and 518r performed using Primer Designer (Scientific & Educational Software, Durham, N.C.) did not show primer self-annealing or hairpin formation. Combined with the results obtained when the V1 region alone was amplified, a different forward primer (e.g., 27f, 5′-AGA GTT TGA TCM TGG CTC AG-3′ [23]) may improve the amplification and DGGE profiles of the V1 to V3 region.

It is interesting to notice the difference in results obtained when two different primer sets were used to amplify the V6 to

V8 region (Fig. 2 and Table 3). The primer set consisting of F-968-GC and R-1401 set has been frequently used for PCR-DGGE analysis of community DNA samples from human and animal gastrointestinal tracts (11, 21, 39, 42), while the primer set consisting of GC-954f and 1369r has been used with other types of environmental samples (18, 19). The annealing sites for these primers are in high proximity to each other: the F-968-GC and GC-954f primers are 30 nucleotides apart, and the R-1401 and 1369r primers are 16 nucleotides apart. However, the primer set consisting of GC-954f and 1369r is more universal than that consisting of F-968-GC and R1401, because when compared to all 16S *rrs* sequences in RDP II (release 8.1), 1369r matches many more (8,595 perfect matches and 4,482 nearly perfect matches [$0.9 < S_{ab} < 1.0$]) than does R-1401 (2,049 perfect matches and 495 nearly perfect matches). The improved DGGE profiles derived with GC-954f and 1369r are qualitatively consistent with the above in silico analysis, and the different DGGE profiles generated from these two primer sets suggest detection of different populations in the samples. For these reasons, we propose that, if the V6 to V8 region is chosen for PCR-DGGE analyses, a more informative analysis of gastrointestinal microbiomes will be produced by using GC-954 and 1369r rather than F-968-GC and R-1401.

In conclusion, given the similarities among the microbial communities present in the gastrointestinal tracts of humans and other herbivores, in terms of the dominance of these communities by members of the phylum *Bacteroidetes* and class *Clostridia*, we recommend that the V3 region be routinely used in PCR-DGGE analyses with such samples. Alternatively, the V3 to V5 or V6 to V8 (with the use of GC-954f and 1369r) region can be chosen if a longer amplicon is preferred.

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