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Use of a Species-Specific DNA Hybridization Probe for Enumerating *Bacteroides vulgatus* in Human Feces

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pBV-1, a recombinant plasmid that contains a chromosomal DNA fragment from *Bacteroides vulgatus*, hybridized to DNA from *B. vulgatus* but not to DNA from other colonic *Bacteroides* species. This plasmid was used as a DNA probe to detect and enumerate *B. vulgatus* in pure culture, in mixed cultures, and in a bacterial fraction from human feces. Bacteria in a pure or mixed culture were lysed by heating the culture in NaOH. The DNA in the disrupted cell suspension was then trapped on nitrocellulose paper by vacuum filtration. If fecal samples were used instead of pure or mixed cultures, it was first necessary to partially purify the DNA by low-speed centrifugation (2,000 × *g*) and phenol-chloroform extraction before filtering. When ^{32}P-labeled pBV-1 was incubated with filters containing *B. vulgatus* DNA, the amount of radioactivity that bound to the filters was proportional to the number of *B. vulgatus* filtered as long as the filtering capacity of the nitrocellulose was not exceeded. Using this procedure, we obtained a value for the concentration of *B. vulgatus* in human feces (2×10^{10} to 3×10^{10} per g of dry weight) that is similar to values obtained by other investigators using conventional bacteriological techniques (3×10^{10} to 6×10^{10} per g of dry weight). The advantage of the DNA hybridization method over conventional techniques is that it is not necessary to isolate pure cultures of bacteria from complex specimens such as feces. Furthermore, our method bypasses the cumbersome set of biochemical tests normally used to identify anaerobic bacteria. The major limitation of our method is its sensitivity. Our results indicate that this method can only be used to detect and enumerate those species which make up at least 2% of the organisms in a complex microbial population.

The human colon harbors a complex bacterial flora (5). Many interesting questions have been raised about the effect of the diet, age, or other factors of the host on the species composition of colonic flora. However, it has been virtually impossible to answer these questions because of considerable methodological difficulties involved in identifying and enumerating even the predominant species of colon bacteria, all of which are obligate anaerobes (8). Cultivation of colonic anaerobes from fecal specimens requires stringent anaerobic techniques and special equipment. Once the organisms have been isolated in pure culture, an elaborate series of metabolic tests must be performed to identify each isolate (4). A possible solution to these difficulties would be to develop an identification method that is based on DNA-DNA hybridization. Since the phenotypic differences which distinguish species are based ultimately on differences in chromosomal DNA sequences, a test designed to detect specific DNA sequences by DNA-DNA hybridization should be at least as reliable as conventional biochemical testing for identifying organisms. Furthermore, a method based on DNA-DNA hybridization might not require prior isolation of bacteria in pure culture if the hybridization probes were sensitive and specific enough to detect a particular DNA sequence in a mixture of organisms.

We have developed a method, based on DNA-DNA hybridization with a species-specific DNA probe, for simultaneously detecting and enumerating *Bacteroides* species. *Bacteroides* spp. are gram-negative obligate anaerobes that are among the most numerous bacteria in the human colon, accounting for up to 30% of all isolates (5, 8). Since these bacteria can utilize dietary polysaccharides for carbon and energy, the levels of these bacteria might be affected by the diet of the host (11). At least 10 major *Bacteroides* species have been isolated from human feces. Although these species differ significantly at the genetic level, as shown by DNA-DNA homology measurements (see Table 1 and reference 6), they are very similar phenotypically and are thus particularly difficult to differentiate by conventional biochemical tests (4). Recently, Salyers et al. (9) showed that it was feasible to construct species-specific DNA probes by cloning and screening random fragments of chromosomal DNA from *B. thetaiotaomicron* and to use these probes to detect this species in pure culture. In the present report, we describe a method for using a DNA probe that is specific for *B. vulgatus* to estimate the concentration of this species in a pure culture, in mixed cultures, and in feces. We chose *B. vulgatus* rather than one of the other colonic *Bacteroides* species because this is one of the numerically predominant *Bacteroides* species in the colon, accounting for approximately 12% of all colon isolates. The approximate concentration of *B. vulgatus* in human feces has been determined by Holdeman et al. (5) and Moore and Holdeman (8) by conventional bacteriological methods, and this gives us a standard for comparison. Furthermore, this species is one of the few former subspecies of *B. fragilis* which remained intact after the recent taxonomic reclassification of colonic *Bacteroides* spp. based on DNA-DNA homology studies (1). Thus, the concentration of *B. fragilis* subsp. *vulgatus* (3×10^{10} to 6×10^{10} per g of dry weight) which has been determined previously (5, 8) should be an accurate assessment of the concentration of *B. vulgatus* in human feces.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Bacteroides* strains used in these experiments (Table 1) were obtained from the culture collection of the anaerobe laboratory of the Virginia Polytechnic Institute and State University, Blacksburg. Unless otherwise indicated, all strain numbers

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TABLE 1. *Bacteroides* strains

Bacteroides species	Strain no.[a]	% DNA homology with *B. vulgatus* type strain[b]
B. vulgatus	4245, type strain (ATCC 8482)	100
	2277	87
	2365	86
	4025	80
	4506-1	86
	5710	87
	6186	82
	6598-B	80
	C1–13	78
	3776-A	
B. thetaiotaomicron	5482 (ATCC 29148)	9
B. ovatus	0038 (ATCC 8483)	8
B. uniformis	0061 (ATCC 8492)	19
B. fragilis group I[c]	2553 (ATCC 25285)	20
B. fragilis group II[c]	2393	14
Bacteroides sp. strain 3452-A[d]	3452-A	4
B. distasonis	4243 (ATCC 8503)	9
Bacteroides sp. strain B5–21[e]	B5–21	19
B. eggerthii	B8–51	14

[a] Unless otherwise noted, all strain numbers are Virginia Polytechnic Institute numbers.
[b] Data from reference 6.
[c] Groups based on DNA homology studies (6).
[d] An unnamed DNA homology group (6).
[e] Formerly *B. fragilis* subsp. A (6).

are Virginia Polytechnic Institute numbers. *Bacteroides* strains were grown in a defined medium with 0.5% glucose as the carbon source (10).

Construction of the DNA probe specific for *B. vulgatus*. Random fragments of chromosomal DNA from *B. vulgatus* 4245 were cloned in pBR322 as described previously (9). They were tested for specificity against the strains in Table 1 by the filtration and hybridization procedures described below. For hybridization experiments, recombinant plasmids were labeled with [α-^{32}P]dCTP by nick translation (2).

Filtering and hybridization procedure. Our filtration procedures were based on the method of Gillespie and Spiegelman (3) for trapping purified single-stranded DNA on nitrocellulose filters. *Bacteroides* spp. were grown to an optical density (at 650 nm) of 0.8 (approximately 10^9 to 2×10^9 bacteria per ml). The concentration of bacteria was determined by direct microscopic enumeration with a Petroff-Hausser counting chamber. Bacteria (10 ml) were harvested by centrifugation at 16,000 $\times$ *g* for 15 min at 4°C and suspended in 10 ml of distilled water. NaOH was added to a final concentration of 0.5 N, and the mixture was heated to 90°C for 20 to 30 min. This treatment lyses the bacteria, denatures the DNA, and destroys much of the cellular RNA. The disrupted cell suspension was neutralized with HCl, diluted with an equal volume of ice-cold 20× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate [pH 7.0]), and chilled on ice. In experiments to determine optimum conditions for binding single-stranded bacterial DNA to nitrocellulose, the final SSC concentration was varied from 2 to 20×. We found that a final concentration of 10× SSC gave the highest recovery of DNA on nitrocellulose filters, as indicated by the amount of added [^{32}P]DNA that was trapped on the filters (see below).

Single-stranded DNA was then trapped on nitrocellulose as follows. Duplicate samples of the neutralized and diluted cell suspension, brought up to a 4.0-ml final volume with 10× SSC, were filtered onto adjacent spots of a 2-in (5.08-cm) nitrocellulose strip (Millipore Corp. [Bedford, Mass.] HAWP, type HA; 0.45 μm) by vacuum aspiration through a glass funnel (Millipore; 2.5-cm diameter, 15-ml capacity). The nitrocellulose spots were prewetted with 4.0 ml of 10× SSC before filtration of a sample. All samples from one cell preparation were filtered side by side on the same strip of nitrocellulose paper, which had been marked with a soft pencil to facilitate orientation of the filtered samples. After filtration, the sample spots were rinsed with 4.0 ml of 10× SSC and allowed to air dry, and the filters were then heated for at least 6 h at 85°C to fix the DNA to the filter.

In experiments to determine the efficiency with which DNA bound to nitrocellulose under these filtration conditions, purified *B. vulgatus* DNA that had been labeled with ^{32}P by nick translation was added to undisrupted cell suspensions before NaOH treatment. The amount of radioactivity that bound to the filter was compared with the amount of radioactivity detected when a comparable amount of [^{32}P]DNA was spotted directly onto prewetted nitrocellulose, dried, and heat fixed as described above. The conditions used in this study were optimized for Millipore HAWP paper and may not work well on other types of nitrocellulose paper or on GeneScreen (New England Nuclear Corp., Boston, Mass.).

Nitrocellulose strips containing fixed DNA were placed side by side into a sealable plastic bag. Prehybridization solution (2) containing 50% formamide (vol/vol) and salmon sperm DNA (200 μg/ml) was added, and the bag was heat sealed and incubated at 42°C for 2 to 3 h. The bag was then cut open, the prehybridization solution was extruded, and hybridization solution (2) containing 50% formamide, 10% (wt/vol) dextran sulfate and approximately 10^6 cpm of ^{32}P-labeled recombinant plasmid was added to the bag. The bag was resealed and incubated at 42°C for 12 to 24 h. In some experiments, dextran sulfate was omitted from the hybridization solution, and times of hybridization were varied from 2 to 72 h. In each hybridization experiment, a nitrocellulose strip that contained known amounts of disrupted *B. vulgatus* 4245 was included as a positive control. Uninoculated medium or bacteria which did not hybridize with the DNA probe were used as a negative control.

After hybridization, nitrocellulose strips were washed once in 0.2% (vol/wt) sodium dodecyl sulfate (SDS)-2× SSPE (1× SSPE is 0.2 M NaCl-10 mM NaH_2PO_4-1 mM EDTA [pH 7.0]) and twice in 0.2× SDS-0.2× SSPE. Roughly 8 to 10 ml of wash buffer was used per in^2 (6.45 cm^2) of filter paper. Each wash lasted 20 min at 60°C. After washing, filter strips were dried briefly at 60 to 70°C to remove excess moisture. The strips were cut into squares (approximately 1 in^2) to separate the sample spots, and individual squares were placed into scintillation vials containing Aqueous Counting Scintillant (Amersham Corp. Arlington Heights, Ill.). The amount of radioactivity that had bound to the filtered samples, measured with a Tri-Carb 460 liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.) reflected the amount of probe that had hybridized to the samples.

In the initial experiments to screen the randomly cloned fragments of *B. vulgatus* DNA for species specificity, each filter spot contained DNA from roughly 10^8 lysed bacteria. In experiments designed to quantitate *B. vulgatus* in pure and mixed cultures, several serial dilutions of the lysed bacterial suspension (diluted with 10× SSC) were filtered.

Fecal specimens. Freshly passed stool samples were ob-

tained from a healthy adult volunteer and processed immediately. A sample was collected in a plastic bag and kneaded thoroughly. Approximately 2 g of feces was weighed out in a tared glass tube, lyophilized for 48 to 72 h, and then reweighed to obtain a dry weight. The remainder of the stool specimen was suspended thoroughly in ice-cold 0.05 M potassium phosphate buffer (pH 7.0) (approximately 50 ml of buffer was used to suspend each gram of feces). The suspended feces were centrifuged at 16,000 × *g* for 15 min at 4°C to pellet bacteria. The supernatant fluid was discarded, and the pellet, which contained bacteria and particulate matter, was washed again with cold phosphate buffer. The final pellet was suspended in distilled water, and the suspension was dispensed into aliquots and kept frozen at −20°C until use.

The procedure that was used to trap DNA from fecal bacteria on nitrocellulose was similar to that described above for trapping bacterial DNA from a pure culture, although several additional steps were necessary to remove interfering material from the fecal sample. Samples of the fecal suspension were heated in 0.5 N NaOH, neutralized, and diluted with 20× SSC as described above. The disrupted fecal bacterial suspension was centrifuged at low speed (2,000 × *g* for 10 min) at 4°C to remove particulate matter and cell debris. The supernatant solution was decanted and extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol) and once with ether (7). The aqueous bacterial DNA solution obtained from the extractions was filtered onto nitrocellulose as described above. In experiments to determine whether the DNA probe was binding to material in feces other than DNA, the bacterial DNA solution was incubated with DNase I (200 μg/ml in 5 mM $MgCl_2$) for 4 to 6 h at 37°C before filtration. In experiments to determine the efficiency with which DNA was recovered from bacteria in feces, purified chromosomal DNA from *B. vulgatus* 4245 that had been labeled with ^{32}P by nick translation was added to a fecal sample before processing.

In experiments to estimate the concentration of *B. vulgatus* in feces, known amounts of *B. vulgatus*, grown in pure culture, were added to a fecal sample before processing as an internal standard. The amount of ^{32}P-labeled probe that hybridized to such filtered samples was then compared with the amount that hybridized to filtered fecal samples to which no *B. vulgatus* had been added and to the amount that hybridized to filtered samples of a pure culture of *B. vulgatus*. Four different fecal samples, obtained from the same subject on different days, were analyzed by this technique.

RESULTS

Specificity of DNA probe. Recombinant plasmids that contained chromosomal DNA inserts from *B. vulgatus* 4245 were screened for hybridization to DNA from the various species listed in Table 1. One of these plasmids, pBV-1, which contained a *B. vulgatus* DNA fragment of approximately 700 base pairs, was specific for *B. vulgatus*, i.e., the amount of ^{32}P-labeled pBV-1 that hybridized to DNA from other *Bacteroides* species when DNA from 10^8 disrupted bacteria was trapped on a filter was consistently less than 10% of the amount that hybridized to DNA from a comparable number of disrupted *B. vulgatus* 4245. In addition, pBV-1 did not hybridize to DNA from *Fusobacterium prausnitzii* ATCC 27768. The *Bacteroides* species listed in Table 1, together with *F. prausnitzii*, represent all of the predominant gram-negative colon bacteria and 35 to 40% of all colon isolates (5, 8). ^{32}P-labeled pBR322 did not hybridize to *B. vulgatus* DNA. Thus, the cloned *B. vulgatus* DNA fragment was responsible for the specificity of pBV-1 for *B. vulgatus*. This fragment is most likely chromosomal DNA, because we found no evidence of plasmids in *B. vulgatus* 4245 (A. Kuritza, personal observation).

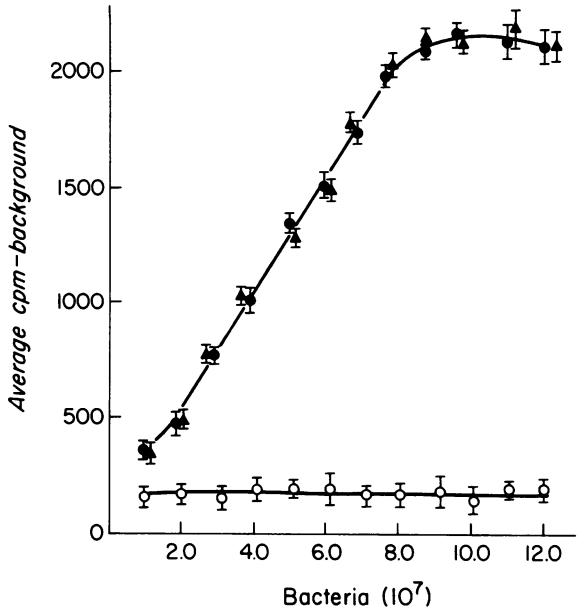

FIG. 1. Relationship of ^{32}P-labeled pBV-1 bound to a filtered sample and the number of disrupted bacteria in the sample. ● and ▲ indicate average values for two separate experiments in which a pure culture of *B. vulgatus* was used. ○ indicates the average values obtained when *B. thetaiotaomicron* 5482 was used instead of *B. vulgatus*. Error bars show the amount of variation within each experiment.

Use of pBV-1 to quantitate *B. vulgatus* in pure culture. The amount of ^{32}P-labeled pBV-1 that hybridized to a filtered sample of *B. vulgatus* 4245 was proportional to the number of bacteria in the sample within the range of 10^7 to 10^8 bacteria (Fig. 1). By contrast, the amount of ^{32}P-labeled pBV-1 that hybridized to filtered samples of other *Bacteroides* species remained near background levels, background being defined as the amount of radioactivity that bound to nitrocellulose through which only media had been filtered. Although the amount of radioactivity that was detected varied with the specific activity of the ^{32}P-labeled pBV-1, the slope of the linear region in the hybridization curve (cf. Fig. 1), i.e., the counts per minute bound per number of disrupted bacteria filtered, was reproducible to within 10% from one experiment to another if corrected for differences in specific activity of the ^{32}P-labeled probe. When strains of *B. vulgatus* other than the type strain 4245 were filtered, the slopes of the hybridization curves were similar to that obtained with strain 4245 (Table 2). These results indicated that pBV-1 could be used to quantitate *B. vulgatus* in mixtures containing more than one strain of this species.

When known amounts of ^{32}P-labeled chromosomal DNA from *B. vulgatus* (10^4 to 10^5 cpm) were added to a pure culture of *B. vulgatus* before lysing the cells, 65 to 70% of the added radioactivity was recovered on the nitrocellulose filters if the number of disrupted bacteria filtered was within the range of 10^7 to 10^8. If the number of disrupted bacteria in a filtered sample exceeded 10^8, the percentage of added [^{32}P]DNA that was recovered on the filters decreased. By comparison, when equivalent amounts of [^{32}P]DNA were

TABLE 2. Hybridization of pBV-1 to different strains of *B. vulgatus*

B. vulgatus strain	kcpm bound to filter per 10^8 disrupted bacteria[a]
4245	2.4
2277	2.5
2365	2.6
4025	2.2
4506-1	2.1
5710	2.7
6186	2.2
6598-B	2.0
C1-13	2.0
3776-A	2.3

[a] kcpm bound, Counts per minute in thousands bound to filter minus background. Each value is the average of four determinations. Background was determined as the counts per minute bound to nitrocellulose through which media had been filtered. The average standard deviation was ±0.3 kcpm.

filtered without added bacteria, 75 to 80% of the [^{32}P]DNA was recovered on the nitrocellulose filters. Thus non-DNA components in a disrupted cell preparation did not significantly interfere with the binding of DNA to nitrocellulose as long as the number of bacteria filtered did not exceed 10^8.

It has been reported that dextran sulfate accelerates the rate of hybridization (12). To test this in our system, we prepared several replicate nitrocellulose strips containing equivalent known amounts of disrupted *B. vulgatus* and incubated the filters with ^{32}P-labeled pBV-1 for 2 to 72 h with and without dextran sulfate. The results of these experiments (Fig. 2) indicated that dextran sulfate increased both the rate of hybridization and the amount of probe that hybridized to a filtered sample. If dextran sulfate was included in the hybridization mixture, the amount of ^{32}P-labeled pBV-1 that hybridized to the samples reached a maximum after 12 h of incubation and remained stable for an additional 12 h. We therefore included dextran sulfate in all hybridizations and allowed hybridization to proceed for 12 to 24 h.

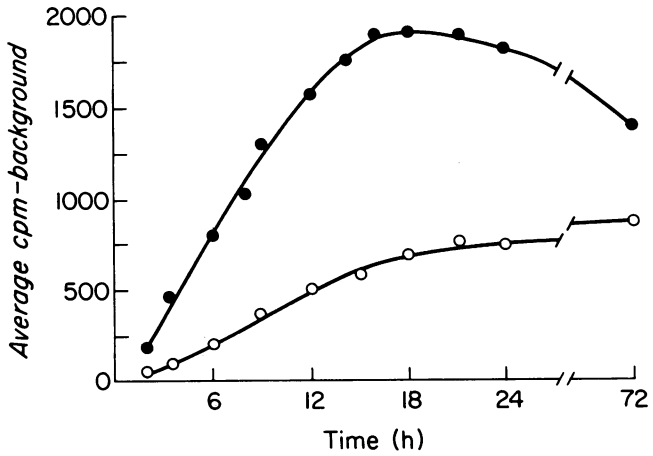

FIG. 2. Effect of time and dextran sulfate (10%) on the hybridization of ^{32}P-labeled pBV-1 to DNA from *B. vulgatus* 4245 that had been trapped on nitrocellulose. Each sample contained approximately 10^8 disrupted bacteria. Hybridization was allowed to proceed for 2 to 72 h with (●) or without (○) dextran sulfate.

Quantitation of bacteria in mixed cultures. Since a fecal sample would contain many organisms other than *B. vulgatus*, it was necessary to determine whether pBV-1 could be used to quantitate this species if it was mixed with other bacteria. When pure cultures of *B. vulgatus* and *B. thetaiotaomicron* were mixed in various proportions so that the total number of bacteria in each mixture was the same (approximately 10^8), the amount of pBV-1 that hybridized to a filtered sample corresponded with the amount of *B. vulgatus* in the sample (Fig. 3). Moreover, the slope of the hybridization curve obtained with the *B. vulgatus-B. thetaiotaomicron* mixtures was the same as that obtained with *B. vulgatus* alone.

In a similar series of experiments, pure cultures of the two organisms were mixed so that *B. vulgatus* made up 25, 50, or 75% of a mixture. The bacteria were lysed, and several different amounts of each mixture were filtered. In these experiments, unlike the previous experiment, the percentage of a mixture that was *B. vulgatus* remained constant, whereas the total number of disrupted bacteria changed in each filtered sample. The results of these experiments (Fig. 4) indicated that although the amount of pBV-1 that hybridized to a sample was dependent on the number of *B. vulgatus* in the sample, the range over which the hybridization curve

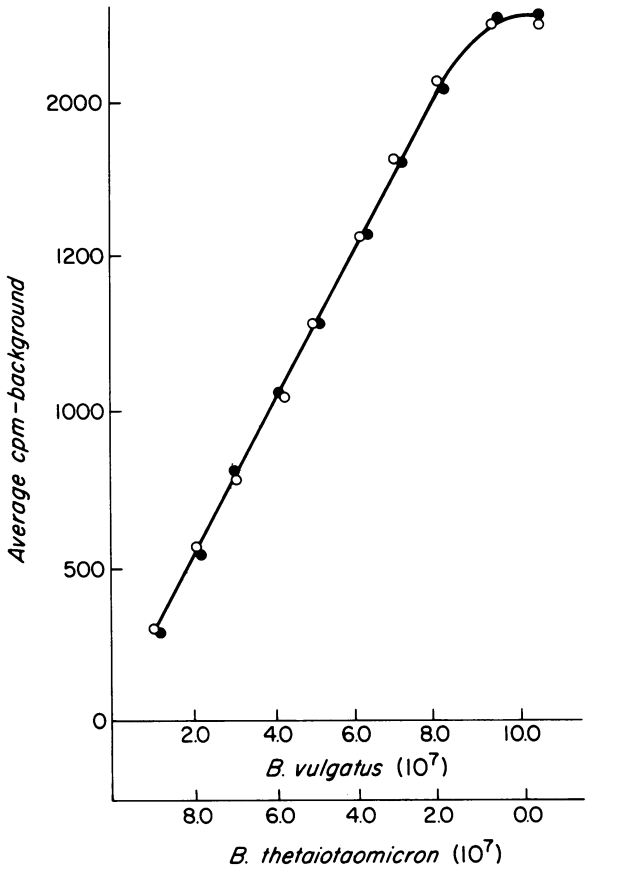

FIG. 3. Hybridization of ^{32}P-labeled pBV-1 to samples containing a pure culture of *B. vulgatus* 4245 (●) or a mixture of *B. vulgatus* 4245 and *B. thetaiotaomicron* 5482 (○). In samples containing a mixture of the two species, the total number of bacteria in each sample was the same (approximately 10^8), whereas the proportions of the two species in each sample varied.

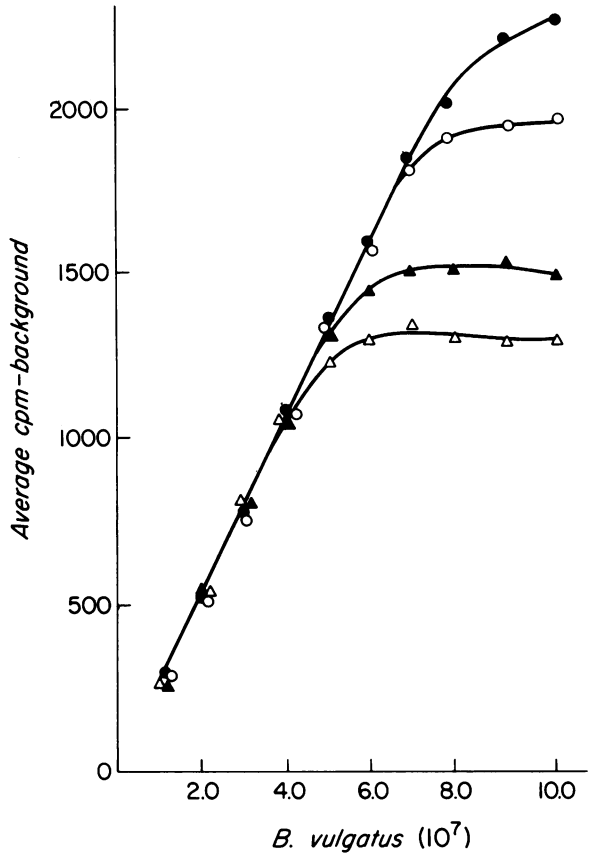

FIG. 4. Hybridization of ^{32}P-labeled pBV-1 to samples containing a pure culture of *B. vulgatus* 4245 (●) or mixtures of *B. vulgatus* 4245 and *B. thetaiotaomicron* 5482 in which *B. vulgatus* accounted for 25 (△), 50 (▲), or 75% (○) of a mixture. The total number of bacteria in each filtered sample varied, whereas the relative proportions of the two species remained constant.

was linear decreased as the relative proportion of *B. vulgatus* in a mixture decreased; i.e., saturation of nitrocellulose was dependent on the total number of bacteria filtered rather than on the number of *B. vulgatus* filtered. Thus, saturation of nitrocellulose may become a serious problem in the detection of a species that makes up a low relative percentage of the total bacteria in a complex mixture of organisms.

Quantitating *B. vulgatus* in human feces. Our initial attempts to detect *B. vulgatus* in feces by using the procedures we had developed for pure cultures were unsuccessful. It was difficult to filter a disrupted fecal cell suspension, because particulate matter in the feces clogged the nitrocellulose. Consequently, the amount of pBV-1 that hybridized to filtered fecal samples varied within the same experiment and from one experiment to another. We were also concerned about the possibility that proteins, lipids, or other cellular components could be competing with DNA for binding sites on the filters. To overcome these difficulties, we introduced two additional steps to our procedure. First, after a fecal sample had been heated in 0.5 N NaOH, neutralized, and diluted with SSC, the sample was centrifuged briefly at low speed (2,000 × *g*, 10 min). This step served to pellet much of the cellular debris and insoluble particulate matter. The supernatant fluid was then extracted twice with phenol-chloroform-isoamyl alcohol and once with ether. Thereby, we obtained a clear aqueous solution, free of particulate matter, that could be easily filtered through nitrocellulose. When we added ^{32}P-labeled chromosomal DNA to a fecal sample before the addition of NaOH, we recovered 90 to 95% of the radioactivity in the aqueous solution after the final extraction.

When we included these two purification steps in our filtration procedure, we were able to detect reproducibly the binding of ^{32}P-labeled pBV-1 to filtered fecal samples (Table 3). The amount of radioactivity that bound to the DNA trapped on the nitrocellulose increased linearly with the amount of the fecal solution filtered (given in Table 3 as equivalent amounts of feces). In addition, if we incubated partially purified fecal DNA solution with DNase before filtration (see above), the amount of radioactivity that bound to such samples decreased to background levels. For the concentrations of feces indicated in Table 3, no hybridization of ^{32}P-labeled pBR322 to the DNA on the filters was detectable. These results indicated that pBV-1 was binding specifically to *B. vulgatus* DNA in the fecal specimen rather than to non-DNA material or to pBR322 sequences in enteric bacteria.

TABLE 3. Quantitation of *B. vulgatus* in human feces

Amount of feces filtered (mg of wet wt)	Amount of ^{32}P-labeled pBV-1 bound (cpm)[a]						Avg cpm per added 10^8 bacteria[d]	% Pure culture[e]	*B. vulgatus* (×10^9) per g of wet wt of feces[f]	*B. vulgatus* (×10^{10}) per g of dry wt of feces[g]
	Feces alone		Feces + 1× cells[b]		Feces + 2× cells[b]					
	Trial I[c]	Trial II[c]	Trial I[c]	Trial II[c]	Trial I[c]	Trial II[c]				
2.5	100	114	250	236	475	502	820	34	5.7	2.8
5.0	177	185	467	513	872	914	862	36	4.2	2.1
7.5	312	382	738	722	1,450	1,413	778	32	5.8	2.9
10.0	406	398	912	1,060	1,765	1,800	805	34	5.0	2.5
15.0	635	690	1,410	1,397	2,685	2,685	705	30	6.3	3.1

[a] Values reported are averages of duplicate determinations. cpm, Counts per minute.

[b] The values 1× and 2× refer to amounts of pure culture added to the fecal suspension before processing to final concentrations of 10^9 and 2×10^9 added cells per ml, respectively.

[c] Trials I and II were performed in parallel on two different stool samples collected several days apart.

[d] Average of (feces + 1× cells − feces alone)/number of added *B. vulgatus* in the sample and (feces + 2× cells − feces alone)/number of added *B. vulgatus* in the sample.

[e] Percentage of counts per minute per 10^8 bacteria obtained when a pure culture containing the same number of *B. vulgatus* was used.

[f] *B. vulgatus* per g of wet weight feces = (counts per minute from fecal samples/[counts per minute from fecal sample + *B. vulgatus* − counts per minute from fecal sample]) × (10^9 added cells per ml/0.15 g of wet weight feces per ml).

[g] Based on an average value of fecal dry weight/fecal wet weight = 20%.

When known amounts of a pure culture of *B. vulgatus* were added to feces, the amount of ^{32}P-labeled pBV-1 that bound to the filtered samples rose proportionately (Table 3). However, the amount of pBV-1 that hybridized to samples of feces plus *B. vulgatus* was only 30 to 35% of the amount that hybridized to a comparable number of *B. vulgatus* in pure culture. Because of this, we used *B. vulgatus* added to feces, rather than *B. vulgatus* in pure culture, as a standard to calculate the number of *B. vulgatus* in feces.

To compute the concentration of *B. vulgatus* in feces, we measured the amount of probe that hybridized to samples of feces plus added bacteria, then we used this value to extrapolate back to determine how many *B. vulgatus* were present in feces plus no added bacteria. We first determined the additional counts per minute per 10^8 added bacteria (Table 3, column 8). The counts per minute from the fecal specimen to which no bacteria had been added (an average of columns 2 and 3 in Table 3) was then divided by this value to give the number of bacteria placed on the filter. This last value was then multiplied by the appropriate dilution to give the number of bacteria in the original specimen. A simple formula for making these computations is given in the footnote to Table 3. The concentration of *B. vulgatus* in feces that we calculated by this technique with values that were obtained from four different stool specimens was 2.1×10^{10} to 3.1×10^{10}/g of dry feces.

DISCUSSION

We have shown that it is possible to use a species-specific DNA probe to estimate the concentration of a particular species in a mixture as complex as human feces. Our estimates for the concentration of *B. vulgatus* (2×10^{10} to 3×10^{10}/g of dry weight) agree well with estimates obtained previously by conventional methods (3×10^{10} to 6×10^{10}/g of dry weight; references 5 and 8). Our approach offers several advantages over conventional methods for bacterial identification and enumeration. Since our method does not require isolation of bacteria, it bypasses the special equipment and procedures that are needed to cultivate anaerobic bacteria. Our method also provides identification and enumeration results simultaneously. In cases in which plating efficiency is low, our method would give more accurate results than those obtained by plate counts. In addition, our method can be used to quantitate bacteria in samples that have been frozen. This last attribute may be of importance for dietary studies involving a large number of fecal specimens, since it would not be necessary to have a properly equipped and staffed microbiology laboratory available to process fresh specimens immediately. It should be kept in mind, however, that our method detects DNA from nonviable as well as viable bacteria and therefore may not be applicable in cases in which the percentage of nonviable bacteria would be high. This is probably not the case in the colon, because Moore and Holdeman (8) claimed that direct microscopic counts were comparable to viable counts in fecal specimens.

pBV-1 appears to be specific for *B. vulgatus*, since it hybridized to *B. vulgatus* DNA, but not to DNA from other strains of predominant gram-negative colon bacteria that we tested (other *Bacteroides* species and *F. prausnitzii*). These organisms account for 35 to 40% of all colon isolates that are cultivable by current bacteriological techniques (5, 8). Gram-positive bacteria, notably *Bifidobacterium*, *Eubacterium*, and *Peptostreptococcus* spp., are also present in high numbers in the colon, although it is unlikely that our probe would cross-hybridize with DNAs from these organisms. It is possible, however, that our probe might react with colon bacteria that have not yet been isolated and, consequently, about which nothing is known.

An identification method based on DNA-DNA hybridization is particularly useful when studying species, such as *Bacteroides* spp., that are difficult to distinguish by standard biochemical tests. In a classic study of human fecal flora, Moore and Holdeman (8) designated most of the *Bacteroides* isolates as subspecies of *B. fragilis* because of their phenotypic similarities. As a result of subsequent DNA homology studies (1, 6), however, these subspecies were elevated to species rank, and some organisms were reassigned to newly established species. For example, *B. uniformis* and *B. thetaiotaomicron*, two species that have been established as a result of DNA homology studies, include many strains that had been phenotypically identified as *B. fragilis* subsp. *thetaiotaomicron*. It is, therefore, unclear whether the *B. fragilis* subsp. *thetaiotaomicron* that accounted for 5 to 10% of the isolates in the Moore and Holdeman study were actually members of *B. thetaiotaomicron*, *B. uniformis*, or a combination of both (6). We are currently using our method to address this and similar questions.

The main limitation of our method is its sensitivity. DNA hybridization probes can be used to detect very small amounts of purified DNA. However, the segment of DNA that we are detecting is only a small portion of the bacterial chromosome, and the chromosome from one organism may make up only a small proportion of the total DNA in a complex mixture. Since nitrocellulose has a limited capacity for binding DNA, we can only expect to detect DNA from those organisms that account for a high enough proportion of a population that they will be well represented in the mixture of DNA that binds to the nitrocellulose. From the results we obtained with pure cultures (cf. Fig. 4) and with feces (cf. Table 3), we predict that this method can only be used to quantitate species that account for at least 2% of the total population, e.g., the 10 to 12 most numerous species in the human colon. It may be possible to improve sensitivity by further purification of DNA from fecal bacteria. However, such additional steps make the method more time consuming and may involve a decrease in the recovery of DNA. With pure cultures, we were able to trap 65 to 70% of the DNA on the filters. Since this amount is similar to the amount obtained with purified DNA, it may represent an upper limit of recovery. With fecal samples, it appears that we are trapping only 30% of the *B. vulgatus* DNA as indicated by comparing the amount of pBV-1 that hybridized to pure cultures of *B. vulgatus* to the amount that hybridized to a comparable number of *B. vulgatus* added to feces (cf. Table 3). This could be due to competition from DNA of other organisms or interference from non-DNA components in feces. If the latter is true, further DNA purification would yield at most a twofold increase in sensitivity. It may be possible to detect less numerous species by using solution hybridization. In this method, a single-stranded probe is allowed to hybridize to DNA in solution rather than on nitrocellulose paper. Unhybridized DNA can be removed by digestion with nucleases, and the double-stranded hybrids can then be recovered by precipitation.

A second limitation of our approach is that it is necessary to filter many different dilutions of bacterial DNA from feces to find concentrations at which hybridization with the probe increases linearly when increasing amounts of DNA are filtered and at which the addition of bacteria (the internal standard) to the fecal specimen before processing also gives a proportional increase in the amount of hybridization (i.e., concentrations at which the added bacteria do not exceed

the capacity of the nitrocellulose). This range of concentrations will be different for each species and, possibly, each stool sample.

A third limitation of our method is that it employs short-lived radioisotopes. Consequently, it was necessary to relabel our probe frequently and to correct the amounts of radioactivity measured for differences in the specific activity of the probe. Recent work on methods for labeling DNA without the use of radioisotopes encourages us to think that this limitation is not a serious one, and it should soon be possible to obtain stable, nonisotopic DNA probes to quantitate bacteria in complex mixtures.

The vector in which our *B. vulgatus*-specific DNA fragment was cloned is pBR322, a plasmid derived from the *Enterobacteriaceae*. Although the facultative bacteria that harbor pBR322-like plasmids represent only a small fraction of the microbial population in the human colon, the plasmids themselves can be present in high copy number. Since there can potentially be background hybridization to such plasmids in a fecal specimen, it is important to check each fecal specimen for hybridization with pBR322 as well as with the species-specific probe.

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