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Predominant Gram-Positive Bacteria in Human Feces: Numbers, Variety, and Persistence

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The predominant gram-positive bacteria in 47 fecal specimens from 10 healthy men were studied by microscopic and cultural counts, by the characterization and tentative identification of isolates, and by the use of fluorescein isothiocyanate (FITC)-conjugated globulins prepared using some of the isolates. Gram-positive bacteria averaged $10^{10.5 \pm 0.4\,(SD)}$/g (wet weight) of feces with significant variation from host to host. Characterization of 865 isolates, all strict anaerobes and carbohydrate fermenters, showed 12 to 39 distinguishable strains from each host and indicated that some strains were present the full period of about 18 months. Sixty percent of the isolates belonged to one of five types, tentatively identified with five species—*Bifidobacterium adolescentis*, *Eubacterium aerofaciens*, *E. rectale*, *Peptostreptococcus productus*, and *Ruminococcus bromii*. There was distinct host idiosyncrasy in the pattern of estimated counts of these five types. Certain strains resembling *B. adolescentis*, *E. aerofaciens*, and *P. productus*, distinguished with FITC conjugates, were resident in their hosts for many months. In direct smears each strain constituted about 1% of the total bacteria.

There have been many surveys of the human fecal flora which have shown that anaerobic gram-positive bacteria are very numerous, numbering more than 10^9/g (wet weight) of feces (5, 9, 14, 15, 20; S. M. Finegold and L. G. Miller, Bacteriol. Proc., p. 93, 1968). In certain of the surveys some or all of these bacteria were shown to be acid tolerant and were described simply as bifidobacteria or anaerobic lactobacilli. Other investigators have reported a variety of gram-positive bacteria isolated in large numbers from human feces and have identified them as species of *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Propionibacterium*, and *Ruminococcus* (1, 11, 16).

Some of the surveys showed that, for certain types of bacteria at least, there is a tendency to host idiosyncrasy in the composition of the fecal flora which persists over a period of weeks or months (7, 24). Other investigations have indicated stability by demonstrating that particular strains of *Escherichia coli* may persist in the human intestine for long periods (2, 6, 19, 23).

The major purpose of this investigation was to characterize the predominant gram-positive bacteria in fecal samples from 10 hosts and to determine the extent of quantitative and qualitative variation both between hosts and within hosts over a period of more than a year.

MATERIALS AND METHODS

Subjects. Ten healthy men, 20 to 40 years old, consuming an average American diet, were studied over a period of about 18 months. Two had had appendectomies, and the remainder had no history of major gastrointestinal tract disorder. No specimens were collected until at least 3 weeks after any antimicrobial treatment or diarrheal disease.

Media. The ingredients for the rumen fluid agar (RF), peptone yeast sugar (PYS) agar and broth, fermentation end-products broth (FEP), diluting fluid (DF), antigen broth (AB), and fermentation test medium (FTM) are given in Table 1. The first five media were prereduced and anaerobically sterilized (11). Before autoclaving they were dispensed into hard glass tubes or flasks which were sealed with notched black rubber stoppers; whenever a tube or flask was opened, a stream of sterile oxygen-free gas (11) was used to maintain anaerobic conditions. PYS agar and FTM were prepared as agar deeps by mixing and boiling the ingredients, adding cysteine, and then distributing and autoclaving in the conventional manner. They were used immediately or reheated just before use.

Processing specimens. A 1-g sample was taken from the center of the fecal specimen within 15 min of passage and was suspended in 9 ml of DF. An additional 1-g sample was weighed in a tared pan for dry weight estimation. Serial 10-fold dilutions in DF were prepared from the suspension. Two tubes of molten RF agar were inoculated with 0.1 ml from each of the 10^{-7}, 10^{-8}, and 10^{-9} dilutions. The tubes were

TABLE 1. *Composition of media*

Medium	Quantity used in 1 liter of medium																	
	Glucose (g)	Maltose (g)	Soluble starch (g)	Test carbohydrate (g)	Trypticase (BBL) (g)	Peptone (Difco) (g)	Yeast extract (g)	Agar (g)	Menadione (mg)	Hemin (mg)	Sheep rumen fluid (mg)	Cysteine HCl (g)	Resazurin (mg)	Bromocresol purple (mg)	Ammonium sulfate (g)	Salts solution (11) (ml)	Distilled water (ml)	Gas phase
RF	0.25	0.25	0.5					20	0.5		300	0.5	1.0		1.0	500	200	CO_2
PYS	2.5	2.5			20		10	(20)[a]	0.5	2.0		0.5	1.0			500	500	CO_2
FEP	5.0	5.0				5.0	5.0					0.5	1.0			40	960	CO_2:N_2 1:9
DF												0.5	0.5			500	500	CO_2
AB	1.0	1.0			10		5.0			2.0		0.5	1.0			40	960	CO_2:N_2 1:9
FTM				10	10		5.0	5.0				0.5		40		40	960	Air

[a] PYS was prepared either with 20 g of agar for roll tubes or deeps or without agar as a broth.

rolled while cooling (11) and incubated for 5 days at 39 C. Slides were prepared from the 10^{-3} and 10^{-4} dilutions by spreading 0.01 ml over 1 cm^2 and heat fixing. Two smears were prepared separately on each slide. The slides were stored in the refrigerator and again heat fixed before staining.

Counts. One slide with two smears of the 10^{-4} dilution was stained with freshly filtered rose bengal (rose bengal, 1 g; phenol, 5 g; calcium chloride, 0.02 g; in 100 ml of distilled water) for 3 min. The individual bacteria were counted in 50 fields of each smear at a magnification of ×950. If the counts for the two smears differed by more than 5% of the total, the count was rejected and another slide was stained and counted. Allowing for the dilutions and the size of the field, the bacteria counted were multiplied by 8.414×10^7 to give the number of bacteria per gram (wet weight). Another slide was Gram stained. Gram-negative and -positive rods and cocci were counted in 25 fields in each smear. The ratios of these types were then applied to the total number of bacteria per gram (wet weight) obtained above to estimate the numbers of these different types per gram (wet weight).

Rumen fluid agar roll tubes with 50 to 500 colonies were selected for colony counts. The counts from two replicate cultures were averaged and multiplied by the dilution factors to give counts per gram (wet weight). Fifty unselected colonies were Gram stained and classified as gram positive or gram negative and as rods or cocci. The ratios of the different types were then applied to the total cultural count to give estimates of the cultural counts of these different types per gram (wet weight).

The 1-g sample in the tared pan was dried to constant weight in an evacuated desiccator over anhydrous calcium sulfate. The dry weight of nonmicrobial residue was estimated by subtracting an estimate of the dry weight of the bacteria (direct count/gram [wet weight] × 2 × 10^{-13} [reference 17]) from the total dry weight. Counts per gram (dry weight) and per gram (dry residue) were obtained by dividing the counts per gram (wet weight) by the dry weight and the dry residue, respectively.

All arithmetical and statistical calculations on the raw data from these counts were performed on the West Virginia University IBM 360/75 computer using the Statistical Analysis System (21).

Isolation and characterization of bacteria. Colonies, usually 60, were picked and streaked on rolled PYS agar which was incubated at 35 C. All transfers were made using anaerobic precautions (11). The days required for visible growth on the initial PYS subculture were recorded, and then the colonies were Gram stained. Colonies of gram-positive bacteria were restreaked on PYS agar until pure cultures were obtained, and then a PYS broth was inoculated. This broth culture was examined by Gram stain, and some of it was lyophilized or stored frozen at −60 C. A PYS broth culture was used to inoculate a PYS agar deep, an FEP broth, and one tube each of FTM with glucose, maltose, lactose, sucrose, soluble starch, mannose, xylose, inulin, mannitol, or inositol; FTM with amygdalin was also used for suspected *Eubacterium aerofaciens*.

The PYS agar deeps were incubated for 3 days and examined for depth of growth, reduction of the indicator, and production of acid and gas. The FTM cultures were examined after 2 and 7 days of incubation for acid and gas production. To determine the end products of fermentation, the FEP broth was incubated for 2 days and centrifuged at 800 × *g* for 30 min, and the supernatant fluid was acidified (11) and stored sealed at −60 C until it was examined for the presence of volatile fatty acids, alcohols, lactate, and succinate by gas-liquid chromatography. Three microliters of this fluid was injected into a glass column containing acid-washed Chromosorb W, 80/100 mesh, coated with SP1200/phosphoric acid, 10/1% (Supelco). The injection chamber was at 130 C, and the column was at a constant 120 C with nitrogen flowing

at 40 ml/min. The column was washed three to five times between each sample with distilled water. The effluent was recorded by a flame ionization detector at 140 C with hydrogen at 40 ml/min and air at 400 ml/min. The signal was amplified to give a range of zero to 10^{-9} amp. Volatile fatty acid peaks were identified by comparing their retention times with those obtained with a control solution of 10 meq of C_2-C_6 fatty acids per liter of FEP. The height of a sample peak was recorded as a percentage of the height of the corresponding control peak. Alcohol peaks, identified by comparison with a control solution in FEP, were recorded as present or absent. To determine the presence of lactate and succinate, 1 ml of the acidified supernatant fluid was methylated with 1 ml of BF_3-methanol (Applied Science) overnight at room temperature and extracted with 0.5 ml of chloroform. Two microliters of the chloroform extract was analyzed by using the same system as above and a control solution containing 10 meq of lactate and succinate per liter. For the final analysis, the acid end products were recorded on a 1 to 4+ system on the basis of the relative amounts in a culture as well as the absolute amounts.

The results of the above tests were coded as the presence or absence of 60 characteristics listed in Table 2; 25 of these characteristics are partially dependent on other listed characteristics. For a very few isolates there were no data on one or two characters—for example, some strongly aerogenic isolates repeatedly disrupted the agar deeps.

One aim of the characterization of the isolates by the method described above was to make it possible to group together all bacteria of one strain, that is, of recent common ancestry, and to separate different strains even when they might belong to the same species. An automatic clustering program, modified from Quadling (13), with subroutine LINK (22) for single-link hierarchical clustering, run on the IBM 360/75, was used for initial sorting of the results for all the isolates from each host separately. Grouping was based mainly on the clusters formed by using the 6% coefficient of difference; that is, members of one cluster would differ from members of every other cluster by at least 4 out of 60 characters. However, clusters formed at other levels and taxonomic criteria from Holdeman and Moore's classification (11) were also taken into account in forming the groups. This was done to avoid both groups which were separated by characters that might be caused by technical variation or changes in a single protein, and groups which included members that differed in characters considered critical by taxonomists. The characters of the groups obtained were compared with the characters given for species listed by Holdeman and Moore.

Detection of bacteria with fluorescent antibody. Antisera were prepared against two or more isolates of gram-positive bacteria from each host by the following method. The bacteria were grown in AB at 35 C, with or without shaking, to maximal turbidity, harvested by centrifugation at 4,000 × *g* for 30 min, washed three times in half-strength fluorescent treponemal antigen buffer (FTA; BBL) with 0.5% Formalin, and resuspended in this buffer to match the turbidity of McFarland tube no. 4. Young albino rabbits were injected intravenously with 1 ml twice a week for 4 weeks and for another week after an interval of 3 weeks. Seven to 10 days after the last injection, the rabbit was bled and the serum was harvested.

TABLE 2. *Morphological and cultural characteristics of isolates listed as present, absent, or no data for automatic clustering*

No.	Characteristic
1	Colonies grew in one day on initial subculture.
2	Round coccal forms in Gram stain from agar or broth.
3	Oval coccal forms in Gram stain from agar or broth.
4	Rod forms in Gram stain from agar or broth.
5	Filamentous forms in Gram stain from agar or broth.
6	Branching forms in Gram stain from agar or broth.
7	Chains of bacteria in Gram stain from agar or broth.
8	Clumps of bacteria in Gram stain from agar or broth.
9	Anaerobe, only grew more than 2 mm below surface of PYS agar deep.
10	Reduced resorufin in PYS agar deep.
11	Produced acid from glucose.
12	Produced marked acid from glucose in 2 days.
13	Produced gas from glucose.
14	Produced marked gas from glucose in 2 days.
15–18	As 11 to 14 for maltose in place of glucose.
19–22	As 11 to 14 for lactose in place of glucose.
23–26	As 11 to 14 for sucrose in place of glucose.
27–30	As 11 to 14 for soluble starch in place of glucose.
31	Produced acid from mannose.
32	Produced gas from mannose.
33–34	As 31 to 32 for xylose in place of mannose.
35–36	As 31 to 32 for inulin in place of mannose.
37	Produced acid from mannitol.
38	Produced acid from inositol.
39	Produced ethanol in FEP.
40	Produced butanol in FEP.
41	Produced 1+ to 4+ acetate in FEP.
42	Produced 2+ to 4+ acetate in FEP.
43	Produced 3+ to 4+ acetate in FEP.
44	Produced 4+ acetate in FEP.
45–48	As 41 to 44 for propionate in place of acetate.
49–52	As 41 to 44, butyrate in place of acetate.
53–56	As 41 to 44, lactate in place of acetate.
57–60	As 41 to 44 for succinate in place of acetate.

The globulin was separated (3, 10) and the concentration was estimated by using a refractometer; if necessary the solution was diluted to a protein concentration of 20 mg/ml. The solution was brought to pH 9 with 10 × concentrated pH 9 buffer (4), and fluorescein isothiocyanate (FITC), isomer I (BBL), was added to give 25 μg of FITC to 1 mg of protein.

The mixture was stirred thoroughly and refrigerated overnight. Excess FITC was removed by dialysis against FTA buffer. The conjugate was filtered and stored at −20 C.

Smears of pure cultures were stained with undiluted FITC conjugate for 30 min at room temperature, washed twice with FTA buffer and twice with pH 9.18 buffer (Fisher Scientific), and mounted in glycerol with pH 9 buffer. The slides were examined with a ×95 objective on a Leitz Ortholux microscope equipped for use with fluorescein stains. As a partial estimate of specificity, the FITC conjugates were tested against all isolates from the same specimen (including many of those which were lost before they were fully characterized), and they were tested for cross-reactions with other isolates of the same type. A satisfactory conjugate gave an even stain of the cell walls of the isolate used in its preparation, and it also stained isolates of the same group from the same specimen. However, it did not stain dissimilar isolates or isolates from other hosts even if they were of the same cultural type. The conjugates were then tested against all isolates from the same host.

The FITC conjugates were also used to stain the smears prepared from the 10^{-3} dilution of feces from the corresponding hosts following a blocking technique to increase specificity. To prepare the blocking sera, 1 ml of each antiserum, from the same lots used to prepare the conjugates, was sorbed with 0.1 ml of packed cells of the antigen used in its production at 45 C for 2 h, with occasional shaking, and in the refrigerator overnight. The cells were removed by centrifugation at 800 × *g* for 30 min. This sorption was repeated twice and checked by staining a smear of the corresponding strain with the sorbed serum 30 min at room temperature, washing with FTA, staining for 30 min with goat anti-rabbit globulin 1:8 (Microbiological Associates), washing, and mounting as above. The sorption was repeated until there was no cell wall fluorescence.

The fecal smears were stained at room temperature. They were covered with a drop of the sorbed antiserum for 30 min. The antiserum was removed with a capillary pipette applied to one corner; this corner was not used for counting. A drop of the specific FITC conjugate containing rhodamine-conjugated bovine serum albumin (NBCo) as a counterstain (the exact amount was adjusted empirically for each conjugate) was then placed on the smear for 30 min and withdrawn as before. The smears were washed three times with FTA buffer and twice with pH 9.18 buffer by dropping the buffer and withdrawing it as above, and then were mounted in buffered glycerol.

The fluorescing bacteria were counted in 50 fields in each smear in the same manner as used for the total microscope counts. The total bacteria were counted in five fields in each smear by using white light and darkfield illumination. If the count was less than 80% of the original total count, the results were discarded and another slide was stained. The fluorescent bacteria counted were multiplied by 8.414×10^6 to give the count of such bacteria per gram (wet weight).

RESULTS

The direct microscopic counts and total cultural counts were calculated on the basis of wet weight of specimen, dry weight of specimen, and dry weight of nonmicrobial residue (Table 3). The counts estimated on each basis were equally variable. Therefore the wet weight basis, which requires the minimum of calculation, was chosen for all further analyses.

Microscopic counts. The 47 specimens averaged $10^{11.1}$ organisms/g (wet weight) as estimated by direct microscopic counts (Table 3). By using the Gram stain, gram-positive rods accounted for 21% of the organisms seen, and gram-positive cocci accounted for 5% (Table 4). There was a positive correlation between the counts of gram-positive cocci and gram-positive rods (correlation coefficient = 0.76; $P = 0.0001$). Altogether, gram-positive bacteria accounted for 25% of organisms seen, varying from 2 to 45% in individual specimens.

Cultural counts. The total counts on RF agar are given in Table 3, and the estimated differen-

TABLE 3. *Comparison of total bacterial counts calculated on different bases*

Type of count	Basis	Geometric mean ($\log_{10}$)[a]	SD ($\log_{10}$)	Coefficient of variation (%)
Microscopic	Per g (wet wt)	11.13	0.25	2.3
	Per g (dry wt)	11.66	0.23	1.9
	Per g (dry residue)	11.70	0.25	2.1
Cultural	Per g (wet wt)	10.86	0.30	2.8
	Per g (dry wt)	11.39	0.31	2.7
	Per g (dry residue)	11.44	0.31	2.7

[a] Total of 47 specimens from 10 hosts: 3 from one and 4 from another and 5 from each of the remaining 8.

TABLE 4. *Estimated counts of bacteria differentiated by Gram reaction and microscopic morphology*

Gram reaction	Morphology	Microscopic counts ($\log_{10}$)			Cultural counts ($\log_{10}$)		
		Geometric mean[a]	SD	Range	Geometric mean	SD	Range
+	Rods	10.37	0.38	9.3–10.9	10.26	0.40	9.1–11.1
+	Cocci	9.75[b]	0.42	8.5–10.6	10.04	0.50	9.0–11.0
−	Rods	10.93	0.28	10.3–11.4	10.47	0.27	9.9–11.1
−	Cocci	9.88	0.47	8.5–10.5	9.60[c]	0.49	8.6–10.6

[a] Total of 47 fecal specimens from 10 hosts: 3 from one, 4 from another, and 5 from each of the remaining 8.

[b] Only seen in 46 specimens.

[c] Only seen in 40 specimens.

tial counts are given in Table 4. The total counts of bacteria cultured averaged 55% (for 46 specimens, with a range of 8 to 175%) of those seen on direct microscopic examination. Differential counts showed higher proportions of gram-positive bacteria and of cocci than the direct microscopic examination: 29% of the colonies were of gram-positive rods and 19% were of gram-positive cocci. The mean apparent recovery rates for the different groups were as follows: gram-positive rods, 128%; gram-positive cocci, 334%; gram-negative rods, 47%; and gram-negative cocci, 93%. Altogether, 47% of the bacteria grown were gram positive; they varied from 16 to 84% in individual specimens. The host was a significant source of variation in the number of gram-positive bacteria cultured ($P = 0.02$).

Isolation and characterization. Seventy-two percent of the colonies picked for isolation grew on subculture; the recovery rate varied greatly between specimens, ranging from 55 to 98% for the 43 specimens from which 50 or more colonies were picked. Fifty-seven percent of the isolates stained gram positive; this percentage varied from 25 to 85% for individual specimens. The proportion of bacteria picked which grew and were gram positive was nearly the same as the proportion of colonies on RF agar which were gram positive, indicating that few gram-positive bacteria were lost on subculturing. However, 24% of these gram-positive bacteria were lost on subsequent processing, leaving 865 isolates which were characterized (Table 5).

TABLE 5. *Bacterial isolates subcultured, characterized, and grouped on the basis of cultural characteristics*

Host	No. of specimens cultured	No. of bacterial isolates				No. of groups distinguished on basis of cultural characteristics
		Subcultured	Grew on subculture	Gram + on subculture	Characterized	
I	3	185	149	97	78	12
II	5	268	174	94	55	24
III	5	432	281	91	58	28
IV	5	300	235	162	130	34
V	4	184	147	72	53	19
VI	5	306	251	173	152	27
VII	5	245	163	112	96	23
VIII	5	295	214	95	82	39
IX	5	295	212	136	103	27
X	5	263	184	104	58	26
Total	47	2,773	2,018	1,137	865	

The majority of the tests used in characterizing the complete set of isolates examined carbohydrate fermentation (fermentation products and range of carbohydrates attacked) because a preliminary survey of 92 isolates had shown that 67 of them were more active in carbohydrate media than in media containing peptone, lactate, or lipid as an energy source, and that 86 of them produced acid from sugars in 7 days.

After characterization, it was apparent that there was a large variety of bacteria, even among isolates from a single specimen. The number of groups of isolates distinguished in each host is given in Table 5. The average number was 28.5 (range 24 to 39) per host for the eight hosts from which five specimens were examined. The average number of groups distinguished from individual specimens was 10.0 (range 3 to 15) per specimen for the 25 specimens from which 15 or more isolates were characterized. Many of the groups could be tentatively identified with species described by Holdeman and Moore (11). All groups thought to belong to a single species are referred to together as a type. Thus, by the definition given under methods, isolates in the same group come from the same host and are thought to belong to the same strain, but isolates of the same type may come from different hosts and are thought to belong to the same species. The types found in more than one individual are listed in Table 6. The characters of these types are those listed by Holdeman and Moore (11) for the species indicated, but the types were not definitely identified because the tests used did not include all the definitive tests for these genera and species. Each type was readily distinguishable from the others except that type 3 (resembling *Eubacterium contortum*) was close to some groups of type 9 (resembling *Peptostreptococcus productus*). Seventy-four percent of the isolates belonged to groups which could be tentatively identified, and 60% were tentatively identified as *Bifidobacterium adolescentis*, *Eubacterium aerofaciens*, *Eubacterium rectale*, *P. productus*, or *Ruminococcus bromii*.

The isolates of type 1 are all bifidobacteria but might include some *Bifidobacterium longum* as well as *B. adolescentis*. The isolates resembling *E. aerofaciens* are not all clearly distinguished from *Clostridium ramosum*, but most of the isolates resembled *E. aerofaciens* more closely because of coccal forms, anaerogensis, or failure to ferment amygdalin. The isolates resembling *E. rectale* might include some clostridia. The isolates resembling *P. productus* had a characteristic morphology but were heterogeneous in

TABLE 6. *Occurrence and tentative identification of predominant types of isolates based on cultural characteristics*

Type	Tentative identification	No. of isolates[a]	% of isolates	Found in:		
				No. of specimens[b]	% of specimens[c]	No. of hosts[d]
1	*Bifidobacterium adolescentis*	84	10	22	50	9
2	*Eubacterium aerofaciens*	160	18	28	68	8
3	*E. contortum*	14	2	5	13	4
4	*E. cylindroides*	36	4	10	26	6
5	*E. rectale*	80	9	24	58	10
6	*E. tortuosum*	7	1	4	11	3
7	*E. ventriosum*	13	1	9	21	5
8	*Propionibacterium acnes*	10	1	3	8	3
9	*Peptostreptococcus productus*	77	9	27	68	9
10	*Ruminococcus albus*	9	1	5	11	3
11	*R. bromii*	115	13	21	55	10
12	*Sarcina ventriculi*	12	1	2	5	2

[a] Total isolates—865.
[b] Total specimens—47.
[c] Total specimens (excluding those from which less than 9 isolates were characterized)—38.
[d] Total hosts—10.

fermentative characters. More than one group from some specimens resembled *P. productus* as described by Holdeman and Moore (11). The isolates resembling *R. bromii* were quite distinctive, and representative isolates from each host all stained with a single FITC conjugate.

Estimated geometric mean counts of these five types were as follows: bifidobacteria, $10^{9.5}$/g (wet weight); *E. aerofaciens*-like, $10^{9.7}$/g (wet weight); *E. rectale*-like, $10^{9.4}$/g (wet weight); *P. productus*-like, $10^{9.4}$/g (wet weight); and *R. bromii*-like $10^{9.7}$/g (wet weight). Analysis of variance of the counts by host and by type of bacteria, and the interaction of these two factors, indicated that the counts varied with the host and the host-type interaction but did not vary with the bacterial type (Table 7). The host-type interaction was obvious with the *E. aerofaciens* type, which was absent or rare in three hosts and common in seven. Strains of this type, distinguished by FITC conjugates, accounted for 5 to 6% of the total direct count in specimens I/1, VI/4, and X/3, a much higher proportion than normally found (compare Tables 3 and 9).

Further analysis of the estimated counts suggested that the counts of bifidobacteria (type 1), *E. rectale*-like (type 5), *R. bromii*-like (type 11), and gram-negative bacteria were intercorrelated. Correlation coefficients ($P < 0.05$) for the 43 specimens from which 50 or more colonies were picked were as follows: between 1 and 5, 0.72; between 1 and 11, 0.73; between 1 and gram-negative rods, 0.56; between 1 and gram-negative cocci, 0.72; between 5 and 11, 0.67; between 5 and gram-negative rods, 0.64; and between 5 and gram-negative cocci, 0.70. Also, estimated counts of type 4, which resembled *E. cylindroides*, and types 9 and 11 were negatively correlated with the residual water content of the feces, obtained by subtracting the dry weight and the estimated water content of the bacteria seen in direct smear (17) from the wet weight. The correlation coefficients ($P < 0.05$) were as follows: for residual water with 4, −0.67; with 9, −0.42; and with 11, −0.65.

The 29% of isolates not included in Table 6 were mostly grouped in ones or twos and did not obviously resemble isolates from other hosts. It is certain that there was no unidentified type comparable in importance with the five described above.

Persistence of bacterial strains as indicated by grouping bacteria on cultural characters. Within individual hosts, many groups of bacteria were found to be present in successive specimens. When there were two consecutive specimens from the same host from which 15 or more isolates had been characterized, an average of 35% (range 14 to 55) of the isolates from the second specimen were grouped with isolates from the first. From the eight hosts from which five specimens were collected, an average of 46% (range 21 to 80) of the isolates from the final specimen were grouped with isolates from previous specimens. The persistence of individual groups is listed in Table 8.

Persistence of bacterial strains as indicated by serological grouping of isolates and direct staining of fecal smears with FITC

TABLE 7. *Analysis of variance of estimated cultural counts of five types of gram-positive bacteria from 43 fecal specimens from which 50 or more colonies were isolated*

Source of variation	Degrees of freedom	Probability of the null hypothesis[a]
Host	9	0.0022
Type of bacteria[b]	4	0.2126
Interaction between host and type of bacteria	36	0.0176
Error	185	

[a] That the given source of variation did not influence the count obtained.
[b] Types resembling *B. adolescentis*, *E. aerofaciens*, *E. rectale*, *P. productus*, and *R. bromii*.

TABLE 8. *Persistence of culturally defined groups of gram-positive bacteria in individual hosts*

Host	No. of specimens cultured	No. of isolates characterized	No. of groups distinguished on cultural characteristics	No. of these groups found in more than one specimen	Days between first and last specimen from which members of the same group were isolated
I	3	78	12	1	281
II	5	55	24	5	59, 344, 419, 578, 578
III	5	58	28	5	51, 380, 480, 580, 580
IV	5	130	34	11	77, 77, 147, 147, 253, 343, 343, 449, 449, 519, 596
V	4	53	19	3	70, 84, 84
VI	5	152	27	10	98, 314, 446, 446, 446, 446, 591, 591, 591, 591
VII	5	96	23	7	84, 238, 345, 350, 350, 588, 588
VIII	5	82	39	8	116, 237, 237, 237, 237, 311, 548
IX	5	103	27	8	135, 238, 382, 485, 485, 620, 620, 620
X	5	58	26	4	222, 510, 510, 638

conjugates. Eighteen FITC conjugates distinguished between isolates of the same cultural type, that is, isolates resembling a single species, and were therefore used for serological grouping. Six conjugates were prepared by using isolates of bifidobacteria; there was no cross-reaction between these conjugate isolate systems, nor were these isolates stained by conjugates prepared with ATCC *B. adolescentis* strains 15703, 15704, 15705, and 15706. Thus, these conjugates defined six serological groups of bifidobacteria. In fecal smears these conjugates stained large irregular rods, but actual bifid forms were rarely seen; only one bifid form in 500 cells was observed in one specimen where this was recorded.

Seven conjugates were prepared by using *E. aerofaciens* type isolates; two of these conjugate isolate systems cross-reacted. Thus, six serological groups were distinguished, one of which was found in two hosts. On fecal smears they stained small rods of regular diameter, cocci, and occasional chains and filaments up to 20 μm long. A conjugate prepared by using an *E. cylindroides*-type isolate and tested against such isolates from five other hosts stained one isolate from another host. On fecal smears it stained long regular rods of constant diameter. Three conjugates were prepared by using *P. productus*-type isolates; there were no cross-reactions, and thus three serological groups were defined. On fecal smears these conjugates stained large oval or pointed cocci, often in pairs, and sometimes elongated into cigar-shaped rods. A conjugate prepared by using one unidentified isolate was tested against similar isolates from two other hosts and stained one of them well. On fecal smears it stained small irregular rods.

Fluorescein isothiocyanate conjugates prepared by using *R. bromii*-type isolates and *Propionibacterium acnes*-type isolates were of no use for distinguishing individual strains because they stained all isolates of the same type. Nevertheless, fecal smears from one host (I) were stained with a conjugate prepared by using a *P. acnes*-type isolate in order to verify that propionibacteria were actually present in the feces; small rods in the fecal smears were stained (Table 9).

The results of staining the isolates and direct fecal smears from each host with FITC conjugates prepared by using isolates from the same host are shown in Table 9. The correspondence between the counts estimated by staining direct smears and those estimated by staining isolates is not good, but both methods showed persistence of serologically defined groups. One group resembling *B. adolescentis*, five resembling *E. aerofaciens*, and three resembling *P. productus* were present for more than 18 months. The similarity of counts on direct smears from a single specimen made by using different conjugates might suggest that the same nonspecifically stained organisms were counted repeatedly, but this was not so since the different types had distinct morphologies as described above.

DISCUSSION

In this study the gram-positive bacteria cultured averaged $10^{10.5}$/g (wet weight) of feces, which is more than that reported in most surveys of fecal flora but the same as that reported by Drasar et al. (5), who also used a strict anaerobic technique. In determining the proportion of the fecal flora that was gram positive, the figure indicated by direct examination (average 25%) was probably too low, since some individual gram-positive bacteria may stain gram negative. The figure (average 47%)

TABLE 9. *Persistence of serologically defined groups of gram-positive bacteria in individual hosts*

Host	Tentative identification of isolate used in the preparation of FITC conjugate	Day of specimen collection as counted from first collection from the host listed	Log_{10} of count from direct smear stained with conjugate	Log_{10} of count estimated from proportion of isolates which stained with conjugate
I	*Eubacterium aerofaciens*	0	9.8	10.7
		112	9.2	9.3
		281	8.5	9.2
	Propionibacterium acnes[b]	0	8.0	—[a]
		112	8.3	10.2
		281	±[c]	—
II	*Eubacterium aerofaciens*[e]	0	—[d]	10.1
		59	8.5	—
		293	—	—
		419	8.0	9.7
		637	±	—
	Eubacterium aerofaciens[e]	0	9.3	9.8
		59	9.3	9.5
		293	9.0	—
		419	9.7	—
		637	9.7	—
III	*Peptostreptococcus productus*	0	9.0	—
		100	9.1	8.9
		329	9.5	9.8
		380	8.3	—
		580	8.0	—
IV	*Eubacterium cylindroides*	0	8.7	10.2
		147	±	—
		253	±	—
		519	—	—
		596	±	—
V	*Bifidobacterium adolescentis*[f]	0	8.6	10.9
		84	8.7	9.3
		328	±	—
		329	±	—
	Bifidobacterium adolescentis[f]	0	±	—
		84	±	9.3
		328	—	9.1
		398	—	—
VI	*Eubacterium aerofaciens*	0	9.3	9.9
		145	9.3	9.9
		216	9.5	10.6
		314	10.0	10.1
		591	9.7	10.1
	Peptostreptococcus productus	0	9.2	—
		145	9.5	9.1
		216	9.3	—
		314	9.8	9.6
		591	9.8	10.2
VII	*Bifidobacterium adolescentis*	0	9.4	10.7
		84	9.6	—
		322	9.1	9.1
		429	9.5	—
		672	9.3	—
	Eubacterium aerofaciens	0	9.1	—
		84	9.1	9.9
		322	9.2	9.1
		429	9.6	9.4
		672	9.1	—
	Peptostreptococcus productus	0	9.1	—
		84	9.4	9.9
		322	9.1	—
		429	9.4	—
		672	9.1	—
VIII	*Bifidobacterium adolescentis*	0	±	9.8
		116	±	—
		220	—	—
		311	±	—
		548	±	—
IX	*Bifidobacterium adolescentis*	0	±	9.8
		135	—	10.1
		266	—	—
		382	—	—
		620	—	9.6
	Eubacterium aerofaciens	0	±	9.9
		135	±	9.6
		266	±	9.4
		382	±	—
		620	9.1	10.6
	Unidentified bacillus	0	10.1	10.1
		135	8.2	—
		266	9.0	—
		382	±	9.3
		620	9.7	10.0
X	*Bifidobacterium adolescentis*	0	—	9.9
		128	±	9.3
		303	—	9.3
		416	—	—
		638	—	—
	Eubacterium aerofaciens	0	9.7	9.6
		128	9.3	9.9
		303	10.0	10.2
		416	9.6	9.3
		638	8.6	9.5

[a] No isolates stained.
[b] With the techniques used in this study, all *P. acnes* stain with one conjugate.
[c] Between 2 and 10 stained bacteria in 100 fields, representing 10^7 to 10^8/g (wet weight).
[d] Zero or one stained bacteria in 100 fields, representing less than 10^7/g (wet weight).
[e] Two serologically distinct groups.
[f] Two serologically distinct groups.

indicated by the cultural counts was probably too high, since it appeared that many of the gram-negative bacteria did not grow on culture. It is estimated that the true proportion is probably between 10 and 50% in most specimens, with the host being a significant factor in determining the actual proportion. It is to be noted that in this investigation the host variable includes diet and other habits which were not controlled.

The gram-positive bacteria isolated were all anaerobes and fermented carbohydrates, but otherwise they were a heterogeneous group not readily represented by a single metabolic type such as bifidobacteria or any one species of *Eubacterium*. This heterogeneity has been previously suggested by others studying smaller groups of subjects (1, 16).

The variety of types found in individual specimens meant that any one type accounted for only a small proportion of the flora. The count of individual strains distinguished by FITC conjugates on direct smears was usually about 1% of the total count. The average estimated cultural count of the five major types was from 2 to 5%, indicating cultural enrichment. Since the sampling procedures were designed on the assumption that predominant strains would constitute 20% of the flora, the data obtained were less useful than was anticipated. Nevertheless, it was shown that there were significant host idiosyncrasies in the composition of the gram-positive fecal flora, and some interrelationships were suggested. It was interesting that the bifidobacteria counts correlated with those of gram-negative anaerobes since bifidobacteria have been considered an alternative to a putrefactive or gram-negative flora (8).

Five types of bacteria were distinguished on the basis of cultural characteristics which accounted for 60% of the bacteria isolated; each type resembles a species which has been reported previously from human feces (11). Three of these types, resembling *B. adolescentis*, *E. rectale*, and *P. productus*, ferment a wide range of carbohydrates and in the gut probably make use of carbohydrates that the host cannot digest or absorb. Another type, resembling *R. bromii*, fermented primarily maltose but also soluble starch, glucose, and sometimes mannose; in the gut it probably makes use of starch which was not digested fast enough for the products to be absorbed by the host. The other type, resembling *E. aerofaciens*, ferments several carbohydrates but does not fall clearly into either of the above categories. This type was most responsible for the variation between hosts. In a few specimens it accounted for a relatively high proportion of the flora.

A major purpose of this study was to distinguish the predominant strains or populations of the gram-positive bacteria within each host and to determine whether they were resident or transient. Two methods were used to distinguish bacteria representing particular strains. The first method, grouping of isolates on the results of cultural tests, presented problems in the selection of tests, the recording of results, and the method of clustering. Only a short series of tests could be handled and, after preliminary tests, tests were selected which were reproducible and distinguished between isolates. This series was inadequate for taxonomic purposes, and therefore subsequent identification of the groups was only tentative. In recording the results of the tests an attempt was made to record some of the features that are used in visual recognition of strains when small numbers of isolates are examined. Such features were the pattern of acid and gas production and the pattern of peaks produced by chromatography of the fermentation end products. In both recording and clustering, it is necessary to use uniform methods for all isolates, but these methods may produce distortion when applied to a wide range of organisms because some types show more phenotypic variation than others. In clustering, the single-link method is sensitive to the numbers in a potential cluster. Large clusters will commonly be recognized at a lower level of difference than small ones. Together, these problems resulted in some anomalous clusters which were edited by using taxonomic criteria from more extensive studies (11). In every host this editing resulted in fewer groups than there were clusters at the 6% level of difference.

A particular problem of the second method for detecting strains, the use of FITC conjugates, was in determining the specificity of the conjugates. Strain-specific conjugates should not cross-react with unrelated bacteria and should distinguish between strains of a single species. Testing for cross-reactions with unrelated bacteria has always been a problem when fluorescent antibody techniques are used in the direct examination of feces since there are so many unknown bacteria in feces. Nevertheless, such techiques are used in the detection of pathogens. In this study three factors suggested that the organisms stained were homologous with those used in preparing the conjugate. First, the conjugates used did not cross-react with a sample of gram-positive bacteria isolated from the same specimen as the isolate used in

preparing the conjugate. Secondly, the organism stained had a characteristic morphology. Thirdly, significant numbers of stained organisms were only seen in fecal smears from hosts from which bacteria of the same serological and cultural type had been isolated. Even if conjugates do not cross-react with unrelated bacteria, they are not necessarily suitable for tracing strains. Serological methods have been used to trace strains of *E. coli* in the fecal flora (2, 19, 23), but *E. coli* was already known to be serologically diverse. The bacteria isolated here, apart from bifidobacteria (18) and propionibacteria (12), have not been previously studied serologically. Therefore the conjugates were tested against isolates of the same type from other hosts. Such tests indicated that the bifidobacteria and the isolates resembling *E. aerofaciens* could be divided into several serological groups so the conjugates would be useful in tracing strains, that strains of *R. bromii* and *P. acnes* could definitely not be distinguished by these conjugates, and that some other types, *E. cylindroides*-like, *P. productus*-like, and an unidentified bacillus, could at least be subdivided by using the conjugates; but there were too few conjugates to establish the degree of heterogeneity.

Grouping on the basis of cultural characteristics allowed all isolates to be grouped. Serological grouping is more definitive but could only group a few isolates (22%). The serological groups correspond largely with cultural groups except that one cultural group of bifidobacteria encompassed two serological groups.

The cultural grouping suggested that there were resident strains in each host and that, allowing for sampling deficiencies, probably at least half of the strains were resident. When the FITC conjugates were used to detect strains, it was found that *E. aerofaciens*-like bacteria were generally represented by a single strain which was a long-term resident. Resident strains of bifidobacteria were also found, but they appeared to change more frequently than the *E. aerofaciens*-like strains. Resident strains of *P. productus* and an unidentified bacillus were suggested.

This technique of staining fecal smears with FITC conjugates for species or strains of bacteria might find useful application as a quick method of obtaining counts in dietary and medical surveys. In most cases the staining was very sharp and specific and would have been suitable for automated counting.

In conclusion, gram-positive bacteria form a large portion of the human fecal flora. Although they all ferment carbohydrate, they cannot be considered as a metabolic unit in studying colonic ecology since they belong to various physiological types, each with a distinctive metabolism, and the relative numbers of these types vary from host to host. There is also variation within each host over time, but some stability of the flora is indicated by both the idiosyncratic patterns of counts detected over the 18-month period and by the persistence of some serologically defined strains over the same period.

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