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Distribution of Bifidobacterial Species in Human Intestinal Microflora Examined with 16S rRNA-Gene-Targeted Species-Specific Primers

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In order to clarify the distribution of bifidobacterial species in the human intestinal tract, a 16S rRNA-gene-targeted species-specific PCR technique was developed and used with DNAs extracted from fecal samples obtained from 48 healthy adults and 27 breast-fed infants. To cover all of the bifidobacterial species that have been isolated from and identified in the human intestinal tract, species-specific primers for *Bifidobacterium longum*, *B. infantis*, *B. dentium*, and *B. gallicum* were developed and used with primers for *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, and the *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*) that were developed in a previous study (T. Matsuki, K. Watanabe, R. Tanaka, and H. Oyaizu, FEMS Microbiol. Lett. 167:113–121, 1998). The specificity of the nine primers was confirmed by PCR, and the species-specific PCR method was found to be a useful means for identifying *Bifidobacterium* strains isolated from human feces. The results of an examination of bifidobacterial species distribution showed that the *B. catenulatum* group was the most commonly found taxon (detected in 44 of 48 samples [92%]), followed by *B. longum* and *B. adolescentis*, in the adult intestinal bifidobacterial flora and that *B. breve*, *B. infantis*, and *B. longum* were frequently found in the intestinal tracts of infants. The present study demonstrated that qualitative detection of the bifidobacterial species present in human feces can be accomplished rapidly and accurately.

The human intestinal tract harbors a large, active, and complex community of microbes. The intestinal microflora plays several significant roles in the digestion of food, the metabolism of endogenous and exogenous compounds, the production of essential vitamins, immunopotentiation, and the prevention of colonization by pathogens in the gastrointestinal tract and hence is involved in maintaining human health (7, 8).

Members of the genus *Bifidobacterium* are some of the most common organisms in the human intestinal tract (26). It has been suggested that *Bifidobacterium* species are important in maintaining general health because they contribute to a beneficial microflora in the intestinal tract and that the diversity and number of *Bifidobacterium* species provide a marker for the stability of the human intestinal microflora (28). Therefore, many attempts have been made to increase the number of *Bifidobacterium* cells in the intestinal tract by supplying certain bifidobacterial strains and food ingredients that stimulate the growth of bifidobacteria as food additives (7, 8, 11, 15). Hence, the distribution of bifidobacteria in the human intestinal microflora is of major interest. Using classical culture methods, workers have found that *Bifidobacterium adolescentis* and *B. longum* are major bifidobacterial species in the adult intestinal microflora (4, 5, 17, 19, 20) and that *B. infantis* and *B. breve* are predominant species in the intestinal tracts of human infants (2, 3, 17, 20). In addition, *B. catenulatum*, *B. pseudocatenulatum*, *B. angulatum*, and *B. dentium* have been also reported to be human intestinal bifidobacteria (24, 25), and *B. gallicum* has been reported to be a rarely isolated species (14). However, the classical culture methods, including isolation, identification, and enumeration of these species, are labor-intensive and time-consuming. Moreover, identification based on phenotypic traits does not always provide clear-cut results and is sometimes unreliable.

For some years, 16S rRNA sequence comparison has attracted attention as a reliable method for classification and identification of several bacterial species (22, 31). 16S rRNA-targeted hybridization probes or PCR primers enable rapid and specific detection of a wide range of bacterial species, and procedures in which these probes and primers are used have become key procedures for detecting microorganisms (6, 10, 12, 23, 30, 32).

In order to develop an accurate and convenient method for characterization of bifidobacteria in the intestinal microflora, we prepared 16S rRNA-gene (rDNA)-targeted species-specific and group-specific primers for all known species of bifidobacteria that inhabit the human intestinal tract. In the present study, a species-specific PCR technique performed with fecal DNA was also used to investigate the distribution of bifidobacteria in the intestinal microflora of human adults and infants.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains listed in Table 1 were obtained from the American Type Culture Collection (Rockville, Md.), the Japan Collection of Microorganisms (Wako, Japan), the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the National Collection of Food Bacteria (Reading, United Kingdom), the National Collection of Type Cultures (London, United Kingdom), the National Institute of Biosciences and Human Technology (Tsukuba, Japan), and the Yakult Central Institute for Microbiological Research (Tokyo, Japan). Most of the strains were cultured anaerobically in GAM broth (Nissui Seiyaku, Tokyo, Japan) supplemented with 1% glucose at 37°C overnight; *Escherichia coli* was cultured aerobically in Trypticase soy broth (Difco, Detroit, Mich.) at 37°C overnight. Direct microscopic counts of pure cultured bifidobacteria were obtained by using duplicate smears of 0.01 ml of a 10^2-fold dilution spread over 1 cm^2 of a glass slide. The smears were heat fixed and gently Gram stained. Six edge fields and four center fields were counted, and the counts were then correlated with the actual sample size (9).

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TABLE 1. Bacterial strains and results of PCR assays in which species-specific primers BiLON, BiINF, BiDEN, and BiGAL were used[a]

Species	Strain(s)[b]	Species-specific primer PCR results[c]			
		BiLON	BiINF	BiDEN	BiGAL
B. adolescentis	ATCC 15703^T, NCFB 2229, NCFB 2230, NCFB 2231	−	−	−	−
B. angulatum	ATCC 27535^T, JCM 1252	−	−	−	−
B. bifidum	ATCC 29521^T, ATCC 15696, ATCC 11863, Yakult[d]	−	−	−	−
B. breve	ATCC 15700^T, ATCC 15698, ATCC 15701, Yakult[d]	−	−	−	−
B. catenulatum	ATCC 27539^T, JCM 7130	−	−	−	−
B. pseudocatenulatum	JCM 1200^T, DSM 20439	−	−	−	−
B. longum	ATCC 15707^T, ATCC 15708, FERM P-6548	+	−	−	−
B. infantis	ATCC 15697^T, ATCC 15702, ATCC 25962	−	+	−	−
B. suis	ATCC 27533^T	+	−	−	−
B. dentium	ATCC 27534^T, DSM 20084, DSM 20221	−	−	+	−
B. gallicum	JCM 8224^T	−	−	−	+

[a] In addition to the bacteria listed, negative PCR results with primers BiLON, BiINF, BiDEN, and BiGAL were obtained for the following bacterial species: *B. animalis* ATCC 25527^T, *B. asteroides* ATCC 25910^T, *B. boum* JCM 1211^T, *B. choerinum* ATCC 27686^T, *B. coryneforme* ATCC 25911^T, *B. cuniculi* ATCC 27916^T, *B. denticolens* DSM 10105^T, *B. gallinarum* JCM 6291^T, *B. indicum* ATCC 25912^T, *B. inopinatum* DSM 10107^T, *B. lactis* DSM 10140^T, *B. magnum* JCM 1218^T, *B. merycicum* JCM 8219^T, *B. minimum* ATCC 27538^T, *B. pseudolongum* subsp. *globosum* ATCC 25864^T, *B. pseudolongum* subsp. *pseudolongum* JCM 1205^T, *B. pullorum* JCM 1214^T, *B. ruminantium* JCM 8222^T, *B. saeculare* DSM 6531^T, *B. subtile* DSM 20096^T, *B. thermophilium* ATCC 25866^T, *E. coli* ATCC 11775^T, *Bacteroides fragilis* NCTC 9343^T, *Bacteroides ovatus* JCM 5824^T, *Bacteroides vulgatus* ATCC 8424^T, *Clostridium bifermentans* JCM 1386^T, *Clostridium perfringens* JCM 1290^T, *Enterococcus faecalis* ATCC 19433^T, *Enterococcus faecium* ATCC 19434^T, *Eubacterium aerofaciens* ATCC 25986^T, *Eubacterium biforme* ATCC 27806^T, *Gardnerella vaginalis* DSM 4944^T, *Lactobacillus acidophilus* ATCC 4356^T, *Propionibacterium acnes* ATCC 6919^T, *Peptostreptococcus prevotii* ATCC 9321^T, and *Ruminococcus productus* ATCC 27340^T.

[b] ATCC, American Type Culture Collection; JCM, Japan Collection of Microorganisms; DSM, German Collection of Microorganisms and Cell Cultures; NCFB, National Collection of Food Bacteria; NCTC, National Collection of Type Cultures; FERM, National Institute of Biosciences and Human Technology.

[c] The PCR specificities of other species-specific primers, such as BiADO, BiANG, BiBIF, BiBRE, and BiCATg, have been reported previously (16).

[d] These strains are used in a probiotic culture in dairy products and were obtained from Yakult Central Institute for Microbiological Research.

Development of 16S rDNA-targeted species-specific primers. Using 31 bifidobacterial 16S rDNA sequences whose accession numbers were described previously (16), we prepared a multiple alignment with the program Clustal W (29). Then potential primer target sites for species-specific detection were identified for all species except *B. catenulatum* and *B. pseudocatenulatum*, which were treated as the *B. catenulatum* group. We then designed eight species-specific primers and a group-specific primer for *Bifidobacterium* species that have been detected in human intestinal tracts (Table 2). These primers were synthesized commercially by Greiner Japan or Rikaken (Tokyo, Japan).

PCR amplification. Each PCR mixture (25 μl) was composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, each species-specific primer (Table 2) at a concentration of 0.25 μM, template DNA, and 0.9 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). The PCR was carried out with a Touchdown thermal cycler (Hybaid, Middlesex, United Kingdom). The following amplification program was used: one cycle consisting of 94°C for 5 min, followed by 35 cycles consisting of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s and finally one cycle consisting of 72°C for 5 min. The amplification products were subjected to gel electrophoresis in 1% agarose, followed by ethidium bromide staining.

Isolation and identification of *Bifidobacterium* strains. Isolation of the *Bifidobacterium* strains listed in Table 3 from human feces and identification based on DNA-DNA homology tests were carried out by using the methods described previously (16). Carbohydrate fermentation patterns were determined by using the API 50 CHL system (API, La Balme les Grottes, France).

Fecal sampling. The fecal samples used in this study were obtained from 51 healthy male adults ranging from 23 to 54 years old (mean, 38.8 ± 8.9 years) and 27 healthy breast-fed babies ranging from 22 to 46 days old (mean, 31.2 ± 4.5 days). The babies were born in Nagasaki University Hospital and had been

TABLE 2. *Bifidobacterium* species- and group-specific primers based on 16S rDNA sequences

Target human intestinal bifidobacterium	Primer[a]	Sequence	Length (bp)	Target site[b]	Product size (bp)
B. adolescentis	BiADO-1	CTCCAGTTGGATGCATGTC	19	182–200	279
	BiADO-2	CGAAGGCTTGCTCCCAGT	18	474–442	
B. angulatum	BiANG-1	CAGTCCATCGCATGGTGGT	19	185–203	275
	BiANG-2	GAAGGCTTGCTCCCCAAC	18	473–441	
B. bifidum	BiBIF-1	CCACATGATCGCATGTGATTG	21	184–204	278
	BiBIF-2	CCGAAGGCTTGCTCCCAAA	19	475–442	
B. breve	BiBRE-1	CCGGATGCTCCATCACAC	18	175–192	288
	BiBRE-2	ACAAAGTGCCTTGCTCCCT	19	475–444	
B. catenulatum group[c]	BiCATg-1	CGGATGCTCCGACTCCT	17	176–192	285
	BiCATg-2	CGAAGGCTTGCTCCCGAT	18	474–442	
B. longum	BiLON-1	TTCCAGTTGATCGCATGGTC	20	182–201	831
	BiLON-2	GGGAAGCCGTATCTCTACGA	20	1028–1008	
B. infantis	BiINF-1	TTCCAGTTGATCGCATGGTC	20	182–201	828
	BiINF–2	GGAAACCCCATCTCTGGGAT	20	1027–1007	
B. dentium	BiDEN-1	ATCCCGGGGGTTCGCCT	17	72–89	387
	BiDEN-2	GAAGGGCTTGCTCCCGA	17	473–443	
B. gallicum	BiGAL-1	TAATACCGGATGTTCCGCTC	20	170–189	303
	BiGAL-2	ACATCCCCGAAAGGACGC	18	479–454	

[a] The standardized primer names are as follows: BiADO-1, S-S-B.ado-0182-a-S-19; BiADO-2, S-S-B.ado-0442-a-A-18; BiANG-1, S-S-B.ang-0185-a-S-19; BiANG-2, S-S-B.ang-0441-a-A-18; BiBIF-1, S-S-B.bif-0184-a-S-21; BiBIF-2, S-S-B.bif-0442-a-A-19; BiBRE-1, S-S-B.bre-0175-a-S-18; BiBRE-2, S-S-B.bre-0444-a-A-19; BiCATg-1, S-S-B.cat-0176-a-S-17; BiCATg-2, S-S-B.cat-0442-a-A-18; BiLON-1 and BiINF-1, S-S-B.lon-0182-a-S-20; BiLON-2, S-S-B.lon-1008-a-A-20; BiINF-2, S-S-B.inf-1007-a-A-20; BiDEN-1, S-S-B.den-0072-a-S-17; BiDEN-2, S-S-B.den-0443-a-A-17; BiGAL-1, S-S-B.gal-0170-a-S-20; BiGAL-2, S-S-B.gal-0454-a-A-18 (1).

[b] The numbers correspond to numbers in the structure model of *E. coli* 16S rRNA (21).

[c] The *B. catenulatum* group consists of *B. catenulatum* and *B. pseudocatenulatum*.

TABLE 3. Identification of isolated *Bifidobacterium* strains with species-specific primers

Strains[a]	Reactions with species-specific primers[b]				Identity[c]
	BiGAL	BiDEN	BiLON	BiINF	
MC-10, MC-11, MC-12, MC-23, MC-24, MC-25, MC-26, MC-27, MC-28, MC-29, MC-30	−	−	+	−	*B. longum*
MC-8, MC-9	−	−	−	+	*B. infantis*

[a] The strains were isolated from human adult and infant feces.
[b] +, positive; −, negative. In addition to the results obtained with specific primers BiGAL, BiDEN, BiLON, and BiINF, negative PCR results were obtained with BiADO, BiANG, BiBIF, BiBRE, and BiCATg (16).
[c] Identification was based on the results of DNA-DNA homology tests and the arabinose and melezitose fermentation patterns.

delivered by the vaginal route. The wet weight of each fecal sample was 10 mg. Each sample was washed three times by suspending it in 1.5 ml of distilled water and centrifuging it at 15,000 rpm in order to reduce the amount of PCR inhibitors, and the pellets were then stored at −70°C until they were used for DNA preparation.

DNA extraction from fecal samples. DNA was extracted from fecal samples essentially by the methods of Zhu et al. (33). Briefly, a fecal sample was suspended in a solution containing 250 μl of extraction buffer (100 mM Tris-HCl, 40 mM EDTA; pH 9.0) and 50 μl of 10% sodium dodecyl sulfate and then subjected to freeze-thawing. Benzyl chloride (150 μl) was added to the suspension, and the mixture was vortexed vigorously at 50°C for 30 min by using a MicroIncubator M-36 apparatus (TAITECH, Tokyo, Japan). Then 150 μl of 3 M sodium acetate was added, and the mixture was cooled on ice for 15 min. After centrifugation at 15,000 × *g* for 10 min, the supernatant was collected, and DNA was obtained by isopropanol precipitation. Finally, the DNA was suspended in 100 μl of TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Contamination with PCR inhibitors was checked for by PCR amplification by using a mixture containing 10 ng of DNA extracted from *B. gallicum* JCM 8224^T, the specific BiGAL primers, and 1 μl of fecal DNA solution. When the extracted DNA was found to be contaminated by PCR-inhibiting substances, further purification was performed by using a MicroSpin S-400 column (Amersham Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturer. Routinely, 1 μl of a fecal DNA solution was used for PCR analysis.

Enumeration of the bifidobacterial population by classical culture methods. The population of *Bifidobacterium* species in fecal samples was enumerated as follows. Fecal samples from adults were collected anaerobically, and serial 10-fold dilutions were prepared with prereduced dilution buffer with vigorous shaking (9). Then, 0.05-ml samples of the 10^5 to 10^8 dilutions were plated onto *Bifidobacterium*-specific TOS agar (27). This medium contained (per liter) 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1 g of yeast extract (Difco), 3 g of KH_2PO_4, 4.8 g of K_2HPO_4, 3 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.5 g of L-cysteine, 10 g of TOS-S (Yakult Honsha Co., Tokyo, Japan), and 15 g of powdered agar (Difco). The plates were incubated anaerobically at 37°C for 3 days, and the colonies that appeared on the samples with the highest dilution were picked in succession into GAM broth (Nissui Seiyaku, Tokyo, Japan). The isolates were identified by using species-specific PCR primers. The total population level of each species was calculated by determining the number of CFU per gram of feces.

Statistical analysis. To determine the statistical significance of the results, Student's *t* test was used.

RESULTS

Specificity of primers. Figure 1 shows the electrophoresis patterns of the PCR products obtained for 10 *Bifidobacterium* species when their specific primers were used. The specificity of the primers was confirmed by PCR in which we used both chromosomal DNAs extracted from 50 different strains belonging to 31 *Bifidobacterium* species and DNAs extracted from 15 non-*Bifidobacterium* species which are commonly found in the human intestinal microflora (Table 1). The PCR specificity of other primers, such as BiADO, BiANG, BiBIF, BiBRE, and BiCATg, has been reported previously (16). Most of the primers detected the target species specifically; the only exceptions were the BiLON primers, which cross-reacted with *B. suis*.

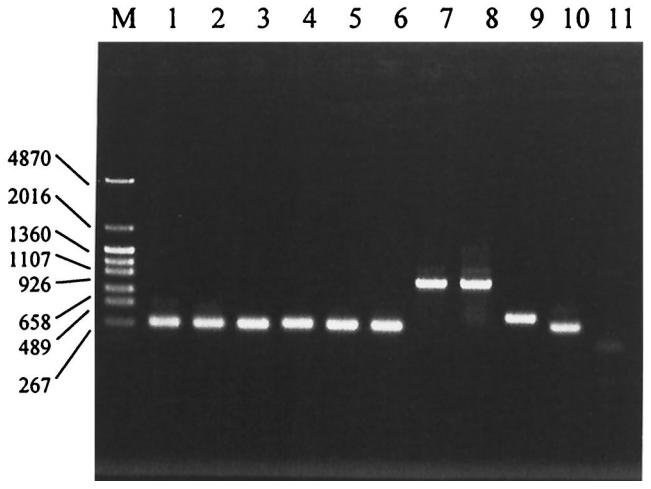

FIG. 1. PCR products obtained for 10 *Bifidobacterium* species with their specific primers. Lane M, DNA size markers (sizes [in bases] are indicated on the left); lane 1, *B. adolescentis* ATCC 15703^T; lane 2, *B. angulatum* ATCC 27535^T; lane 3, *B. bifidum* ATCC 29521^T; lane 4, *B. breve* ATCC 15700^T; lane 5, *B. catenulatum* ATCC 27539^T; lane 6, *B. pseudocatenulatum* JCM 1200^T; lane 7, *B. longum* ATCC 157071^T; lane 8, *B. infantis* ATCC 15697^T; lane 9, *B. dentium* ATCC 27534^T; lane 10, *B. gallicum* JCM 8224^T; lane 11, negative control (PCR performed with primer BiADO and *E. coli* ATCC 11775^T).

Identification of isolated *Bifidobacterium* strains. The species-specific PCR technique was used to identify *Bifidobacterium* strains isolated from human feces. As shown in Table 3, 13 isolates were clearly identified as 11 strains of *B. longum* (MC-10, MC-11, MC-12, MC-23, MC-24, MC-25, MC-26, MC-27, MC-28, MC-29, and MC-30) and 2 strains of *B. infantis* (MC-8 and MC-9) by using the newly developed BiLON and BiINF primers. Using DNA-DNA hybridization tests, we identified these strains as members of *B. longum* or *B. infantis*, but it was difficult to distinguish the two species because the levels of homology of each isolate to the reference strains (*B. longum*

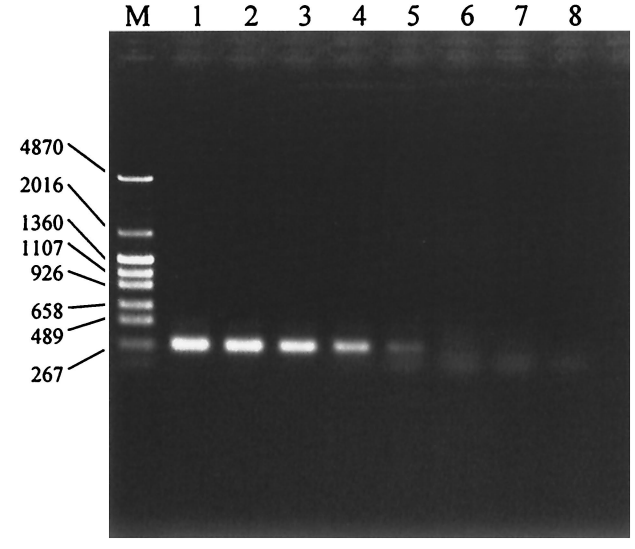

FIG. 2. Detection limits of the species-specific PCR methods, as determined by using DNAs extracted from 10-mg fecal samples mixed with various amounts of *B. angulatum* ATCC 27535^T. Lane M, DNA size markers (sizes [in bases] are indicated on the left); lane 1, 10^6 cells per PCR mixture; lane 2, 10^5 cells; lane 3, 10^4 cells; lane 4, 10^3 cells; lane 5, 10^2 cells; lane 6, 10 cells; lane 7, 1 cell; lane 8, no cells (negative control).

TABLE 4. Distribution of *Bifidobacterium* species in human adult feces

Sample	Reactions with species-specific primers[a]								
	BiADO	BiANG	BiBIF	BiBRE	BiCATg	BiLON	BiINF	BiDEN	BiGAL
AD-1	+	−	−	−	+	−	−	+	−
AD-2	−	−	−	−	−	−	−	−	−
AD-3	+	−	−	−	+	+	−	+	−
AD-4	−	−	+	+	+	+	−	−	−
AD-5	+	−	−	−	+	−	−	−	−
AD-6	+	−	−	−	+	−	−	−	−
AD-7	−	−	−	−	+	+	−	−	−
AD-8	−	−	−	−	+	−	−	−	−
AD-9	+	−	+	−	+	+	−	−	−
AD-10	+	−	+	−	+	+	−	−	−
AD-11	+	−	+	−	+	+	−	−	−
AD-12	−	−	−	−	+	−	−	−	−
AD-13	+	−	−	−	+	+	−	−	−
AD-14	−	−	−	−	−	+	−	−	−
AD-15	−	−	−	−	+	+	−	−	−
AD-16	+	+	+	+	+	+	−	−	−
AD-17	−	−	+	−	+	+	−	−	−
AD-18	−	+	−	−	−	+	−	−	−
AD-19	−	−	−	−	+	+	−	−	−
AD-20	−	−	+	−	+	+	−	−	−
AD-21	+	−	+	−	+	+	−	−	−
AD-22	−	−	−	−	+	+	−	−	−
AD-23	+	−	−	−	+	+	−	−	−
AD-24	+	−	+	+	+	−	−	−	−
AD-25	+	−	+	−	+	+	−	−	−
AD-26	+	−	−	−	+	+	−	−	−
AD-27	+	−	+	−	+	+	−	−	−
AD-28	+	−	−	+	+	+	−	−	−
AD-29	+	−	−	−	−	−	−	−	−
AD-30	+	−	+	−	+	+	−	−	−
AD-31	−	−	+	+	+	+	−	−	−
AD-32	−	−	−	−	+	−	−	−	−
AD-33	+	−	+	−	+	−	−	−	−
AD-34	−	−	−	−	+	−	−	−	−
AD-35	+	−	−	−	+	+	−	−	−
AD-36	+	−	−	−	+	+	−	−	−
AD-37	+	−	−	−	+	+	−	−	−
AD-38	+	−	+	−	+	+	−	−	−
AD-39	+	−	−	−	+	−	−	−	−
AD-40	−	−	−	−	+	−	−	−	−
AD-41	+	−	+	−	+	−	−	−	−
AD-42	−	−	−	−	+	−	−	−	−
AD-43	+	−	−	−	+	+	−	+	−
AD-44	+	−	+	−	+	+	−	−	−
AD-45	+	−	−	−	+	+	−	−	−
AD-46	+	−	−	−	+	+	−	−	−
AD-47	−	−	+	+	+	−	−	−	−
AD-48	−	−	−	−	+	+	−	−	−
No. of positive samples (%)	29 (60)	2 (4.2)	18 (38)	6 (13)	44 (92)	31 (65)	0 (0)	3 (6.3)	0 (0)

[a] +, positive; −, negative. Primers BiADO, BiANG, BiBIF, BiBRE, BiCATg, BiLON, BiINF, BiDEN, and BiGAL are specific for *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, the *B. catenulatum* group, *B. longum*, *B. infantis*, *B. dentium*, and *B. gallicum*, respectively.

ATCC 15707^T and *B. infantis* ATCC 15697^T) were similar, ranging from 60 to 95%. However, identification of these strains was possible because all of the *B. longum* strains fermented arabinose and melezitose, whereas no *B. infantis* strain fermented these sugars (26). Thus, identification of these strains based on DNA-DNA homology data and carbohydrate fermentation patterns gave the same results as identification by the species-specific PCR technique.

DNA preparation. The benzyl chloride extraction method provided sufficient amounts of DNA to perform PCR amplification for both pure cultures of bifidobacteria and fecal samples. The washing steps effectively removed the PCR inhibitors from all 27 infant fecal samples and 36 of 51 adult fecal samples. However, the DNA extracted from 15 samples was still contaminated by PCR-inhibiting substances even after the washing steps. Therefore, further purification was performed with the MicroSpin S-400 column, and the PCR inhibitors were removed from 12 of the 15 samples. Finally, the DNAs extracted from 48 adult and 27 infant fecal samples were subjected to the distribution analysis described below.

Detection limits of the species-specific PCR methods. In order to determine the detection limit of the species-specific PCR, approximately 10 mg (wet weight) of a fecal sample that did not contain *B. angulatum* was mixed with various amounts

TABLE 5. Distribution of *Bifidobacterium* species in human infant feces

Sample	Reactions with species-specific primers[a]								
	BiADO	BiANG	BiBIF	BiBRE	BiCATg	BiLON	BiINF	BiDEN	BiGAL
INF-1	−	−	−	+	−	−	−	−	−
INF-2	−	−	+	−	−	−	−	−	−
INF-3	−	−	+	+	−	+	+	−	−
INF-4	−	−	−	+	−	−	+	+	−
INF-5	−	−	−	−	−	−	−	−	−
INF-6	−	−	−	+	−	−	+	+	−
INF-7	−	−	−	+	−	+	+	−	−
INF-8	−	−	−	−	−	+	−	−	−
INF-9	−	−	−	+	−	+	−	−	−
INF-10	−	−	−	−	−	−	−	−	−
INF-11	−	−	−	+	−	−	−	−	−
INF-12	−	−	−	+	+	−	+	−	−
INF-13	+	−	+	−	−	−	−	−	−
INF-14	−	−	−	+	+	−	−	−	−
INF-15	−	−	−	+	−	+	−	−	−
INF-16	−	−	−	+	+	−	−	−	−
INF-17	−	−	−	−	−	−	−	−	−
INF-18	−	−	−	+	−	−	−	−	−
INF-19	−	−	+	+	+	−	+	−	−
INF-20	−	−	−	−	−	+	−	−	−
INF-21	−	−	+	+	+	+	+	−	−
INF-22	−	−	−	−	−	−	+	−	−
INF-23	−	−	−	+	−	+	+	−	−
INF-24	−	−	−	+	−	−	−	−	−
INF-25	−	−	−	+	−	−	−	−	−
INF-26	−	−	−	+	−	+	+	−	−
INF-27	+	+	+	+	−	+	+	+	−
No. of positive samples (%)	2 (7.4)	1 (3.7)	6 (22)	19 (70)	5 (19)	10 (37)	11 (41)	3 (11)	0 (0)

[a] +, positive; −, negative. Primers BiADO, BiANG, BiBIF, BiBRE, BiCATg, BiLON, BiINF, BiDEN, and BiGAL are specific for *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, the *B. catenulatum* group, *B. longum*, *B. infantis*, *B. dentium*, and *B. gallicum*, respectively.

of *B. angulatum* ATCC 27535^T cells (10^8 to 10^2 cells per 10 mg), and DNAs were isolated from these mixtures. Figure 2 shows that the target species was detected by this procedure at a concentration of 10^2 cells per PCR assay mixture (equivalent to 10^4 cells per 10 mg of feces or 10^6 cells per g of feces). The same results were obtained when diluted samples of the DNA extracted from 10^9 cells of the *B. angulatum* strain were used as the template DNA (data not shown). As other *Bifidobacterium* species are usually present in human fecal samples, the detection limit was examined by using diluted DNA extracted from 10^9 cells of pure cultured bifidobacteria. The results obtained for *B. adolescentis* ATCC 15703^T and NCFB 2229, *B. bifidum* ATCC 29521^T, *B. breve* ATCC 15700^T, *B. catenulatum* ATCC 27539^T, *B. pseudocatenulatum* JCM 1200^T, and *B. dentium* ATCC 27534^T were the same as the results obtained for *B. angulatum* (data not shown). On the other hand, *B. longum* ATCC 157071^T, *B. infantis* ATCC 15697^T, and *B. gallicum* ATCC 15697^T were detected when they were present at a concentration of 10^3 cells per PCR mixture (data not shown).

Distribution of *Bifidobacterium* species in the feces of healthy adults and infants. The distributions of bifidobacterial species in 48 healthy adults and 27 breast-fed infants are shown in Tables 4 and 5, respectively. Table 6 summarizes the bifidobacterial species found, their frequencies, and the numbers of species detected in individuals. In adult intestinal tracts, the *B. catenulatum* group was the most common taxon (detected in 44 samples [92%]), followed by *B. longum* (31 samples [65%]) and *B. adolescentis* (29 samples [60%]). *B. bifidum* (18 samples [38%]) and *B. breve* (6 samples [13%]) were subdominant species. *B. dentium* (three samples [6.3%]) and *B. angulatum*

TABLE 6. Distribution of *Bifidobacterium* species in human adults and infants[a]

Taxon or combination	No. of positive samples (% of total)	
	Adults (n = 48)	Infants (n = 27)
Species		
B. adolescentis	29 (60)	2 (7.4)
B. angulatum	2 (4.2)	1 (3.7)
B. bifidum	18 (38)	6 (22)
B. breve	6 (13)	19 (70)
B. catenulatum group	44 (92)	5 (19)
B. longum	31 (65)	10 (37)
B. infantis	0 (0)	11 (41)
B. dentium	3 (6.3)	3 (11)
B. gallicum	0 (0)	0 (0)
Combinations of:		
One species	8 (17)	9 (33)
Two species	9 (19)	5 (19)
Three species	14 (29)	6 (22)
Four species	15 (31)	2 (7.4)
Five species	0 (0)	1 (3.7)
Six species	1 (2.1)	0 (0)
Seven species	0 (0)	1 (3.7)
No bifidobacteria	1 (2.1)	3 (11)

[a] The adults were 38.8 ± 8.9 years old (mean ± standard deviation), and the infants were 31.2 ± 4.5 days old. The number of species detected per sample was 2.8 ± 1.2 (mean ± standard deviation) for the adults and 2.1 ± 1.6 for the infants.

TABLE 7. Comparison of the species-specific primer method with the classical culture method when the same samples were used

Sample	Taxon	Results of:	
		PCR method[a]	Culture method (log CFU/g)
AD-1	*B. adolescentis*	+	9.5
	B. catenulatum group	+	8.9
	B. dentium	+	ND[b]
AD-2[c]	*Bifidobacterium* species	−	ND
AD-3	*B. adolescentis*	+	9.4
	B. catenulatum group	+	9.6
	B. longum	+	8.3
	B. dentium	+	ND
AD-4	*B. bifidum*	+	9.8
	B. breve	+	ND
	B. catenulatum group	+	10.0
	B. longum	+	ND
AD-5	*B. adolescentis*	+	ND
	B. catenulatum group	+	9.4
AD-6	*B. adolescentis*	+	ND
	B. catenulatum group	+	8.6
AD-7	*B. catenulatum* group	+	8.9
	B. longum	+	ND
AD-8	*B. catenulatum* group	+	10.2
AD-9	*B. adolescentis*	+	ND
	B. bifidum	+	8.9
	B. catenulatum group	+	9.8
	B. longum	+	ND
AD-10	*B. adolescentis*	+	ND
	B. bifidum	+	9.6
	B. catenulatum group	+	ND
	B. longum	+	10.3

[a] +, positive; −, negative.
[b] ND, not detected.
[c] No *Bifidobacterium* species was detected by either the PCR method or the culture method.

(two samples [4.2%]) were minor species. No *B. infantis* or *B. gallicum* was detected in this study. In breast-fed infants, *B. breve* (19 samples [70%]) was the most frequently found species, followed by *B. infantis* (11 samples [41%]) and *B. longum* (10 samples [37%]). *B. bifidum* (six samples [22%]), the *B. catenulatum* group (five samples [19%]), and *B. dentium* (three samples [11%]) were detected occasionally. *B. adolescentis* (two samples [7.4%]) and *B. angulatum* (one sample [3.7%]) were rare species in infants. *B. gallicum* was not detected. In one adult and three infant fecal samples, no bifidobacterial species were detected. The average numbers of species detected per individual were 2.8 ± 1.2 in adults and 2.1 ± 1.6 in infants. The intestinal *Bifidobacterium* flora of adults was more complex and diverse than that of infants ($P < 0.05$).

Comparison of the species-specific primer method with the culture method. The bifidobacterial composition obtained with the new primer method was compared with results obtained with the classical culture method (Table 7). All of the species isolated and identified by the culture method were also detected by the species-specific PCR technique. It should be noted that there were some species that were detected by the PCR method but not by the culture method. This indicates that the PCR method is able to detect a wider range of species than the culture method.

DISCUSSION

In this study, we developed molecular methods to investigate the distribution of bifidobacterial species in human intestinal tracts by using DNAs extracted from fecal samples. We have previously described five different species- and group-specific primers for *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, and the *B. catenulatum* group (16). In addition to these primers, 16S rDNA-targeted species-specific primers for *B. longum*, *B. infantis*, *B. dentium*, and *B. gallicum* were developed and used in this study. The nine different pairs of primers cover all of the bifidobacterial species that have been isolated and identified in the human intestinal tract, and therefore they provide an effective way to analyze *Bifidobacterium* species that inhabit the human intestinal tract. Although the BiLON primers did not distinguish *B. longum* and *B. suis*, we believe that *B. suis* should be taxonomically combined with *B. longum*, because the two species are closely related based on a DNA-DNA homology value of 75 to 78% (13) and on the level of 16S rDNA similarity (more than 99%) (18). We also believe that *B. catenulatum* and *B. pseudocatenulatum* should be treated as the *B. catenulatum* group due to their similarity in the DNA-DNA homology test, murein type, and 16S rDNA sequences, as discussed previously (16). The newly developed BiLON and BiINF primers distinguished *B. longum* and *B. infantis*, even though these taxa are closely related species (13, 18). These primers are effective for identification of the two species, as confirmed with isolated bifidobacteria (Table 3).

The benzyl chloride extraction used in this study was very simple and provided sufficient amounts of DNA for PCR amplification. It has been reported that PCR-based analysis of fecal samples is difficult to perform due to the presence of multiple inhibitors of the polymerase enzyme reaction (23). In the present study, the washing steps and purification with the MicroSpin S-400 column were effective in reducing the amounts of PCR inhibitors found in the fecal DNA solutions for most of the samples. However, additional improvements in the preparation procedures for fecal DNA are required, since 3 of 51 samples still contained inhibitors. Targeted *Bifidobacterium* species were detected when they were present at a concentration of at least 10^2 or 10^3 cells per PCR mixture, indicating that the detection limit for the procedures used was 10^6 or 10^7 cells per g of feces. In contrast, the sensitivity of the analysis based on the conventional culture method in which *Bifidobacterium*-specific selective medium is used is limited because it is difficult to detect minor species from a cultivated plate among the numerous predominant species. As the predominant *Bifidobacterium* species are usually present at a level of 10^9 to 10^{10} cells per g of human feces (2, 5, 17, 19, 20), the detection limit of the culture method for minor bifidobacterial species is about 10^8 cells per g. Therefore, the differences between the PCR method and the culture method shown in Table 7 account for the different detection limits. The present species-specific PCR detection method for bifidobacteria is about 10 to 100 times more sensitive than the classical culture method.

Examination of the bifidobacterial species distribution in the human intestinal tract revealed that the *B. catenulatum* group is the most common taxon inhabiting the human adult intestinal tract. This is a notable finding because it has frequently been reported that *B. adolescentis* is the most common species (5, 17, 19, 20). The difference may be due to the use of different identification techniques. It has been reported that it is difficult to differentiate *B. adolescentis*, *B. catenulatum*, and *B. pseudocatenulatum* based on the usual carbohydrate fermentation pattern (24, 25). Therefore, the *B. catenulatum* group may have been confused with *B. adolescentis* in some studies. On the other hand, some previous studies showed that *B. catenulatum* and *B. pseudocatenulatum* are members of the human adult intestinal microflora (4, 24, 25), but the frequencies in these studies

were not as high as the frequencies in our study. The difference may be due to the difference in detection limits between the conventional culture method and the 16S rDNA-targeted PCR technique or to regional differences in microfloras. *B. infantis* has a unique host specificity, even though this species is closely related to *B. longum*, as indicated by DNA-DNA homology and 16S rDNA sequence similarity (13, 18). It is interesting that *B. breve* was detected in adult fecal samples even though it has been recognized as a typical infantile bifidobacterial species (4, 5, 17, 19, 20). This may have been due to the difference in the detection limits of the techniques used, as described above. *B. gallicum* was not detected in this study, suggesting that *B. gallicum* should not be recognized as a member of the human intestinal microflora because the type strain is the only strain that has been isolated from human feces so far (14).

In the present study, the distributions of bifidobacterial species were basically consistent with the results obtained by the classical culture methods (2–5, 17, 19, 20) except for the *B. catenulatum* group, suggesting that the species-specific PCR method is a reliable technique for investigating intestinal floral components. For further investigation, an improved quantitative PCR method is necessary. In the near future, the quantitative PCR method combined with the species-specific primers for intestinal floral components is expected to lead to new opportunities for noncultivation studies of intestinal microflora.

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