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Human Colonic Biota Studied by Ribosomal DNA Sequence Analysis

KENNETH H. WILSON* AND RHONDA B. BLITCHINGTON

Infectious Diseases Section, VA Medical Center, and Duke University Medical Center, Durham, North Carolina

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Human colonic biota is a complex microbial ecosystem that serves as a host defense. Unlike most microbial ecosystems, its composition has been studied extensively by relatively efficient culture methods. We have compared an established culture-based method with direct amplification and partial sequencing of cloned 16S rRNA genes from a human fecal specimen. Nine cycles of PCR were also compared with 35 cycles. Colonies and cloned amplicons were classified by comparing their ribosomal DNA (rDNA; DNA coding for rRNA) sequences with rDNA sequences of known phylogeny. Quantitative culture recovered 58% of the microscopic count. The 48 colonies identified gave 21 rDNA sequences; it was estimated that 72% of the rDNA sequences from the total population of culturable cells would match these 21 sampled sequences (72% coverage). Fifty 9-cycle clones gave 27 sequences and 59% coverage of cloned rDNAs. Thirty-nine rDNAs cloned after 35 cycles of PCR gave 13 sequences for 74% coverage. Thus, the representation of the ecosystem after 35 cycles of PCR was distorted and lacked diversity. However, when the number of temperature cycles was minimized, biodiversity was preserved, and there was good agreement between culturing bacteria and sampling rDNA directly.

Numerous recent studies have reported the results of sampling small subunit ribosomal DNA (rDNA; DNA coding for rRNA) sequences found in various environments to explore the composition of complex natural communities of bacteria (8, 16–19). Uniformly, these investigations have led to the conclusion that traditional methods of culturing natural populations have underestimated biodiversity. Thus, samples of rDNA derived from seawater, soil and the cyanobacterial mat of hot springs appear to represent predominant populations in these ecosystems, while the species that grow on culture plates represent only minor populations. These results have not been surprising given that the vast majority of organisms counted microscopically in samples from these environments cannot be grown.

The human colonic biota presents a somewhat different situation in that extensive past investigations have characterized this ecosystem in more detail than most other natural communities (4, 10, 12). The recovery of fecal bacteria on culture has sometimes been described as approaching the microscopic count. The results of these studies have drawn the picture of a highly complex ecosystem, consisting predominantly of hundreds of species of anaerobic bacteria. However, this picture cannot be considered to be drawn in indelible ink. Most of the work cited in which microscopic counts were performed relied on counting Gram-stained organisms. It is likely that some organisms were washed off slides during the staining procedure and were not counted, artificially raising the calculated recovery rate.

We performed the present study to test the hypothesis that significant populations of bacteria in human biota are missed even when optimal culture methods are used. A freshly passed human fecal sample was mixed, serially diluted, and plated anaerobically on a specialized culture medium. DNA was extracted from the same specimen, and bacterial rDNA was amplified, cloned, and sequenced. rDNA sequences found in the cloned material were compared with those found in colonies. Since the recovery of bacteria from human fecal specimens is relatively efficient when done optimally, the study could also be designed to examine bias introduced by PCR and cloning.

* Corresponding author. Mailing address: Chief, Infectious Diseases Section (111H), VA Medical Center, 508 Fulton St., Durham, NC 27705. Phone: (919) 286-6950. Fax: (919) 286-6895. Electronic mail address: Wilso003@mc.duke.edu.

MATERIALS AND METHODS

Fecal culture. A fecal specimen freshly passed by a 40-year-old man who had not had antibiotics for 5 years was taken immediately into an anaerobic chamber. The specimen was kneaded in a plastic bag, and a 1-ml aliquot was placed in 30 ml of prereduced tryptic soy broth and homogenized for 10 s in a blender. Serial dilutions were performed in tryptic soy broth, and 0.1-ml samples of the appropriate dilutions were plated on anaerobically sterilized modified medium 10 (3). Appropriate dilutions were also counted microscopically in a Petroff-Hausser chamber. After 3 days of anaerobic incubation at 37°C, colonies were transferred to medium 10 stab cultures and to buffer for amplification of rDNA.

Extraction and amplification of nucleic acids from fecal specimen. A second 1-ml aliquot of the same fecal homogenate was placed in a 25-ml Corex tube for extraction of DNA. It was vortexed until dispersed in 20 ml of phosphate-buffered saline, and the debris was removed by centrifugation at $30 \times g$ for 2 min. The supernatant was spun at $12{,}000 \times g$ for 5 min. The final pellet was resuspended in 500 ml of Tris-EDTA buffer (pH 8.0). The DNA was then extracted by adding 500 ml of buffer-saturated phenol with 1/10 volume of 25- to 50-μm glass beads and shaking for 1 min on a minibead beater (Biospec Corp., Bartlesville, Okla.). DNA was further extracted three times with phenol-chloroform and then precipitated with 1/10 volume of 3 M sodium acetate and 3 volumes of ethanol. Amplification with primers PC5B (*Escherichia coli* positions 1507 to 1492) and P3mod (*E. coli* positions 787 to 802) has been described previously (7). For the nine-cycle PCR, the fecal DNA was gel purified in agarose and used at the maximal concentration possible.

Amplification of DNA from colonies. Primers PC5B and P3mod were used in PCRs to amplify eubacterial 16S rDNA directly from the transferred colonies as described previously (7). The amplicons were sequenced directly with fluorescent-labelled dideoxynucleotides and an ABI model 373A automated sequencer (Applied Biosystems, Foster City, Calif.).

Cloning of amplicon from fecal DNA. The rDNA amplified from the fecal specimen was cloned with the TA cloning kit (Invitrogen, San Diego, Calif.). Individual clones were then transferred directly into the PCR mix and shaken with 1/10 volume of glass beads to liberate the DNA. *Taq* polymerase (AmpliTaq; Roche Molecular Systems, Inc., Branchburg, N.J.) was added, and the DNA was amplified for 35 cycles with primers directed at the SP6 and T7 promoters located on either side of the plasmid's insert. Amplicons of the appropriate size were reamplified with the original PCR primers to generate an adequate amount of sequencing template. The amplicons were directly sequenced with primers directed at conserved sites.

Identification of rDNA sequences. The assembled partial rDNA sequences corresponded to *E. coli* 16S rRNA bases 1197 to 1492. Each sequence was compared with sequences in GenBank and in the Ribosomal Database Project

(RDP) (14). If a sequence was within 2 bases of matching a known sequence, the clone or colony was placed into the corresponding taxon. It was thus assumed that a 2-base mismatch represented *Taq* polymerase error or strain variation. If a new sequence was not within 2 bases of a previously sampled sequence, it was determined which sequences in RDP were the most similar. The clone or colony was then named for the most specific branch of the phylogenetic tree (RDP) in which these similar sequences occurred (Table 1).

Dot blot hybridizations. Nucleic acids from the fecal specimen and from PCR were blotted onto a nylon membrane (GeneScreen; NEN Research Products, Boston, Mass.) and fixed with UV light. Three probes were used. BacP4 (GAG GAAGGTGGGGATGACGTCAA; *E. coli* positions 1175 to 1197) was complementary to bacterial rRNA in general. ErecP (GGCTTCACAGCTTTGCTTCC) was designed to be species-specific for *Eubacterium rectale* rRNA. Lowgc2P (GGACGTTGTTTCTGAGT) was designed to be specific for the rRNA sequence of the organism designated low G+C #2. Neither probe site matched any sequence found in GenBank or RDP.

Probes were labelled at the 3′ end with digoxigenin-11-ddUTP. Membranes were prehybridized in a mixture of 5× SET (20× SET is 0.5 M NaCl, 0.03 M Tris [pH 7.4], 2 mM EDTA), 0.1% sodium PP_i, 0.2% sodium dodecyl sulfate (SDS), and 2 mg of heparin per ml for 2 h at 45°C. One hundred picomoles of labelled probe was added to the prehybridization solution, and the mixture was incubated at 45°C overnight. The membrane was washed three times at 45°C for 15 min in 0.2% SDS–5× SET. Detection of digoxigenin was accomplished by chemiluminescence.

Calculation of coverage. Coverage refers to the estimated proportion of colonies or clones in the population under study that could be assigned to a category detected in a given sample. It could also be described as the probability that the next colony or clone sampled would fall into a category already found. It was calculated by the method of Good (9). Because the populations represented by cloning and culturing were potentially different, coverage was calculated separately for each method.

RESULTS

Identity of culturable organisms. The plate count was 58% of the microscopic count. Fifty colonies were picked at random for partial sequencing of 16S rDNA. The procedure could be completed for 48 colonies. The colonies gave 21 distinct rDNA sequences corresponding to phylogenetic categories shown in Table 1 and Fig. 1. Only 31% of the isolates corresponded to a known species for which the 16S rDNA sequence was available. Organisms that could be named, such as identifiable species of *Bacteroides* and *Eubacterium* as well as *Bifidobacterium pseudocatenulatum*, were well-described members of the predominant anaerobic bacterial community of the human colon. Thirteen sequences were found only once. When the data were analyzed to determine coverage, it was found that the 16S rDNA sequence categories detected accounted for 72% of the culturable cells. This proportion is consistent with previous data on identification of colonies from predominant biota by conventional techniques for taxonomy (10). Two organisms accounted for 16% each of the cultured cells: one was *Eubacterium rectale*, and the other belonged to the RDP group containing *Clostridium leptum* and relatives (a subgroup of clostridium group 1 in Table 1). Both were gram-positive bacteria with low G+C contents. If recovery of each of these two organisms was absolutely efficient, each would be expected to account for 9% of all cells present (correcting for a recovery rate of 58%). Of course, if recovery were less than absolutely efficient for these particular species, they would account for an even larger proportion of the total cells present.

rDNA clones after nine cycles of PCR. Twenty-seven distinct sequences were found in rDNA that had been amplified for only nine cycles. Only a little over a quarter of sequences corresponded to established taxa for which rRNA sequences were available. All of these species were known to be part of predominant human biota. The sequence for the organism designated beta proteobacterium 1 was similar to that of a common contaminant in this laboratory and was eliminated from further consideration. Aside from the beta proteobacterium 1 sequence, 20 sequences were seen only once. The coverage was 59%. As indicated by the larger number of different

TABLE 1. Comparison of rDNA sequence frequencies[a]

Organism	No. of rDNA sequences found:		
	In colonies	After PCR	
		9 cycles	35 cycles
Bacteroides ovatus	3	4	1
Bacteroides ovatus variant[b]	0	1	0
Bacteroides uniformis	1	6	1
Bacteroides vulgatus	1	1	0
Escherichia coli	0	1	0
Bacteroides #1	0	1	0
Bacteroides #2	0	1	0
Bacteroides #3	2	3	9
Bacteroides #4	1	0	0
Bacteroides #5	0	0	1
Beta proteobacterium #1[c]	0	1	0
Enteric gram-negative #1	0	0	1
Planctomyces grp #1	0	1	1
Bifidobacterium pseudocatenulatum	1	0	0
Eubacterium rectale[d]	8	0	0
Eubacterium sirauem	1	1	0
Lachnospira pectinoschiza	0	0	1
Ruminococcus lactaris	1	0	0
Ruminococcus obeum	3	0	0
Bifidobacterium #1	3	0	0
Clostridium grp 1 #1	0	2	0
Clostridium grp 1 #2	0	1	1
Clostridium grp 1 #3	0	1	0
Clostridium grp 1 #4	0	1	0
Clostridium grp 1 #5	0	1	0
Clostridium grp 1 #6	8	1	0
Clostridium grp 1 #7	0	0	1
Clostridium grp 2 #1	1	0	0
Clostridium grp 2 #2	6	2	2
Clostridium grp 2 #3	0	1	0
Clostridium grp 2 #5	1	0	0
Clostridium grp 2 #6	1	0	0
Clostridium grp 2 #7	1	0	0
Clostridium grp 2 #8	1	0	0
Clostridium grp 2 #10	0	0	1
Clostridium grp 2 #11	1	0	0
Clostridium grp 2 #12	0	1	1
Clostridium grp 2 #13	0	1	0
Clostridium grp 2 #14	1	0	0
Clostridium grp 2 #15	0	1	0
Low G+C #1	0	1	0
Low G+C #2[d]	2	12	16
Low G+C #3	0	0	2
Low G+C #4	0	1	0
Low G+C #5	0	1	0
Low G+C #6	0	1	0
Total	48	50	39

[a] Frequencies of rDNA sequences found in bacterial colonies isolated from a human fecal specimen were compared with sequences from clones of amplified rDNA. Sequences after 9 cycles of PCR were also compared with sequences after 35 cycles. If sequences matched an entry in GenBank or the RDP, they were named for the corresponding species; if they did not match a known organism, sequence names correspond to approximate placement in the phylogenetic tree of the RDP May 1995 release. Clostridium group (grp) 1 is described further in the legend to Fig. 1. It contained *Eubacterium sirauem* and seven other unnamed organisms. Clostridium group 2 contained *Eubacterium rectale*, *Lachnospira pectinoschiza*, *Ruminococcus lactaris*, and *Ruminococcus obeum* as well as 13 unnamed organisms. The locations of clostridial groups 1 and 2 are shown in Fig. 1. Six organisms were gram-positive bacteria with low G+C contents that could not be phylogenetically placed more specifically.

[b] *B. ovatus* variant mismatches *B. ovatus* rRNA sequence by 4 bases.

[c] This organism was considered a probable laboratory contaminant.

[d] $P < 0.01$ for nine-cycle clones compared with colonies by the Fisher exact test.

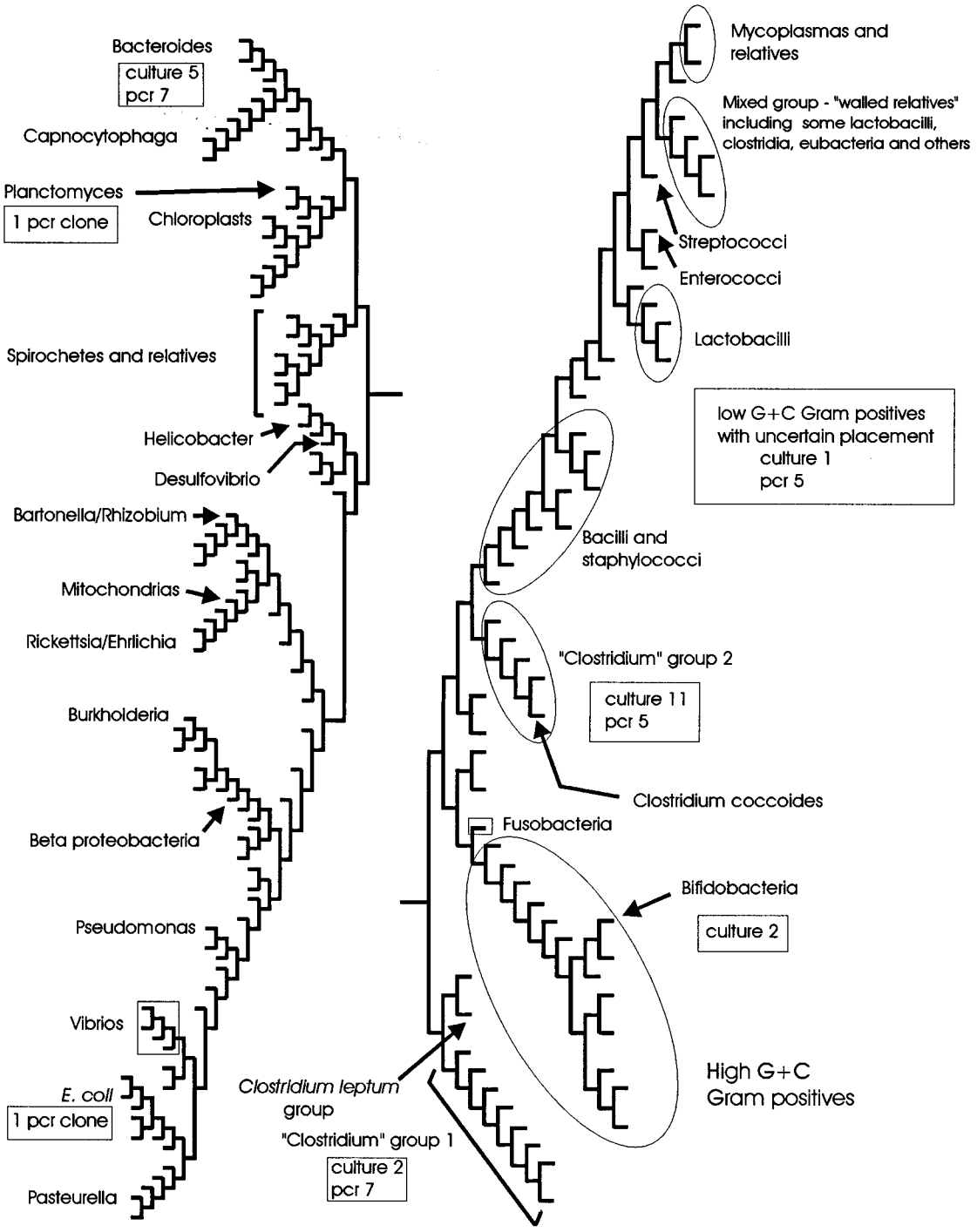

FIG. 1. Phylogenetic placement of sequences found by culturing and by nine cycles of PCR. Clostridium group 1 is our own designation for the branch of low-G+C gram-positive bacteria that contains *Clostridium butyricum*, the type species of the genus *Clostridium*. This group consists mostly of anaerobic spore-forming rods such as *Clostridium perfringens*, *Clostridium leptum*, *Clostridium sporogenes*, and *Clostridium novyii*. Clostridium group 2 is a highly heterogeneous group of organisms that contains members of *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Lachnospira*, and *Epulopiscium* spp. Organisms were placed according to the location of their nearest relatives as determined by the rank order of the most similar sequences found in the RDP (14). Therefore, except for known species, placement is approximate. In particular, the *C. leptum* group of clostridium group 1 lies near the root of this branch, and many of the organisms in this study that fell into clostridium group 1 were most closely related to the *C. leptum* group. Most organisms in clostridium group 2, on the other hand, were most similar to the *Clostridium coccoides* group, which does not fall near the origin of the branch, making placement on the branch more certain. The tree is derived from the RDP and shows branching order but not phylogenetic distance. Numbers indicate the locations of unique sequences; total numbers are shown in the table.

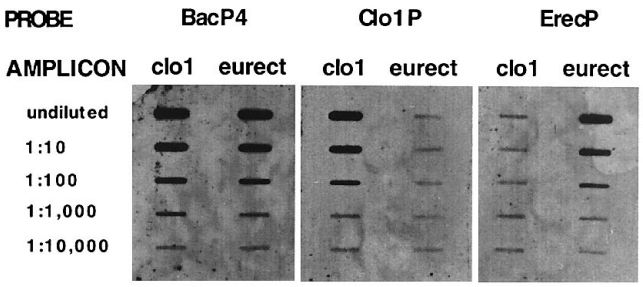

FIG. 2. Serial 10-fold dilutions starting with 0.5 ml of each amplicon probed with oligonucleotides directed at universal bacterial rRNA (BacP4), low G+C #2 (Clo1P), or *Eubacterium rectale* (ErecP). Both specific probes give a signal intensity slightly less than that given by the broad-spectrum probe BacP4 when hybridized to rDNA from the respective target organisms. However, BacP4 could serve as a point of reference for signal strength expected from total bacterial rRNA in the sample.

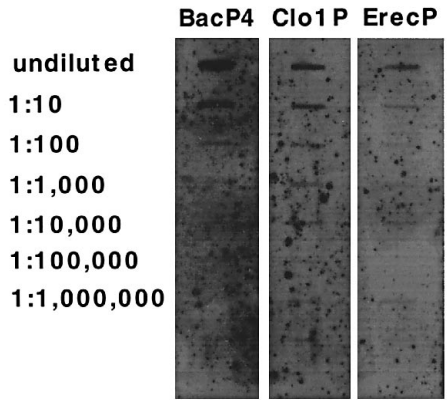

FIG. 3. Relative signal strengths for BacP4, ErecP, and Lowgc2P (Clo1P) when hybridized to serial dilutions of nucleic acids extracted from the fecal specimen. The signal for Lowgc2P (Clo1P) is greater than that from ErecP. Each accounts for between 1 and 10% of 16S rRNA.

sequences seen and the lower coverage, the representation of the ecosystem appeared slightly more complex by direct sampling of rDNA than by culturing.

Despite the fact that they have a low G+C content and would be expected to be amplified efficiently, *Eubacterium rectale* was not detected ($P < 0.01$ by the Fisher exact test) and the organism called clostridium grp 1 #6 occurred only once. On the other hand, the organism designated low G+C #2 represented 25% of clones and only 4% of colonies ($P < 0.01$). This organism did not grow well, appearing as a clear, pinpoint colony, and was a gram-variable, tortuous rod. In enriched brain heart infusion broth, it produced a small amount of butyric acid. It did not produce spores. It was not possible to establish its identity because of its poor growth in broth media.

rDNA clones after 35 cycles of PCR. Because it is a common practice to amplify samples of DNA from natural ecosystems more extensively than 9 cycles, we performed 35 cycles to determine whether more extensive amplification led to distortion of the populations detected. With only 38 clones sequenced, the coverage was 74%; in other words, only 13 sequences accounted for a large proportion of the rDNAs represented. The organism designated low G+C #2 now accounted for 42% of the total ($P = 0.009$ by chi-square analysis compared with 25% of nine-cycle clones), and far fewer of the sequences found in the cultured cells were detected in the clones (Table 1). This phenomenon was not simply a matter of detecting fewer organisms with a high G+C content, since only four of the colonies fell into this category.

Oligonucleotide probe experiments. The data given above indicated that PCR significantly distorted the representation of some organisms in the ecosystem. Thus, it could not be concluded that the organism called low G+C #2, abundant by rDNA analysis, was actually an important component that was underrepresented by culture. We therefore designed oligonucleotide probes to estimate the actual population sizes of that organism as well as *Eubacterium rectale*, an organism represented abundantly on culture but not found in the sample of amplified rDNA. Figure 3 shows that both probes give a signal intensity only slightly less than that of the broad-spectrum probe BacP4 when hybridized to rDNA from the respective targets. Therefore, BacP4 could serve as a point of reference for the strength of the signal from total bacterial rRNA. Figure 2 shows the relative signal strengths for BacP4, ErecP, and Lowgc2P (Clo1P) when hybridized to rRNA extracted from the fecal specimen. Both species-specific probes gave a signal on the order of 1 to 10% of the total bacterial signal of BacP4, and the Lowgc2P (Clo1P) signal was more intense than that of ErecP. In Fig. 4, the same probes are hybridized to the nine-cycle amplicon. Relative to the signal from Lowgc2P (Clo1P), the ErecP signal was weaker when hybridized to the amplicon than when hybridized to the rRNA. This finding implicates the PCR rather than cell lysis or the cloning step as a cause of the observed distortion. The sequence of *Eubacterium rectale* 16S rRNA in RDP matched the 5′ bacterial primer exactly. Further limited sequencing through the 3′ priming site and into the intergenic spacer region between 16S and 23S rDNA confirmed that the 3′ priming site was also a perfect match. Thus, mismatch of a primer did not explain the decreased representation of *Eubacterium rectale* in the PCR product.

Overall agreement of results. After 35 cycles of amplification, the distortions in the ecosystem's representation were extensive and obvious. However, given the nature of the data, it was less obvious how much difference existed between nine cycles of PCR and culturing. Clearly, there were significant discrepancies involving the detected populations of *Eubacterium rectale* and low G+C #2. But putting these differences aside, how well did the methods agree overall? The coverage data provided a useful tool for answering this question. Within the total population of sequences represented by nine cycles of PCR, the proportion of all sequences that would fall into the detected sequence categories for clones was calculated to be 0.59. Within the population of sequences from colonies, the proportion of all sequences that would fall into the detected sequence categories for colonies was 0.72. Thus, if clones and

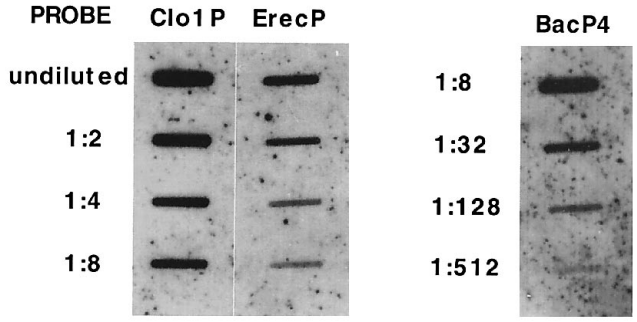

FIG. 4. The same probes as those described in the Fig. 3 legend hybridized to serial dilutions of the nine-cycle amplicon. Relative to BacP4, the signal of Lowgc2P is stronger when probing the amplicon than when probing rRNA.

colonies represented the same population, the proportion of sequences falling into categories that were detected by both methods would be expected to be 0.42 (or 0.72×0.59). The observed proportion of shared sequences was in fact 0.55, significantly higher than expected ($P < 0.05$ by chi-square analysis). The higher-than-expected overlap can probably be attributed to two factors. First, the coverage calculation is, after all, only an estimate. Second, the populations being sampled were known to be different, and this difference could paradoxically increase overlap. For instance, the demonstrated bias of PCR favoring low G+C #2 significantly increased the number of sequences in this shared category. A similar analysis of overlap between sequences found by culturing and those found after 35 cycles of PCR showed that while the proportion of shared sequences was expected to be 0.55, the observed proportion was 0.30. It would have been even lower had the sequence of low G+C #2 not constituted such a large proportion of cloned sequences, all counted as being shared. Thus, the results from culturing agreed reasonably well with the data from nine cycles of PCR but as expected did not mirror the 35-cycle data.

DISCUSSION

The colonic biota is a complex ecosystem with the important function of controlling the population sizes of many potentially pathogenic microbes (1, 5, 6, 20). In humans, it appears that the composition of this community changes little from the cecum to the distal colon (11). Extensive efforts have been made in the past to cultivate the bacteria found in human feces, making human colonic biota one of the most successfully studied natural communities of bacteria. Despite a great deal of overlap, the exact makeup of this ecosystem appears to vary significantly from individual to individual. However, the community always has been found to consist of hundreds of strictly anaerobic species from many genera coexisting with smaller populations of facultative organisms.

The results of rDNA analysis gave a picture that was very similar to that of the culture-based method in this study and to well-done culture-based studies in the past. However, the differences were noteworthy. At least one very numerous organism, low G+C #2, grew poorly on the culture plates and appeared to be underrepresented among the colonies. The difficulty in identifying this organism points up an important aspect of research on the colonic ecosystem. Hundreds of indigenous species have been described, but relatively few rDNA sequences from these organisms are available. Identifying these organisms accurately by classical methods is very difficult, and the number of laboratories capable of doing so is dwindling. Thus, it is not yet possible to determine whether any of the rDNA sequences from clones in this study were derived from organisms that have never been cultivated.

Two organisms detected by rDNA analysis were not previously known to colonize the human intestine. *Lachnospira pectinoschiza* is readily culturable from the rumen (2) but has not been detected in tens of thousands of bacterial colonies studied from human colonic biota. It seems likely, then, that it was a small subpopulation not usually detected and was amplified more efficiently than most organisms to become a large enough proportion of the total to be sampled after 35 cycles of PCR. The other unexpected organism, seen after nine cycles of PCR, was most similar to planktomycetes. No known predominant community members in the human gut have such a phylogenetic placement. It is uncertain whether this organism was a community member or was ingested in food or water.

Two organisms that were highly represented on the culture plates were virtually missing among the clones of rDNA. There were probably several factors at play. As noted above, lack of processivity in DNA replication may have played a role, i.e., it appears that *Taq* polymerase did not always completely copy some templates. Differential amplification of rDNA molecules from mixed populations has been observed previously (15). Prior data are consistent with the explanation that DNA from organisms with a high content of G+C amplifies relatively poorly. However, the G+C content of *Eubacterium rectale* is thought to be around 30% (13), and the organism clostridium grp 1 #6 is also a low-G+C gram-positive organism. Furthermore, the G+C contents of all sequences in this study were between 50 and 60%. Thus, a failure to denature DNA of certain species is unlikely to explain differential amplification after the first two cycles (when the entire genomes are being denatured), yet the proportion of low G+C #2 increased substantially between 9 and 35 cycles. The distortion of relative numbers caused by PCR of 16S rDNA from complex communities is not yet adequately understood. Until better methods are developed, keeping the number of cycles to a minimum appears to be a reasonable strategy if large amounts of adequately pure DNA are available. Another factor may have played a role in the relative abundance of *Eubacterium rectale* on the culture plates. It differed morphologically from low G+C #2 in that its cells were smaller and it had less of a tendency to form chains. Both of these properties would cause a given cell volume (corresponding to a quantity of rRNA probed in Fig. 3) to yield relatively more colonies of *Eubacterium rectale.*

Despite the differences in results from culturing compared with PCR and cloning, these methods were in good overall agreement. The ecosystem represented by nine cycles of PCR was slightly more complex than that represented by culture, suggesting that PCR may have introduced slightly less bias. However, this study strongly supports the value of the extensive information available concerning the predominant culturable human biota. It appears that these methods complement one another and that further investigation of rDNAs in human colonic biota will be a helpful approach.

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