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Quantification of Bacterial Groups within Human Fecal Flora by Oligonucleotide Probe Hybridization

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To investigate the population structure of the predominant phylogenetic groups within the human adult fecal microbiota, a new oligonucleotide probe designated S-G-Clept-1240-a-A-18 was designed, validated, and used with a set of five 16S rRNA-targeted oligonucleotide probes. Application of the six probes to fecal samples from 27 human adults showed additivity of 70% of the total 16S rRNA detected by the bacterial domain probe. The *Bacteroides* group-specific probe accounted for 37% ± 16% of the total rRNA, while the enteric group probe accounted for less than 1%. *Clostridium leptum* subgroup and *Clostridium coccoides* group-specific probes accounted for 16% ± 7% and 14% ± 6%, respectively, while *Bifidobacterium* and *Lactobacillus* groups made up less than 2%.

The presence of an extremely complex microbial population adapted to live within the human host must have a significant impact on human health. Knowledge concerning numbers and species of bacteria found in the human colon is important because it provides an index of the metabolic potential of the colonic microbiota. Early work analyzing the colonic microbiota has focussed on enumeration and identification of numerically predominant cultivable species (4, 6, 14). Nevertheless, evidence has accumulated indicating that a significant fraction of the microbiota can escape cultivation (4, 13, 19, 21).

In recent years, there has been an increasing effort to describe complex environments by using genetic tools (2, 16, 18). Regarding the human intestinal gut microbiota, the rRNA approach has been used to study specific groups of bacteria by using various molecular techniques (5, 10, 12, 19, 20, 23). None of these techniques provided thorough quantitative information on the different microbial groups and their contribution in terms of activity to the whole microbial community.

In the present study, we describe the use of a set of six 16S rRNA-targeted oligonucleotide probes and quantitative dot blot hybridization technique for analyzing the human adult fecal microbiota composition from frozen fecal samples.

Probe design included a search of target and nontarget group complementarity by using the rRNA database (containing 10,700 aligned sequences as of September 1999) (11). A computer-assisted specificity control of the different probes was performed with the check probe function available from the Ribosomal Database Project facility (11). Based on human fecal rDNA sequence analysis (19), probe Clept1240 was designed to target the *Clostridium leptum* subgroup, which is presented in Table 1 and corresponds to the semiconserved region between nucleotides 1240 and 1257 (*Escherichia coli* consensus numbering). Probes specific for the *Bifidobacterium* group (Bif1412), *Bacteroides* group (Bacto1080), enteric group (Enter1432), *Clostridium coccoides* group (Erec482), and *C. leptum* subgroup (Clept1240) were used in conjunction with *Lactobacillus* group probe (Lacto722) to assess RNA proportions of these populations with respect to the total microbiota as determined by using the bacterial domain probe (Bact338) (see Table 2 for probe sequences).

RNA extraction and dot blot hybridization were performed as previously described by Stahl et al. (18) and modified by Doré et al. (3). The 16S rRNA-targeted oligonucleotide probes and the corresponding washing temperatures used in this study are listed in Table 2. The specificity of each probe was experimentally tested against RNA extracts from strains representing 46 different bacterial species. All probes hybridized to their corresponding target bacteria and did not cross hybridize with any of the nontarget microorganisms.

The following 46 reference species were used for dot blot hybridization: *Actinomyces naeslundii* (ATCC 12104), *Bacteroides vulgatus* (ATCC 8482), *Bacteroides fragilis* (ATCC 25285), *Bacteroides eggerthii* (ATCC 27754), *Bacteroides uniformis* (ATCC 8492), *Bacteroides putredinis* (ATCC 29800), *Bifidobacterium adolescentis* (ATCC 15703), *Bifidobacterium pseudolongum* (ATCC 25526), *Bifidobacterium dentium* (ATCC 15423), *Bifidobacterium infantis* (ATCC 15697), *Bifidobacterium longum* (ATCC 15707), *Clostridium perfringens* (ATCC 13124), *Clostridium beijerinckii* (ATCC 25752), *Clostridium butyricum* (ATCC 43755), *Clostridium putrificum* (ATCC 25784), *Clostridium coccoides* (ATCC 29236), *Clostridium nexile* (ATCC 27757), *Clostridium absonum* (ATCC 27555), *Clostridium bifermentans* (ATCC 638), *Clostridium clostridiiforme* (ATCC 25537), *Coprococcus eutactus* (ATCC 27759), *Escherichia coli* (ATCC 11775), *Eubacterium siraeum* (ATCC 29066), *Eubacterium tenue* (ATCC 25553), *Eubacterium ventriosum* (ATCC 27560), *Enterococcus faecalis* (CIP 76117), *Enterococcus faecium* (CIP 54.32), *Fusobacterium prausnitzii* (ATCC 27766), *Klebsiella pneumoniae* (CIP 54.45), *Lactobacillus acidophilus* (ATCC 4356), *Lactobacillus fermentum* (ATCC 14931), *Lactobacillus reuteri* (ATCC 23272), *Leuconostoc lactis* (ATCC 19256), *Propionibacterium acnes* (ATCC 6919), *Proteus mirabilis* (CIP 54.15), *Pseudomonas aeruginosa* (CIP 102240), *Ruminococcus hansenii* (ATCC 27752), *Ruminococcus flavefaciens* (ATCC 19208), *Ruminococcus gnavus* (ATCC 29149), *Ruminococcus productus* (ATCC 27340), *Salmonella paratyphoides* (CIP 54.136), *Serratia marcescens* (CIP 67.55), *Staphylococcus aureus* (CIP 65.15), *Strep-*

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TABLE 1. Partial nucleotide sequence of the region between nucleotides 1240 and 1257 of the 16S rRNA (*E. coli* numbering) from various bacterial species showing the target region for the Clept1240 (underlined)[a]

Probe or organism	16S rRNA sequence
Clostridium leptum probe	3′ CTGACGGCAACTRTTTTG 5′
Clostridium leptum	5′ GACUGCCGUUGACAAAAC 3′
Eubacterium siraeum	5′ ---------- 3′
Ruminococcus albus	5′ ---------- 3′
Clostridium cellulosi	5′ ---------- 3′
Lactobacillus acidophilus	5′ --------G-------C- 3′
Bacteroides fragilis	5′ ---------C--U--G-- 3′
Clostridium coccoides	5′ --------GG--U--CC- 3′
Bifidobacterium bifidum	5′ ---C----GG-U---CU- 3′

[a] Alignment of the probe sequence, its target site, and the sequence of corresponding sites for *C. leptum* subgroup S-G-Clept-1240-a-A-18. R is an (A/G) wobble nucleotide. The probe name is in accordance with the oligonucleotide probe database nomenclature (1). Dashes indicate full complementarity with the probe sequence.

tococcus bovis (ATCC 33317), *Streptococcus sanguinis* (ATCC 10556), and *Streptococcus intermedius* (ATCC 27335).

Fresh fecal samples were collected from 27 healthy human adults (13 males and 14 females). The individuals were 20 to 45 years old and received unrestricted western-type diets. They had not received antibiotic therapy during the preceding 6 months and were free of known metabolic or gastrointestinal diseases, including diabetes, ulcerative colitis, Crohn's disease, peptic ulcers, and cancer. The collected samples were immediately frozen and stored at −20°C for future analysis.

Dilution series of control RNA (4 to 500 ng) from pure cultures and triplicates of 250 ng of total RNA extracted from frozen fecal samples were blotted and hybridized as described previously by Doré et al. (3). Quantification of hybridization signals on dot blots was achieved by using radio imaging with the Instant Imager (Packard Instruments). All quantitative rRNA measurements were standardized by using rRNA extracts from pure cultures of *B. vulgatus* (ATCC 8482), *B. longum* (ATCC 15707), *E. coli* (Boehringer Mannheim rRNA standards), *F. prausnitzii* (ATCC 27760), *L. acidophilus* (ATCC 4356), and *R. productus* (ATCC 27340). The universal probe was used for normalizing rRNA concentrations. Results are expressed as percent of total bacterial 16S rRNA that is detected by the Bact338 and are given as means ± standard deviations from triplicate measurements.

Total *Bacteroides* and enteric group rRNAs accounted for 37% ± 16% and 1% of total bacterial rRNA, respectively. Low-guanine-plus-cytosine-content gram-positive organisms, including those of the genera *Lactobacillus*, *Streptococcus*, and *Enterococcus*, accounted for by using Lacto722 comprised up to 1% of the total 16S rRNA. *Clostridium*, *Eubacterium*, *Peptostreptococcus*, *Ruminococcus*, and *Fusobacterium* speciestargeted using both Erec482 and Clept1240 represented 16% ± 7% and 14% ± 6% of the total bacterial rRNA, respectively. The *Bifidobacterium* group represented less than 1% of the total. Thus, by using a panel of six probes, an average of 70% of all bacterial 16S rRNA was accounted for in the present study (Table 3).

Our observations are consistent with previous work using molecular techniques for studying human adult fecal microbiota. Franks et al. (5) developed and applied six 16S rRNA-targeted probes for major species and groups of anaerobic intestinal bacteria to enumerate bacterial populations in fresh feces of healthy human volunteers by using a fluorescent in situ hybridization (FISH) technique. They accounted for two-thirds (64%) of the total fecal microbiota. A direct comparison with our results by using quantitative dot blot hybridization is difficult since probe specificity was different and the dot blot hybridization technique measures proportions of rRNA representing numbers as well as activities of the different groups of bacteria, while the fluorescent in situ hybridization technique measures the number of cells containing a sufficient number of ribosome to be detected.

A *B. fragilis* group-specific probe and a *Bacteroides distasonis* species-specific probe used in combination accounted for 20% of the fecal flora in the study conducted by Franks et al. (5). Our Bacto1080 probe is broader in target and encompasses the *Bacteroides*, *Prevotella*, and *Porphyromonas* subgroups, hence the 37% accounted for.

The *C. coccoides* phylogenetic group did not account for more than 16% of the total bacterial 16S rRNA. The same probe, Erec482, was used in the study conducted by Franks et al. (5), and target cells accounted for 29% of the microbiota. They also used a probe called Lowgc2P which is specific for an uncultivated group of gram-positive bacteria within the *C. leptum* subgroup. Its target represented 12% of the total human fecal microbiota. This result was in accordance with the 1 to 10% proportion of the total human fecal 16S rDNA detected with this probe in the study conducted by Wilson and Blitchington (21). Our novel Clept1240 probe is broader in terms of specificity and accounted for 14% of the total 16S rRNA.

Regarding the group *Bifidobacterium*, which was represented by less than 1% total bacterial rRNA, on average (0 to 5%), the proportion appears lower than that obtained by Langendijk et al. (10) (5%) or by Franks et al. (5) (3%). This discrepancy can, in part, be explained by the use of probes

TABLE 2. 16S rRNA targeted oligonucleotide probes and experimental wash temperatures of buffer under experimental conditions[a]

Target group probe	Probe name[b]	Primer sequence (5′ to 3′)[c]	Experimental wash temperature (°C)	Reference
Univ1390	S-*-Univ-1390-a-A-18	GACGGGCGGTGTGTACAA	46	22
Bact338	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	54	2
Bacto1080	S-*-Bacto-1080-a-A-18	GCACTTAAGCCGACACCT	50	3
Enter1432	S-G-Enter-1432-a-A-15	CTTTTGCAACCCACT	43	Chmieliewski et al., submitted for publication
Bif1412	S-G-Bif-1412-b-A-21	CGGGTGCTRCCCACTTTCATG	52	8
Lacto722	S-G-Lacto-722-a-A-25	YCACCGCTACACATGRAGTTCCACT	51.2	17
Clept1240	S-G-Clept-1240-a-A-18	GTTTTRTCAACGGCAGTC	48.5	This study
Erec482	S-*-Erec-0482-a-A-19	GCTTCTTAGTCARGTACCG	47	5

[a] Buffer was comprised of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 1% sodium dodecyl sulfate.
[b] Probe names are in accordance with the Oligonucleotide Probe Database (1).
[c] Y represents a (C/T) wobble nucleotide and R represents an (A/G) wobble nucleotide.

TABLE 3. Ribosomal RNA indexes of 27 individuals measured by quantitative dot blot hybridization by using the Bact338 as reference compared to Bacto1080, Clept1240, Erec482, Bif1412, Enter1432, and Lacto722

Individual	Group						Sum
	Bacto1080	Clept1240	Erec482	Bif1412	Lacto722	Enter1432	
5	34.35	12.20	12.55	0.3	0.73	2.78	63
6	42.18	18.80	12.30	0.3	0.06	0.74	74
13	25.95	23.85	25.72	1.6	0.00	0.91	78
14	18.21	19.05	24.77	1.1	2.55	0.00	66
17	21.02	26.38	6.76	5.0	0.00	0.46	60
18	42.02	7.11	8.63	0.2	0.29	1.00	59
21	52.42	20.15	8.43	3.8	0.00	0.90	86
23	62.02	5.84	12.59	0.2	0.40	0.19	81
38	27.93	13.99	18.27	1.6	0.10	0.28	62
44	39.82	14.69	18.75	0.0	2.28	0.00	76
45	43.84	14.79	15.91	0.0	2.29	0.03	77
47	55.50	19.41	14.05	0.5	0.15	0.20	90
49	9.70	20.91	27.99	0.2	0.04	0.30	59
69	50.42	18.67	9.77	0.3	1.06	3.38	84
19	23.73	7.32	8.65	0.5	0.13	1.47	42
20	41.73	38.69	9.21	0.6	0.00	1.29	92
26	25.36	14.62	13.57	0.0	0.23	0.44	54
33	36.43	16.90	9.54	0.1	0.61	0.26	64
35	43.83	16.84	16.39	0.4	2.09	0.79	80
37	27.85	14.56	15.79	0.5	1.49	0.42	61
48	27.81	18.15	14.80	0.6	0.46	0.32	62
50	18.87	15.76	11.70	0.4	0.15	0.65	48
51	7.37	15.86	21.52	0.1	0.54	0.42	46
56	57.07	11.23	12.39	0.2	0.58	0.39	82
66	73.91	11.35	10.12	0.5	0.46	0.34	97
67	30.05	13.69	14.70	0.6	0.53	0.38	60
71	54.71	10.32	16.31	0.3	0.21	0.00	82
Mean ± SD	36.8 ± 16	16.3 ± 7	14.5 ± 6	0.7 ± 1	0.6 ± 1	0.7 ± 1	70 ± 15

targeting different sequence region of the 16S rRNA molecule. Moreover, after thriving in their respective ecological niches, suboptimal physicochemical conditions encountered in fecal matter, such as loss of contact with the mucosa or depletion of their preferred energy substrates, would yield lower rRNA contents. The same hypothesis can explain the occurrence of low levels of enteric group rRNA, since the ecological niche of organisms in this group is the cecum. In their study using pyxigraphic sampling (15), Marteau et al. (P. Marteau, P. Pochart, J. Doré, A. Bernalier, G. Corthier, and J. C. Rambaud, unpublished data) showed that facultative anaerobes, which represented 35% of the total viable counts in the cecum, did not exceed 1% of the total in feces.

Lactobacilli have complex nutritional requirements such as amino acids, peptides, nucleic acid derivatives, vitamins, salts, fatty acid esters, and fermentable carbohydrates for growth (7). Some of these complex nutrients are probably in very low supply in the colon due to their degradation and absorption in the small intestine, hence the minor contribution of this group.

The estimation of methodological congruence based on the analysis of the fecal microbiota from the same subjects with the same probe panel by using both techniques will prove most valuable in the future.

Interindividual variations in microbiota composition were noticed in our study, but no striking differences between males and females emerged. The *Bacteroides* group was the most variable with a rRNA index varying between 7 and 74%. The *C. leptum* subgroup varied between 6 and 39%, and the *C. coccoides* group varied between 7 and 28% of total bacterial rRNA. This data is consistent with previous work, indicating that each individual harbors his or her own unique microbial community (6, 12).

Considering that many fastidious microorganisms not only require selective media, but also many days for growth, the use of oligonucleotide probes targeting 16S rRNA provides a direct and quantitative estimate of the microbial community without culture-based enumeration and identification. The selectivity of oligonucleotide probes and the ease with which they can be generated make them ideally suited for monitoring the growth dynamics of these microorganisms under different dietary conditions and for tracking probiotics in the gut. Moreover, the use of species-specific probes will enable us to identify and quantify major microbial species directly in the gut. This will help in the design of preventive nutrition strategies aiming to modulate human colonic bacterial populations towards a balanced and healthy microbiota.

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