APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3668–3672 Vol. 62, No. 10
0099-2240/96/$04.00+0

Specific Detection and Analysis of a Probiotic *Bifidobacterium* Strain in Infant Feces

RUBEN G. KOK,[1] ANTHONY DE WAAL,[1] FRITS SCHUT,[2] GJALT W. WELLING,[2] GERARD WEENK,[3] AND KLAAS J. HELLINGWERF[1]*

Department of Microbiology, E. C. Slater Institute, BioCentrum Amsterdam, 1018 WS Amsterdam,[1] Laboratory of Medical Microbiology, University of Groningen, 9700 RB Groningen,[2] and Nutricia Microbiological Research Laboratory, 2700 MA Zoetermeer,[3] The Netherlands

Received 5 March 1996/Accepted 2 August 1996

For specific detection of the probiotic *Bifidobacterium* sp. strain LW420 in infant feces and for rapid quality control of this strain in culture, three strain-specific 16S rRNA gene-targeted primers have been developed. These primers allow specific detection of the organism via PCR. Specificity of the primers was determined in DNA samples isolated from single-strain and mixed cultures of bifidobacteria and in heterogenous fecal samples. The feasibility of this method for use in specific detection of probiotic strains was investigated through addition of *Bifidobacterium* sp. strain LW420 to infant instant milk formula (IMF) and PCR analyses of bacterial DNA isolated from feces of 17 newborn IMF-fed infants. In feces of all nine babies that had been fed with the probiotic IMF, the strain-specific PCR signal could be detected. No signal was found in feces of the eight infants that had been fed with a nonprobiotic IMF, demonstrating the specificity of the PCR method. All 17 infants developed a major fecal *Bifidobacterium* population already after 3 days, as determined through genus-specific and strain-specific PCR. Phenotypical screening of *Bifidobacterium* sp. strain LW420 and analysis of homology of the 16S rRNA gene sequence of this strain with that of other bifidobacteria deposited in databases do not allow positive classification of LW420 among the currently known species of *Bifidobacterium*.

Industrial interest in the use of specific bacterial strains as food additives, for instance, in dairy products, is rapidly growing. This development leads to the requirement for accurate quality control of the probiotic products and hence methods for specific identification of probiotic strains. Such accurate identification procedures also could serve in precisely monitoring the development of the population of the specific probiotic strain during passage through the human gastrointestinal tract.

Identification of bacterial species has been lifted to an advanced level, especially through detailed analysis of rRNA sequences. The work of Woese and many others has shown sequence comparison of rRNAs, especially 16S and 23S rRNAs, to be a reliable method for classification and identification of many bacterial isolates (13, 21). The variable degree of conservation of stretches of nucleotides in these rRNAs even allows the design of oligonucleotide probes, specific at the desired taxonomic level (1, 11), to be used as hybridization probes or as primers in amplification reactions. The rapidity and sensitivity of the latter PCR method are manifest.

Recently, Langendijk et al. (9) have described the generation and analysis of 16S rRNA gene (rDNA) probes, specific for the genus *Bifidobacterium*, which enable the exclusive detection of bifidobacteria in fecal samples through in situ hybridization. In the present study, these probes have been used as primers in PCR for the detection of the genus *Bifidobacterium* in infant feces. In addition, we developed a new set of primers which specifically recognize *Bifidobacterium* sp. strain LW420. LW420 is a probiotic strain that can be used as an additive in baby instant milk formulas (IMFs), particularly because of its high growth rate in complex media, its acid tolerance, and its relative oxygen insensitivity. The strain-specific primers tested positive as reporters for strain LW420 in feces of infants that received this strain as a probiotic supplement to their daily administered IMF. The use of strain-specific 16S rDNA-targeted PCR in monitoring the development of the human fecal flora is discussed. In addition, we discuss the taxonomic identity of strain LW420.

* Corresponding author. Mailing address: Department of Microbiology, E. C. Slater Institute, BioCentrum Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands. Phone: 31-20-5257055. Fax: 31-20-5257056. Electronic mail address: a417hell@chem.uva.nl.

MATERIALS AND METHODS

Strains and culturing methods. The majority of strains of *Bifidobacterium* used in this study were derived from the American Type Culture Collection, Deutsche Sammlung für Mikroorganismen, and National Collection of Industrial and Marine Bacteria strain libraries. *Bifidobacterium* sp. strain LW420 was received from Lab Wiesby (Wiesby Biofermentation GmbH, Niebuell, Germany). Strains of *Bifidobacterium* were routinely grown at 37°C in anaerobic APT (5) broth (Becton Dickinson Microbiology Systems; 25 g/liter) or in DeMan, Rogosa & Sharpe medium (Merck; 25 g/liter). All media were supplemented with 0.5 g of cysteine-HCl per liter and 0.002 g of resazurin per liter in filled glass bottles with airtight screw caps. When required, media were solidified with 1.5% (wt/vol) agar for plates. Plate cultures were grown in anaerobic jars.

Infant feeding and fecal sampling. Seventeen newborn infants were fed with two different experimental infant formulas in a double-blind, parallel, randomized study design. The composition of the formulas was completely in line with legal requirements generated from the European Union directive. The formulas differed from the currently marketed standard formulas in having a lower protein and phosphate content and no added iron. Both formulas were identical, except for the presence of *Bifidobacterium* sp. strain LW420, which was added to one of the two formulas to a concentration of 10^6 viable cells per g of dry powder. All 17 infants were healthy full-term infants (37 to 42 weeks of gestation) of appropriate size for gestational age, with no evidence of disease at the third day of life and no need for the use of antibiotics and born by vaginal delivery. Informed consent was obtained from the parents at about 3 weeks before the expected date of delivery. IMF feeding of infants was on demand of the infants, as advised for breast-fed infants. According to the design of the study, neither the infants' parents nor the involved researchers were informed about the nature of the IMF that the infants received, until completion of the study. Fecal samples from each baby were collected 3 days and 1, 2, 4, 8, and 12 weeks after birth. IMF feeding

TABLE 1. Properties of the rDNA-targeted PCR primers used in this study

rDNA-targeted primer		Nucleotide sequence (5′→3′)	Optimal annealing temp (°C)[a] (primer combination)	Length of generated PCR fragment (bp)[b]
Type and designation	Name (position)[c]			
General				
a	Bif16S3 (8–27)	AGGGTTCGATTCTGGCTCAG		
b	Bif16S4 (1512–1492)	ACGGTTACCTTGTTACGACTT	60–66 (a + b)	1,498
c	Bif23S1	GCCAAGGCATCCACCGT	56–60 (a + c)	2,015
Genus specific				
d	Bif164-PCR (164–181)	GGGTGGTAATGCCGGATG	62–66 (d + e)	523
e	Bif662-PCR (679–662)	CCACCGTTACACCGGGAA		
Strain specific				
f	LW420A (454–470)	GCACGGTTTCGGCCGTG	62–68 (f + h)	567
g	LW420C (177–194)	GGATGCTCCGCTCCATCG	62–68 (g + h)	845
h	LW420D (1028–1011)	GGGAAACCGTGTCTCCAC		

[a] Temperature yielding one specific PCR product of the correct size with LW420 DNA as the template DNA. For primer combination LW420C-LW420D, this is also the annealing temperature at which only *Bifidobacterium* sp. strain LW420 yields a PCR fragment.
[b] LW420 DNA as the template DNA.
[c] Nucleotide numbering based on the *Escherichia coli* 16S rDNA sequence.

occurred throughout the full 12 weeks of sampling. Fecal specimens were directly frozen at −20°C and stored frozen until used for DNA isolation. The experiments that form the basis of this study were approved by the Ethics Committee of Utrecht University Hospital.

Isolation of chromosomal DNA. Chromosomal DNA was isolated from cultured *Bifidobacterium* strains by using the Instagene DNA purification matrix of Bio-Rad, according to the instructions of the supplier. DNA (1 to 3 ng) was routinely used as a template in PCR amplification. For preparation of nucleic acids from infant feces, 50 to 100 mg (wet weight) of feces was used. Disruption of cells was performed using a Mini Bead Beater (Technolab). One milliliter of lysis buffer and 300 mg of zirconium beads (0.1-mm diameter) were added to the feces, and the mixture was shaken twice in the Mini Bead Beater for 1.5 min at 5,000 rpm. The suspension was centrifuged in a microcentrifuge at 18,000 × *g*, and the clear supernatant was used for extraction of nucleic acids, essentially according to the methods of Boom et al. (2) and Van der Hoek et al. (19). Nucleic acids were extracted with 60 μl of Celite suspension (2). TE buffer (100 μl; 10 mM Tris HCl [pH 8], 1 mM EDTA) was used for final elution. The resulting solution contains chromosomal DNA of the fecal bacterial flora. Routinely, 1 μl of the nucleic acid solution was used in a 50-μl PCR mixture.

PCR amplification, primer design, and sequence analysis. PCR amplification of 16S-23S rDNA fragments of chromosomal DNA of *Bifidobacterium* sp. strain LW420 was carried out in an Eppendorf Mastercycler. SuperTaq was used as the thermostable polymerase according to the recommendations of the supplier (Sphaero-Q). Deoxynucleoside triphosphates (dNTPs) and oligonucleotide primers were purchased from Pharmacia Biotech. PCR primers used in this study are listed in Table 1. Primers Bif16S3, Bif16S4, and Bif23S1 were used to amplify 16S rDNA and 16S-23S intergenic spacer DNA of *Bifidobacterium* sp. strain LW420. The last primer is a general 23S rDNA-targeted primer based upon general primers proposed by Frothingham and Wilson (6) and Jensen et al. (7). Amplification reaction mixtures routinely contained 10 pmol of each primer, 200 pmol of each dNTP, 1 ng of template DNA, and 0.1 U of SuperTaq polymerase per 50 μl. The thermocycle program used in genus-specific and strain-specific PCR consisted of the following time and temperature profile: (i) 95°C for 5 min; (ii) 30 cycles of 1 min at 95°C, 45 s at 66°C, and 1 min at 72°C; and (iii) 1 cycle of 1 min at 95°C, 45 s at 66°C, and 5 min at 72°C.

Primers Bif164-PCR and Bif662-PCR were used for specific amplification of *Bifidobacterium* 16S rDNA. These PCR primers were derived from probes Bif164 and Bif662 (9). The target regions of these probes have been previously described as *Bifidobacterium*-specific regions in the 16S rRNA, useful for genus-specific in situ hybridization experiments (9). Strain-specific primers LW420A, LW420C, and LW420D were designed as described in Results and Discussion. PCR products were detected through electrophoresis in 1% (wt/vol) agarose gels.

The nucleotide sequence of amplified 16S rDNA fragments was determined from both strands, with the double-stranded PCR fragment as the template, according to the chain termination method (16) adapted for Sequenase (version 2.0; U.S. Biochemicals). For this purpose, PCR fragments were gel purified and isolated with the Sephaglas Bandprep kit of Pharmacia. In addition to this, single-strand sequencing was performed, using single-stranded DNA of multiple independent M13 clones of the 16S rDNA region. Protocols used for sequencing and cloning of PCR fragments in M13mp18/19 were all standard protocols (15). Sequence primers were developed on the basis of conserved regions of bifidobacterial 16S rDNA, as derived from alignments of sequences extracted from the databases.

Analysis of the fermentative characteristics of strain LW420. The capacity of *Bifidobacterium* sp. strain LW420 to ferment a variety of sugars was investigated according to the method described by Scardovi (17).

Nucleotide sequence accession number. The nucleotide sequence of the 16S rDNA of LW420, including the adjacent 16S-23S spacer region, has been deposited in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number X89111.

RESULTS AND DISCUSSION

Sequence analysis of 16S rDNA of *Bifidobacterium* sp. strain LW420. The nucleotide sequence of the 16S rDNA of *Bifidobacterium* sp. strain LW420 was determined to enable design of strain-specific PCR primers. A fragment of 1,498 bp of 16S rDNA was amplified through PCR with primers Bif16S3 and Bif16S4 (see Materials and Methods and Table 1). We also amplified and sequenced the 16S-23S intergenic spacer region as a potential priming region, using 23S rDNA-targeted primer Bif23S1 in combination with Bif16S3. This spacer region has recently been shown to serve especially well in analysis of intraspecific relationships among bifidobacteria (10). The annealing position of the primers to the 16S-23S rDNA region of *Bifidobacterium* sp. strain LW420 and the DNA fragments resulting from PCR amplification of rDNA using the relevant primer sets are depicted in Table 1.

As expected, 16S rDNA of LW420 showed significant homology with 16S rDNA sequences of bifidobacteria present in the databases (Table 2). Relatively high homology was found with 16S rDNA of *Bifidobacterium animalis* and *Bifidobacterium globosum*, although numerous ambiguities (N) in those sequences make it difficult to decide whether sequence homology is significantly above or below the 97% identity threshold required for positive species assignment, as proposed by Stackebrandt and Goebel (18). If ambiguous nucleotides are assumed to be identical, the level of identity with the *B. animalis* sequence is 98.1%, versus only 96.2% with the 16S rDNA of *B. globosum*. Strain LW420 may therefore belong to *B. animalis*. Also the 16S-23S intergenic spacer region of LW420 is most similar to the equivalent spacer region in two strains of *B. animalis* extracted from GenBank (not shown). However, upon completion of the strain-specific PCR described below, we found that the 16S rDNA of *Bifidobacterium* sp. strain LW420 also shows 98.1% identity with the 16S rDNA of a proposed new species of *Bifidobacterium*, designated *Bifidobacterium lactis* sp. nov. (12). These data raise the possibility that strain LW420 belongs to this new species.

TABLE 2. Percentages of homology of the 16S rDNA sequence of *Bifidobacterium* sp. strain LW420 to 16S rDNA of other bifidobacteria, as extracted from the databases[a]

EMBL/GenBank accession no.	Species of *Bifidobacterium*	% Homology (no. of overlapping residues)
BL16SRRN1	*B. lactis* sp. nov.	98.1 (1,592)
BGRR16SH	*B. globosum*	93.3 (1,480)[b]
BA16SRRN	*B. animalis*	91.7 (1,465)[b]
BBRR16SC1	*B. breve*	91.7 (1,501)
S83624	*B. bifidum*	91.5 (1,503)
BCRR16SE	*B. coryneforme*	91.5 (1,478)
BSRR16SO	*B. suis*	91.5 (1,476)
BMRR16SM	*B. minimum*	91.3 (1,475)
BRRDQ	*B. bifidum*	90.7 (1,501)
BPRR16SN	*B. pseudolongum*	90.4 (1,479)
BARR16SB1	*B. asteroides*	90.2 (1,464)
BARR16SA1	*B. adolescentis*	89.8 (1,476)
BMRR16SL	*B. magnum*	89.4 (1,479)
BIRR16SI	*B. indicum*	89.2 (1,480)
BDRR16SG	*B. dentium*	88.9 (1,480)
BIRR16SJ	*B. infantis*	88.6 (1,478)

[a] Sequences were compared with the program FastA (14).
[b] Ambiguities (N) in the deposited sequences were regarded as different from the LW420 sequence. Homology percentages should therefore be regarded as minimum values. Assuming ambiguous nucleotides as identical to the LW420 sequence, maximal homology for the 16S rDNA of *B. animalis* and *B. globosum* would be 98.1 and 96.2%, respectively.

Phenotypic analysis of *Bifidobacterium* sp. strain LW420. As sequence analysis of the 16S rDNA of *Bifidobacterium* strain LW420 alone could not lead to positive species identification of the strain, its fermentative characteristics were compared with those reported by Scardovi (17) (Table 3). The fermentation properties of LW420 are unique with respect to fermentation of trehalose and inulin. Furthermore, LW420 does not degrade starch, whereas *B. globosum* and *B. animalis* do. The overall fermentation pattern of *B. animalis* differs considerably from that of strain LW420 (Table 3). Like all other bifidobacteria, strain LW420 displays fructose-6-phosphate phosphoketolase activity (not shown).

Comparing the results of the 16S rDNA sequence analyses with fermentation properties, especially those of *B. infantis*, is interesting, since *Bifidobacterium* sp. strain LW420 originally was thought to be identical to the type strain of *B. infantis*, i.e., strain ATCC 15697. As is clear from the 16S rDNA sequence comparison, however, LW420 does not belong to the *B. infantis* cluster. On the other hand, though 16S rDNA homology between LW420 and especially *B. animalis* is high, fermentation patterns differ enough to doubt classification of LW420 among *B. animalis*. Alternatively, LW420 may belong to a new species of *Bifidobacterium*, possibly to the newly identified species *B. lactis* (12). It would be of interest to compare the fermentation characteristics of LW420 with those of *B. lactis*. At present, it is impossible to positively classify strain LW420 among either *B. animalis* or *B. lactis*. Until further analyses have been carried out, we therefore designate this strain *Bifidobacterium* sp. strain LW420, abbreviated to LW420 throughout this paper. Notably, LW420 is a rapidly growing strain that displays a relatively high tolerance to oxygen, compared with many other bifidobacteria (data not shown).

Development of LW420-specific PCR primers. The requirement for rapid and sensitive quality control methods prompted us to investigate whether it was possible to design PCR primers that would specifically recognize the presence of LW420. On the basis of a comparison of the 16S rDNA sequence of LW420 with 16S rDNA of other bifidobacteria retrieved from the databases, using a multiple alignment of 16S sequences created by the program Clustal V, we identified three potential primer target sites, LW420A, LW420C, and LW420D, that were (partly) specific for the LW420 16S rDNA sequence (Table 1). The specificity of the two primer sets (i.e., LW420A-LW420D and LW420C-LW420D) was first tested in PCR performed with chromosomal template DNA isolated from cultures of 20 different strains belonging to 11 *Bifidobacterium* species (Table 4). At annealing temperatures of 62 to 66°C (Table 1), not a single PCR product was formed in any of the strains tested (Table 4) (using 1 to 30 ng of chromosomal DNA as the template), including strains with only one or two nucleotide differences near the 3′ end of the primers. Instead, DNA of LW420 (1 ng) always yielded a single band of the right size, at an annealing temperature of up to 68°C. The same was true for DNA samples isolated from mixed cultures of all 20 *Bifidobacterium* strains. Only when LW420 was added to these mixed cultures could the LW420-specific PCR fragment be detected. These results show that the two primer combinations and the amplification conditions described here are sufficiently stringent for specific detection of LW420, at least in this limited comparative experiment. Calculated from PCR results using the LW420-specific primers and mixed *Bifidobacterium* cultures with variable concentrations of cells of LW420 (fixed total cell concentration, 10^8 to 10^9 cells per ml), strain LW420 could reliably be detected when it was present at above 10^3 cells per ml.

Specific detection of LW420 in infant feces. Since the above-described primers were specific for 16S rDNA of LW420 grown in pure and mixed cultures, we tested their applicability in detection of the probiotic LW420 in infant feces. In addition, we used the two genus-specific probes recently described by Langendijk et al. (9) and here adapted for use as PCR primers (Table 1, Bif164-PCR and Bif662-PCR) in order to

TABLE 3. Analysis of the fermentation characteristics of *Bifidobacterium* sp. strain LW420

Sugar or carbon source	Growth[a] of:			
	LW420	*B. infantis*	*B. animalis*	*B. globosum*
D-Ribose	w	+	+	+
L-Arabinose	w	−	+	d
Lactose	+	+	+	+
Cellobiose	−	−	d	−
Melezitose	−	−	d	−
Raffinose	+	+	+	+
Sorbitol	−	−	−	−
Starch	−	−	+	+
Gluconate	−	−	−	−
Xylose	d	d	+	d
Mannose	−	d	d	−
Fructose	+	+	+	+
Galactose	+	+	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
Trehalose	+	−	d	−
Melibiose	+	+	+	+
Mannitol	−	−	−	−
Inulin	+	d	−	−
Salicin	−	−	+	−

[a] Growth was recorded in media with the indicated carbon source. Fermentation characteristics were determined only for strain LW420 used in this study. Characteristics of *B. infantis*, *B. animalis*, and *B. globosum* were taken from the work of Scardovi (17) and are shown only for comparison. +, growth; −, no growth; w, weak growth; d, doubt, i.e., variable reaction (17).

TABLE 4. Specificity of PCR product formation with LW420-specific primer combinations Bif420C-Bif420D and Bif420A-Bif420D[a]

Bifidobacterium strain	Product formation with:		
	LW420C-LW420D	LW420A-LW420D	Bif164-Bif662
Bifidobacterium sp. strain LW420	+	+	+
B. adolescentis (DSM 20083)	−	−	+
B. adolescentis (ATCC 11146)	−	−	+
B. adolescentis (ATCC 15703)	−	−	+
B. angulatum (ATCC 27535)	−	−	+
B. angulatum (DSM 20098)	−	−	+
B. animalis (DSM 20104)	−	−	+
B. bifidum (DSM 20015)	−	−	+
B. bifidum (DSM 20456)	−	−	+
B. bifidum (NCIMB 8810)	−	−	+
B. bifidum (ATCC 35914)	−	−	+
B. breve (ATCC 15700)	−	−	+
B. catenulatum (DSM 20214)	−	−	+
B. catenulatum (ATCC 27675)	−	−	+
B. gallicum (DSM 20093)	−	−	+
B. globosum (NCC 341)	−	−	+
B. infantis (DSM 20088)	−	−	+
B. infantis (ATCC 25962)	−	−	+
B. longum (NCIMB 8809)	−	−	+
B. pseudocatenulatum (DSM 20438)	−	−	+
B. pseudocatenulatum (ATCC 27917)	−	−	+

[a] Specificity was tested with chromosomal DNA of several species of *Bifidobacterium* as the template in PCRs with primer combinations Bif420C-Bif420D and Bif420A-Bif420D. Results of the genus-specific PCR (with primer combination Bif164-Bif662) are shown for comparison.

detect bifidobacteria in general. This set of primers yielded a product of the expected size in all cultures of *Bifidobacterium* strains used in Table 4. In a preliminary test, nucleic acid extracts were prepared from feces of an entirely breast-fed infant (baby A), collected at 4 and 6 weeks after birth. No PCR signal could be detected in these samples with the two LW420-specific primer sets, whereas the genus-specific primers yielded a clean fragment of the expected size, indicating the presence of bifidobacteria in the fecal flora of this infant (Table 5). The LW420-specific PCR signals could be detected only when cells of LW420 were added to the feces before isolation of total DNA from the sample (not shown). This demonstrates that the primer sets LW420A-LW420D and LW420C-LW420D can be used to specifically detect strain LW420 in samples as heterogeneous as infant feces.

In order to determine the feasibility of monitoring the population of a specific strain of *Bifidobacterium* in infant feces through PCR, we applied the LW420-specific PCR technique to bacterial DNA present in nucleic acid extracts from feces of 17 newborn IMF-fed infants. From this group, nine babies received the LW420-supplemented formula and eight babies received the nonsupplemented feeding (see Materials and Methods). Fecal samples, collected at six time points after birth (ranging from 3 days to 12 weeks), were analyzed through PCR with the LW420-specific primer set LW420C-LW420D for detection of LW420. In addition, the genus-specific primer combination Bif164-Bif662 was used to demonstrate the presence of bifidobacteria in general. As is shown in Table 5, LW420 was detected in feces from all infants that received IMF supplemented with LW420 at all time points (except for the day 3 sample of baby 3), but it was not detected in any fecal samples from those infants not given LW420 in their IMF. Therefore, primer combination LW420C/D can be used to qualitatively monitor the presence of the probiotic strain LW420 in infant feces.

In order to determine the detection limit of the LW420-specific PCR in these experiments, approximately 60 mg (dry weight at 5×10^{10} cells per g [dry weight]) of one of the fecal samples of baby A (Table 5) was mixed with various amounts of LW420 cells (10^2 to 10^8 LW420 cells per 60 mg [dry weight] of feces) and DNA was isolated from these mixtures. With the LW420-specific primer set LW420C-LW420D, LW420 could be detected when it was present at a concentration of 10^3 cells or more per 60-mg sample (detection limit, $\approx 10^4$ cells per g [dry weight] of feces, or 2×10^{-5}% of the total population). Similar results could be obtained when diluted samples of the isolated total DNA were used as template DNA in the PCRs (not shown). This strain-specific detection method is therefore much more sensitive than detection methods based upon in situ hybridization with labelled probes (detection limit, generally $\approx 10^8$ cells per g of feces against a total population background of 10^{11} cells per g, or 0.1% of the total population).

Interestingly, when diluted fecal DNA samples corresponding to day 3 and week 12 samples of two LW420-fed infants (babies 16 and 17) were analyzed, the LW420-specific PCR signal could still be detected in all DNA samples at a dilution of 5×10^4-fold. Since the DNA present in these highly diluted samples is derived from maximally 10^3 to 10^4 cells, the concentration of LW420 cells in these fecal samples can be estimated at around 10% or more of the total bacterial population.

TABLE 5. Detectability through PCR of bifidobacteria in general and of *Bifidobacterium* sp. strain LW420 in particular in feces of newborn IMF-fed infants and scheme of addition of probiotic LW420 to IMF[a]

Baby	Bacteria detectable in all fecal samples		LW420 added to IMF
	Bifidobacteria	LW420	
A[b]	Yes	No	NA
1	Yes	Yes	Yes
2	Yes	No	No
3	Yes[c]	Yes[c]	Yes
4	Yes	No	No
5	Yes	Yes	Yes
6	Yes	Yes	Yes
7[d]	Yes[e]	No	No
8[d]	Yes[e]	No	No
9	Yes	No	No
10	Yes	Yes	Yes
11	Yes[f]	No	No
12	Yes	Yes	Yes
13[g]	Yes	No	No
14	Yes	Yes	Yes
15	Yes	No	No
16	Yes	Yes	Yes
17	Yes	Yes	Yes

[a] Fecal samples, isolated from baby A at weeks 4 and 6 and from babies 1 to 17 at day 3 and weeks 1, 2, 4, 8, and 12, were analyzed with respect to the presence of bifidobacteria in general and LW420 in particular by using primers Bif164 and Bif662 and primers LW420C and LW420D, respectively.

[b] Baby A was an entirely breast-fed infant, independent from the other 17 infants. As this baby did not receive IMF at all, the final column is not applicable to samples from this infant (NA).

[c] No PCR product was obtained from the day 3 fecal sample with either primer set. This was tested in repeated chromosomal DNA isolates.

[d] No fecal samples from weeks 1, 8, and 12 were available from these babies.

[e] A *Bifidobacterium* signal was detected in fecal samples from day 3 and week 4; no signal was detectable in samples from week 2.

[f] No *Bifidobacterium* signal was detectable in samples from day 3 and week 2.

[g] No fecal samples from day 3 and week 12 were available from this infant.

Moreover, the general bifidobacterial signal generated with primers Bif164 and Bif662 showed the same detection limit in this experiment, implying that representatives of the genus *Bifidobacterium* in general constitute at least 10% of the bacterial flora. Similar results were obtained in day 3 and week 12 fecal samples of an LW420-negative infant (baby 9). From the fact that both LW420-specific and genus-specific primer sets yield the same results, it can be concluded that LW420 forms a major part of the *Bifidobacterium* population in feces of the infants fed with LW420-supplemented formula.

Despite the absence of a *Bifidobacterium* signal in day 3 samples from babies 3 and 11 (Table 5), it can be inferred that already 3 days after gestation, bifidobacteria constitute a major part of the fecal flora of the majority of the tested formula-fed infants, regardless of whether bifidobacteria supplement the daily administered formula. Interestingly, representatives of the genus *Bifidobacterium* were indeed found to dominate, constituting over 99% of the bacterial population in feces of several of the above infants, including babies that did not receive the LW420-supplemented formula. This was concluded from a preliminary investigation using genus-specific primer Bif164 as a fluorescein isothiocyanate-labelled rRNA-targeted probe in quantitative in situ hybridizations (data not shown). *Bifidobacterium* counts of 90 to 100% of the entire microflora have been reported for breast-fed infants after 4 to 5 days (3, 4). However, it is generally believed that the development of the *Bifidobacterium* population significantly lags behind in bottle-fed infants compared with that in breast-fed babies. *Bifidobacterium* counts would not exceed 10 to 70% of the total population after 3 days (3, 4). The relatively low sensitivity of classical culturing and enumeration methods versus high sensitivity of the 16S rDNA or rRNA-targeted methods may underlie this discrepancy.

In conclusion, PCR primers LW420C and LW420D can be used to specifically detect the presence of *Bifidobacterium* sp. strain LW420 in infant fecal samples with high sensitivity. This offers possibilities to accurately monitor the development of the population of this organism in the intestinal flora of newborn infants, especially when performed in combination with quantitative in situ hybridization studies. Such rDNA- or rRNA-targeted studies may shed new light on the bacterial colonization of the intestines of newborn infants. Furthermore, PCR with the strain-specific primers, together with phenotypic tests, can well serve as important markers for identification of strain LW420, useful in quality control of cultures of probiotic strains.

In a broader perspective, the results presented in this paper show that 16S rDNA-targeted primers can easily be employed to monitor the presence of one particular strain in human intestines. Recently, a similar approach has been described for the specific detection of species of *Fusobacterium* (20) and *Bacteroides* (8) in human intestines through PCR using species-specific 16S rDNA-targeted primers. The present availability of such highly sensitive strain-monitoring procedures undoubtedly will refine classical data on composition and development of the human intestinal flora.

ACKNOWLEDGMENTS

We are grateful to Klaske van Hoeij for helpful discussions on the development and composition of the fecal flora of infants and to André van Oudenallen for assistance with serial PCR. We thank U-Gene for providing us with the infant fecal samples. We appreciate the contributions of Loes Crielaard to this study.

REFERENCES

1. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
2. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
3. **Bullen, C. L., P. V. Tearle, and M. C. Stewart.** 1977. The effect of humanized milks and supplemented breast feeding on the faecal flora of infants. J. Med. Microbiol. **10:**403–412.
4. **Cooperstock, M. S., and A. J. Zedd.** 1983. Intestinal flora of infants, p. 79–99. *In* D. J. Hentges (ed.), Human intestinal flora in health and disease. Academic Press, Inc., New York.
5. **Deibel, R. H., J. B. Evans, and C. F. Niven, Jr.** 1957. Microbiological assay for thiamin using *Lactobacillus viridescens*. J. Bacteriol. **74:**818–821.
6. **Frothingham, R., and K. H. Wilson.** 1993. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. J. Bacteriol. **175:**2818–2825.
7. **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59:**945–952.
8. **Kraeder, C. A.** 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. **61:**1171–1179.
9. **Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson, and G. W. Welling.** 1995. Quantitative fluorescence in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. **61:**3069–3075.
10. **Leblond-Bourget, N., P. Herve, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. Int. J. Syst. Bacteriol. **46:**102–111.
11. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleiffer.** 1992. Phylogenetic oligonucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
12. **Meile, L., U. Rueger, C. Gut, M. Dasen, and M. Tueber.** 1995. *Bifidobacterium lactis* sp. nov., a new relatively oxygen tolerant species isolated from commercially fermented milk. GenBank accession number X89513.
13. **Olsen, G. J., R. Overbeek, N. Larsen, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W.-M. Kua, T. J. Macke, Y. Xing, and C. Woese.** 1992. The ribosomal RNA database project. Nucleic Acids Res. **20:**2199–2200.
14. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
17. **Scardovi, V.** 1984. Genus *Bifidobacterium* Orla-Jensen, 1924, 472, p. 1418–1434. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. I. The Williams & Wilkins Co., Baltimore.
18. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
19. **Van der Hoek, L., R. Boom, J. Goudsmit, F. Snijders, and C. J. A. Sol.** 1995. Isolation of human immunodeficiency virus type 1 (HIV-1) RNA from feces by a simple method and difference between HIV-1 subpopulation in feces and that in serum. J. Clin. Microbiol. **33:**581–588.
20. **Wang, R.-F., W.-W. Cao, and C. E. Cerniglia.** 1996. Phylogenetic analysis of *Fusobacterium prausnitzii* based upon the 16S rRNA gene sequence and PCR confirmation. Int. J. Syst. Bacteriol. **46:**341–343.
21. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.