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Species-Specific Oligonucleotide Probes for Five *Bifidobacterium* Species Detected in Human Intestinal Microflora

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Portions of the 16S rRNA from closely related species of the genus *Bifidobacterium* that are found in the human intestinal microflora were sequenced in order to design species-specific oligonucleotide probes. Five oligonucleotide probes ranging from 16 to 19 bases in length and complementary to 16S rRNA sequences from *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* were synthesized. With crude high-molecular-weight RNA preparations as targets, these probes showed the desired species specificity, even down to a 1-nucleotide difference. For the practical evaluation of these probes, their specificity and sensitivity were tested against seven strains of the same species and 54 strains of heterologous bacteria with fixed whole cells as targets. The probes for *B. adolescentis*, *B. breve*, and *B. longum* showed efficient and specific hybridization. Although the probes for *B. bifidum* and *B. infantis* cross-reacted with a few bacterial strains not isolated from humans, these probes showed species specificity for human intestinal bacteria. These 16S rRNA probes should prove valuable for the identification and detection of human intestinal *Bifidobacterium* species.

Bifidobacteria are major constituents of the human intestinal microflora and are of considerable interest in studies on the relationship between the intestinal microflora and the host's health. It would be of major interest to develop rapid and simple assays for the characterization of bacterial components of the microflora. To date, differentiation of *Bifidobacterium* spp. has mainly been performed on the basis of phenotypic characteristics, such as carbohydrate fermentation patterns and cellular morphology (9). More recently, a molecular approach based on DNA-DNA hybridization has been used (4).

Bacterial 16S rRNA sequences are prime targets for molecular diagnostic assays because of their high copy number and their well-conserved gene structures. The method for determining 16S rRNA sequences has been facilitated by the use of reverse transcriptase and cDNA sequence analysis (3). These 16S rRNA sequences have proven important in phylogenetic studies (10) and also in the identification of specific microorganisms (2, 5, 7, 11). Additionally, a simple in situ hybridization technique with fixed cells that are permeable to short oligonucleotides has been developed (1).

In this study, we have synthesized DNA probes specific for five *Bifidobacterium* species which are often detected in human feces. The specificity of our probes for *Bifidobacterium adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum* was tested with both extracted bacterial RNAs and fixed whole cells. These probes show promise for use in rapid clinical diagnostic tests for the detection of bifidobacteria.

Crude high-molecular-weight RNAs were extracted from eight different *Bifidobacterium* strains in order to sequence portions of their 16S rRNA. Cells were cultivated overnight in 100 ml of GAM broth (Nissui Seiyaku) supplemented with 1% glucose. After incubation, the cells were washed twice with washing solution (50 mM Tris [pH 7.4], 1 mM EDTA) and suspended in 7.3 ml of washing solution. Next, 2.3 ml of 2 M sucrose, 0.33 ml of 0.5-mg/ml *N*-acetylmuramidase (Seikagaku Kogyo) and 0.07 ml of 50-mg/ml lysozyme were added. The suspension was then incubated for 30 min at 37°C. After the incubation, 925 μl of EDTA solution (250 mM EDTA, 50 mM Tris [pH 8.0]) and 575 μl of sodium dodecyl sulfate (SDS) solution (20% SDS, 50 mM Tris [pH 8.0], 20 mM EDTA) were added and further incubated at 60°C until the mixture became clear. To remove proteins, extraction with TE (10 mM Tris [pH 8.0], 1 mM EDTA)-saturated phenol was performed three times. The aqueous phase was then extracted with an equal volume of chloroform-isoamyl alcohol (24:1). Nucleic acids were ethanol precipitated twice, and the precipitate was dried in a centrifugal evaporator. Crude high-molecular-weight RNAs were isolated by the method of Wilson et al. (11). The RNA was dissolved in TE at a concentration of 2.0 mg/ml or stored in 70% ethanol at −70°C.

The 16S rRNA was sequenced by using reverse transcriptase (RNA sequencing kit; Boehringer Mannheim) under the conditions suggested by the supplier, with some modifications. The universal 16S rRNA primers A [G(A/T)ATTACCGCGGC(G/T)GCTG] and C [ACGGGCGGTGT GT(A/G)C], as described by Lane et al. (3), were used for sequencing. These primers corresponded to the universal sites at positions 519 to 536 and 1392 to 1406, respectively, of the *Escherichia coli* 16S rRNA (6). These regions were selected because they are highly conserved in all bacteria.

TABLE 1. Probes synthesized and their nomenclature

Probe	Sequence	Target strain
PPR	G(A/T)ATTACCGCGGC(G/T)GCTG	Positive control
PAD	GCTCCCAGTCAAAAGCG	*B. adolescentis*
PBI	GCAGGCTCCGATCCGA	*B. bifidum*
PBR	AAGGTACACTCAACACA	*B. breve*
PIN	TCACGCTTGCTCCCCGATA	*B. infantis*
PLO	TCTCGCTTGCTCCCCGATA	*B. longum*

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		428	498
B. adolescentis	ATCC 15703	GUAAACCGCUUUUGACUGGGAGCAACCUCGG	GGUGAGUGUACCUUUCGAAUA
B. angulatum	ATCC 27535	GUAAACCGCUUUUGUUGGGGAGCAAGCUUCG	GGUGAGUGUACCUUUCGAAUA
B. bifidum	ATCC 29521	GUAAACCUCUUUUGUUUGGGAGCAAGCUUCG	GGUGAGUGUACCUUUCGAAUA
B. breve	ATCC 15700	GUAAACCUCUUUUGUUAGGGAGCAAGGCACUUUGU	GUUGAGUGUACCUUUCGAAUA
B. dentium	ATCC 27534	GUAAACCGCUUUUGAUCGGGAGCAACCUCGG	GGUGAGUGUACCCU-CGAAUA
B. infantis	ATCC 15697	GUAAACCUCUUUUAUCGGGGAGCAAGCGUG	AGUGAGUUUACCCGUUGAAUA
B. longum	ATCC 15707	GUAAACCUCUUUUAUCGGGGAGCAAGCGAG	AGUGAGUUUACCCGUUGAAUA
B. pseudolongum	ATCC 25526	GUAAACCGCUUUUGUUCAAGGGCAACACGGUUUCGGCCGUGUUGAGUGGAUUGUUCGAAUA	

		1298	1323
B. adolescentis	ATCC 15703	CAGUUCGGAUUGGAGUCUGCAACCCG	
B. angulatum	ATCC 27535	CAGUUCGGAUUGGAGUCUGCAACUCG	
B. bifidum	ATCC 29521	CAGUUCGGAUCGGAGCCUGCAACCCG	
B. breve	ATCC 15700	CAGUUCGGAUCGCAGUCUGCAACUCG	
B. dentium	ATCC 27534	CAGUUCGGAUUGGAGUCUGCAACCCG	
B. infantis	ATCC 15697	CAGUUCGGAUCGCAGUCUGCAACUCG	
B. longum	ATCC 15707	CAGUUCGGAUCGCAGUCUGCAACUCG	

FIG. 1. Partial 16S rRNA sequences of various *Bifidobacterium* species. Numbering corresponds to the structure model of *E. coli* 16S rRNA (6). The target regions for species-specific oligonucleotide probes are underlined. Universal primers A and C were used for sequencing from positions 428 to 498 and 1298 to 1323, respectively. The sequence of *B. pseudolongum* from positions 1298 to 1323 was not determined.

These probes and the oligonucleotide probes described below were synthesized with a DNA synthesizer (Applied Biosystems model 380B). The annealing mixture was prepared by combining 4.0 μl of RNA solution (2.0 mg/ml), 1.5 μl of primer (20 μg/ml), and 1.5 μl of distilled water. The reaction mixture was incubated at 42°C for 15 min, and 1 μl of chase mixture (a 1 mM concentration of each deoxynucleoside triphosphate) was added. The mixture was incubated at 42°C for an additional 15 min. The mixtures were then electrophoresed through a denaturing 8 M urea–6% polyacrylamide gel.

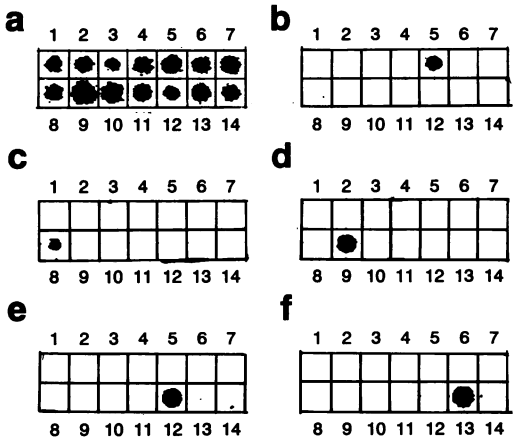

FIG. 2. Dot blot hybridization between crude rRNA and species-specific probes. A total of 20 ng of rRNA was blotted and hybridized to the appropriate probe. The hybridization (nonstringent) and final washing (stringent) temperatures, respectively, used for each probe are: (a) probe PPR, 55 and 65°C; (b) probe PAD, 50 and 60°C; (c) probe PBI, 55 and 65°C; (d) probe PBR, 40 and 50°C; (e) probe PIN, 55 and 70°C; (f) probe PLO, 55 and 70°C. The dot-blotted RNAs (squares 1 through 14, respectively) were from *Escherichia coli* ATCC 11775, *Actinomyces israelii* ATCC 10048, *Eubacterium aerofaciens* ATCC 25986, *Peptostreptococcus productus* ATCC 27340, *B. adolescentis* ATCC 15703, *B. angulatum* ATCC 27535, *B. animalis* ATCC 25527, *B. bifidum* ATCC 29521, *B. breve* ATCC 15700, *B. dentium* ATCC 27534, *B. globosum* ATCC 25864, *B. infantis* ATCC 15697, *B. longum* ATCC 15707, and *B. pseudolongum* ATCC 25526.

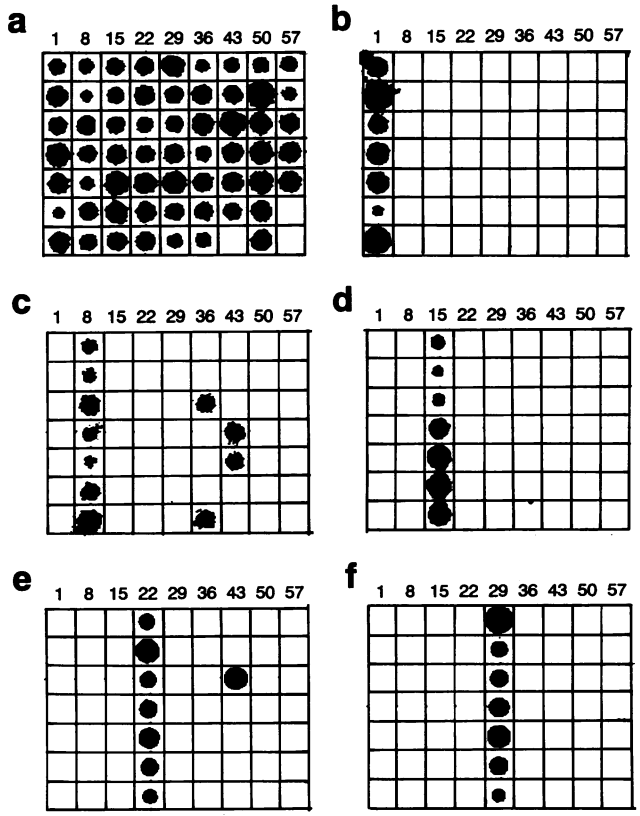

FIG. 3. Dot blot hybridization between fixed cells and probes PPR (a), PAD (b), PBI (c), PBR (d), PIN (e), and PLO (f). Approximately 10^6 fixed cells were dot blotted. The hybridization and washing temperatures for each probe were the same as described in the legend to Fig. 2. For each panel, each square from top to bottom and from left to right contained RNA from each of the strains listed in Table 2 in that order (e.g., in column 1, row 1 is *B. adolescentis* ATCC 15703, row 2 is *B. adolescentis* NCDO 2229, etc.).

TABLE 2. Microorganisms used in this study[a]

No.	Species	Strain no.	No.	Species	Strain no.
1	*Bifidobacterium adolescentis*	ATCC 15703	32	*Bifidobacterium longum*	YMW 7158
2		NCDO 2229	33		YMW 1412
3		NCDO 2230	34		YMW 1512
4		NCDO 2231	35		YMW 2512
5		YMW 727	36	*Bifidobacterium angulatum*	ATCC 27535
6		YMW 1114	37	*Bifidobacterium animalis*	ATCC 25527
7		YMW 1419	38	*Bifidobacterium asteroides*	ATCC 25910
8	*Bifidobacterium bifidum*	ATCC 15696	39	*Bifidobacterium catenulatum*	ATCC 27539
9		ATCC 29521	40	*Bifidobacterium dentium*	ATCC 27534
10		ATCC 11863	41	*Bifidobacterium globosum*	ATCC 25864
11		YMW 511	42	*Bifidobacterium indicum*	ATCC 25912
12		YMW 718	43	*Bifidobacterium pseudocatenulatum*	JCM 1200
13		YMW 164	44	*Bifidobacterium pseudolongum*	ATCC 25526
14		YIT 4007	45	*Bifidobacterium suis*	ATCC 27533
15	*Bifidobacterium breve*	ATCC 15700	46	*Bifidobacterium thermophilum*	ATCC 25525
16		ATCC 15698	47		ATCC 25866
17		YMW 92	48	*Escherichia coli*	ATCC 11775
18		YMW 116	49	*Actinomyces israelii*	ATCC 10048
19		YMW 1333	50	*Bacteroides ovatus*	ATCC 8483
20		YIT 4010	51	*Bacteroides vulgatus*	ATCC 8424
21		YIT 4065	52	*Enterococcus faecalis*	YIT 2031
22	*Bifidobacterium infantis*	ATCC 15697	53	*Enterococcus faecium*	YIT 2004
23		ATCC 15702	54	*Eubacterium aerofaciens*	ATCC 25986
24		ATCC 25962	55	*Eubacterium biforme*	VPI 9218
25		YMW 1610	56	*Lactobacillus acidophilus*	YIT 0070
26		YMW 1737	57	*Lactobacillus casei*	YIT 0078
27		YMW 1695	58	*Peptostreptococcus prevotii*	ATCC 9321
28		YMW 1615	59	*Peptostreptococcus productus*	ATCC 27340
29	*Bifidobacterium longum*	ATCC 15707	60	*Streptococcus lactis*	YIT 2027
30		ATCC 15708	61	*Streptococcus thermophilus*	YIT 2037
31		FERM P6548			

[a] ATCC, American Type Culture Collection; FERM, Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tukuba, Japan; JCM, Japan Collection of Microorganism; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; VPI, Virginia Polytechnic Institute and State University; YIT, Yakult Institute, Tokyo, Japan; YMW, wild-type strains isolated from human feces. Numbers are used to identify strains in Fig. 3.

We were able to obtain approximately 300 nucleotides of sequence information from each of the eight *Bifidobacterium* strains. After comparing these sequences, we synthesized five probes corresponding to unique sites within the 16S rRNAs of different strains (Table 1). The strains and the hybridization sites of the DNA probes used in these studies are shown in Fig. 1.

In order to test the specificity of these probes, we first performed hybridization to crude high-molecular-weight RNAs (Fig. 2). Oligonucleotide probes were 5′-end labeled with ^{32}P by using Megalabel (Takara Shuzo). Denatured RNA (20 ng) was applied to a nylon membrane (GeneScreen Plus; Du Pont) with a microfiltration apparatus (Bio-Dot; Bio-Rad) as described previously (8). The membrane was baked for 30 min at 80°C to remove formaldehyde and then sealed in a polyethylene bag after prehybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's solution, 1% SDS, 0.1 mg of salmon sperm DNA per ml, 10% dextran sulfate; 0.125 ml/cm^2) was added. Prehybridization was performed for 1 h under nonstringent conditions. After the prehybridization solution was replaced by hybridization solution (6× SSC, 2× Denhardt's solution, 1% SDS, 10% dextran sulfate, 10 ng of labeled probes per ml; 0.125 ml/cm^2), incubation was continued for 2 h. The membrane was removed and washed three times with 6× SSC at room temperature for 10 min. The membrane was then washed under stringent conditions for 30 min as described in the legend to Fig. 2. Autoradiograms were obtained by using a Betascope 603 blot analyzer (Betagen Corp.).

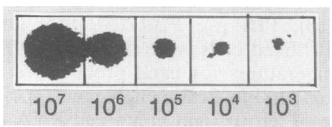

FIG. 4. Sensitivity of detection. Approximately 10^3 to 10^7 fixed cells of *B. longum* ATCC 15707 were dot blotted and hybridized to probe PLO under the conditions described in the legend to Fig. 2.

Probe PPR is the universal primer A that was used for sequencing and provided a positive control for the presence of detectable target sequences. Figure 2a is a dot blot showing hybridization of PPR to RNA from all strains examined. Probes PAD, PBI, PBR, PIN, and PLO were made to detect *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*, respectively. Figures 2b, c, d, e, and f show that these probes hybridized specifically to RNA from the target strains but not from other strains. Although there is only one different base between *B. infantis* and *B. longum* at the target site for their respective probes, PIN and PLO (Fig. 1), we could differentiate *B. longum* from *B. infantis* by using appropriate washing conditions.

In order to test whether the target regions of these probes are species specific and whether the target regions in the same species differ from those in other strains, dot blot analysis of additional strains of the same species and heter-

ologous species were performed (Fig. 3). Because of the rapidity and technical simplicity of the method, whole cells treated with 1% formaldehyde were used as targets instead of extracted RNA. The cells were fixed in 1% formaldehyde solution by the method of Salama et al. (7). The fixed cells were applied to a nylon membrane and baked at 80°C for 30 min. Prehybridization and hybridization were performed in the same way as for RNA dot blot hybridization. The strains used are listed in Table 2.

Probe PPR, used as a positive control for confirming the permeability of the fixed cells, hybridized to all strains tested except *Actinomyces israelii* ATCC 10048 (Fig. 3a). Probe PPR hybridized to RNA extracted from the same strain, as shown in Fig. 2a, indicating that this strain may be resistant to 1% formaldehyde treatment. Probes PAD, PBR, and PLO hybridized exclusively with strains of the same species but not with strains of any of the other *Bifidobacterium* species or any of the non-*Bifidobacterium* reference organisms tested (Fig. 3b, d, and f). Probes PBI and PIN also hybridized with all strains of the same species tested; however, PBI cross-reacted with *B. asteroides* ATCC 25910, *B. indicum* ATCC 25912, and *B. thermophilum* ATCC 25525 and 25866 (Fig. 3c), and PIN cross-reacted with *B. suis* ATCC 27533 (Fig. 3e). By partial sequencing of the 16S rRNA from *B. suis* ATCC 27533 with universal primer A, it was shown that this strain had the same sequence as *B. infantis* ATCC 15697 at the target position for probe PIN (data not shown). As described above, probes PBI and PIN are not precisely species specific. However, these probes did not show cross-reactivity among the strains of human intestinal *Bifidobacterium* species.

We are currently developing nonradioactive methods, such as fluorescent labeling, so that these oligonucleotide probes can be used widely for diagnostic purposes. Preliminary studies with the DIG system (Boehringer Mannheim) indicated that probes PAD, PBI, PBR, PIN, and PLO do not hybridize to the RNAs extracted from *Clostridium bifermentans* NCTC 506, *C. difficile* ATCC 17859, *C. innocuum* ATCC 14501, *C. paraputrificum* ATCC 25780, *C. perfringens* NCTC 4969, and *C. ramosum* JCM 1298 (data not shown). *Clostridium* is one important genus of gram-positive human intestinal bacteria which we had not used in the experiment shown in Fig. 3.

In order to determine the minimum number of fixed cells required to give a detectable hybridization signal, we performed the hybridization to various numbers of fixed cells of *B. longum* with probe PLO (Fig. 4). The lowest number of fixed cells detectable with probe PLO under the conditions used for hybridization (2 h) and detection by Betascope (30 min) was 10^4. A weak signal was detected at the region where 10^3 cells had been applied (Fig. 4). This result indicates that the number of cells in one colony is sufficient to identify the species of the organism.

In this study, we designed five oligonucleotide probes for the detection and identification of *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis*, and *B. longum*. Although the probes for *B. bifidum* and *B. infantis* cross-reacted with a few strains of heterologous *Bifidobacterium* species of nonhuman origin, those five probes were highly species specific against strains of the human intestinal species. This RNA-DNA hybridization technique is more rapid, simple, and sensitive than methods based on phenotypic characteristics or DNA-DNA homology. The autoradiograms shown in this article were obtained after only 30 min of exposure by Betascope and were comparable to those obtained after 2 days of exposure with X-ray films. With the Betascope, the entire test took less than 6 h after pure cell suspensions were obtained.

REFERENCES

1. **Delong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360–1363.
2. **Hoshina, S., S. Kahn, W. Jiang, P. Green, H. Neu, M. Morotomi, P. LoGerfo, and I. B. Weinstein.** 1990. Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene segments from gastric endoscopic biopsies. Diagn. Microbiol. Infect. Dis. **13**:473–479.
3. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82**:6955–6959.
4. **Lauer, E., and O. Kandler.** 1983. DNA-DNA homology, murein types, and enzyme patterns in the type strains of the genus *Bifidobacterium*. Syst. Appl. Microbiol. **4**:42–64.
5. **Morotomi, M., S. Hoshina, P. Green, H. C. Neu, P. LoGerfo, I. Watanabe, M. Mutai, and I. B. Weinstein.** 1989. Oligonucleotide probe for detection and identification of *Campylobacter pylori*. J. Clin. Microbiol. **27**:2652–2655.
6. **Noller, H. F., and C. R. Woese.** 1981. Secondary structure of 16S ribosomal RNA. Science **212**:403–411.
7. **Salama, M., W. Sandine, and S. Giovannoni.** 1991. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **57**:1313–1318.
8. **Sambrook, J., T. Maniatis, and E. Fritsch.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 7.54–7.55. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Scardovi, V.** 1986. Genus *Bifidobacterium* Orla-Jensen 1924, 472^{AL}, p. 1418–1434. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
10. **Stackebrandt, E., and O. Charfreitag.** 1990. Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii*-specific oligonucleotide probe. J. Gen. Microbiol. **136**:37–43.
11. **Wilson, K. H., R. Blitchington, B. Hindenach, and R. C. Greene.** 1988. Species-specific oligonucleotide probes for rRNA of *Clostridium difficile* and related species. J. Clin. Microbiol. **26**:2484–2488.