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# Genus- and Species-Specific PCR Primers for the Detection and Identification of Bifidobacteria

Takahiro Matsuki\*, Koichi Watanabe,  
and Ryuichiro Tanaka

Yakult Central Institute for Microbiological Research,  
1796 Yaho, Kunitachi, Tokyo 186-8650, Japan

## Abstract

**16SrDNA-targeted genus- and species-specific PCR primers have been developed and used for the identification and detection of bifidobacteria. These primers cover all of the described species that inhabit the human gut, or occur in dairy products. Identification of cultured bifidobacteria using PCR primer pairs is rapid and accurate, being based on nucleic acid sequences. Detection of bifidobacteria can be achieved using DNA extracted from human faeces as template in PCR reactions. We have found that, in adult faeces, the *Bifidobacterium catenulatum* group was the most commonly detected species, followed by *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bifidobacterium bifidum*. In breast-fed infants, *Bifidobacterium breve* was the most frequently detected species, followed by *Bifidobacterium infantis*, *B. longum* and *B. bifidum*. It was notable that the *B. catenulatum* group was detected with the highest frequency in adults, although it has often been reported that *B. adolescentis* is the most common species. Real-time, quantitative PCR using primers targeting 16S rDNA shows promise in the enumeration of bifidobacteria in faecal samples. The approach to detect the target bacteria with quantitative PCR described in this review will contribute to future studies of the composition and dynamics of the intestinal microflora.**

## Introduction

In the study of intestinal microflora, it is important to clarify the relationship between the host and the microbial community. The structure of the human intestinal microflora has been investigated in great detail by anaerobic culture techniques (Moore and Holdeman, 1974; Finegold *et al.*, 1974). These intensive investigations provided significant information about the microflora. However traditional culture methods raise the following problems: the methods are labor-intensive and time-consuming; only easily cultivable organisms are counted; and classification and identification based on phenotypic traits does not always provide clear-cut results, and is sometimes unreliable. Therefore there is a need for practical techniques that enable rapid and accurate analysis of the gut microflora. Recent research

has led to rapid advances in the application of molecular techniques based on 16S and 23S rRNA gene sequences to study the microbial diversity in ecosystems (Amann *et al.*, 1995). In the analysis of intestinal microflora, 16S rRNA targeted oligonucleotide probes have been applied in fluorescent *in situ* hybridization (FISH) as a culture independent method (Langendijk *et al.*, 1995; Franks *et al.*, 1998). The 16S rDNA-cloning-library method (Wilson and Blitchington, 1996; Suau *et al.*, 1999) and DGGE/TGGE (denaturing/temperature gradient gel electrophoresis) method (Millar *et al.*, 1996; Zoetendal *et al.*, 1998) have also provided an efficient strategy for exploring the biodiversity of the microflora. Although these studies provide significant information, the approaches are time-consuming and detect 90-99% of the members of the microflora.

On the other hand, PCR with specific 16S rDNA-based oligonucleotide primers is a powerful method for the detection of target bacteria within complex ecosystems. As the method is rapid, accurate and sensitive, it has frequently been used in the detection and identification of pathogenic bacteria (Satake *et al.*, 1997; Gumerlock *et al.*, 1991). Wang *et al.* (1996) first applied the PCR method to the analysis of intestinal microflora. They prepared species-specific primers based on 16S rRNA gene sequences for 12 bacteria that are predominant in the human intestinal tract. Specific oligonucleotide primers have been designed for many bacterial species that are known to be present in the intestinal tract. So far, species-specific primers for *Bifidobacterium* (Matsuki *et al.*, 1999), *Ruminococcus* (Wang *et al.*, 1997), *Eubacterium* (Kageyama and Benno, 2001), and *Lactobacillus* (Walter *et al.*, 2000, Song *et al.*, 2000) have been developed and applied successfully.

The specific PCR techniques do not provide information on bacteria that are not the target of the primers. Therefore it is necessary to derive specific primers for the major genera or groups present in the gut as well as for bacterial species. Moreover, the development of reliable quantitative PCR methods is necessary. Research on quantitative detection is already proceeding, and the establishment of optimal procedures and application of real-time quantitative PCR is expected.

This review details the identification of cultured strains using species-specific PCR primers and the specific detection of bacteria from fecal DNA, with particular emphasis on *Bifidobacterium* species.

## Phylogenetic Relationship of the Genus *Bifidobacterium*

The genus *Bifidobacterium* includes gram-positive pleomorphic strict anaerobes and some species are commonly detected in the human gut. Bifidobacteria are phylogenetically grouped in the actinomycete branch of gram-positive bacteria with high G+C content and presently

\*For correspondence. Email takahiro-matsuki@yakult.co.jp.

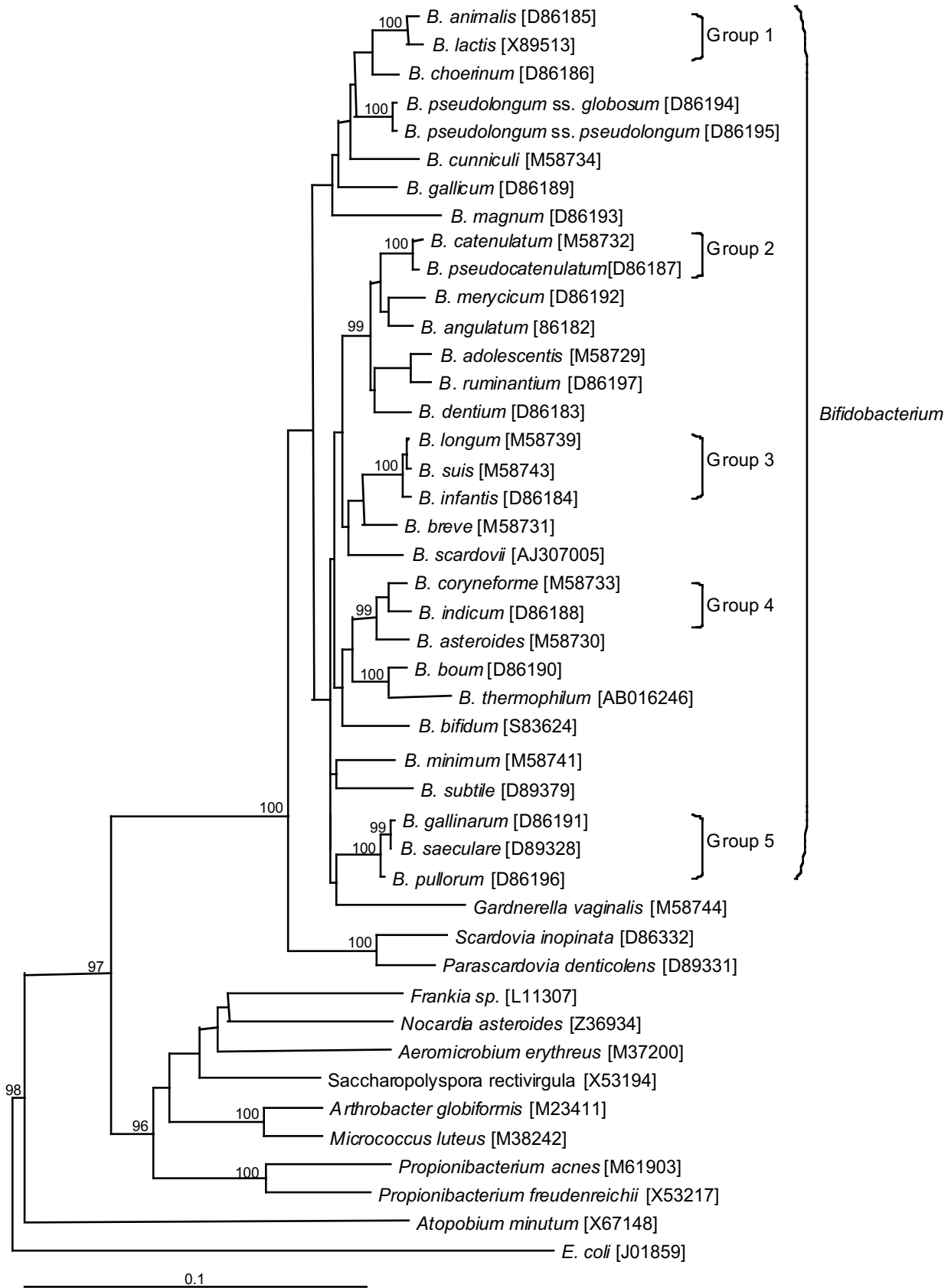


Figure 1. Phylogenetic tree based on 16S rDNA sequences, showing the relationships of *Bifidobacterium* species (Miyake *et al.*, 1998, with some modification). The tree was rooted with *E. coli* and constructed using neighbor-joining method with bootstrap values calculated from 100 trials. Only values above 95% are indicated. Bar = 10% sequence divergence.

are assigned to 30 species (Euzéby, 1997; Hoyles *et al.*, 2002; Jian *et al.*, 2002). The use of rRNA sequences as indicators of phylogenetic analysis is now widespread (Woese, 1987). In the genus *Bifidobacterium*, 16S rDNA (Frothingham *et al.*, 1992; Miyake *et al.*, 1998; Leblond-Bourget *et al.*, 1996), 16S to 23S internal transcribed spacer (ITS) sequences (Leblond-Bourget *et al.*, 1996), and the HSP60 gene sequence (Jian *et al.*, 2001) were used to analyze the phylogenetic relationship. Although some differences were reported, the phylogenetic trees constructed from ITS and HSP60 sequences were basically similar to that of 16S rDNA. Therefore, this section describes the phylogenetic relationship of the genus *Bifidobacterium* on the basis of 16S rDNA sequences.

A phylogenetic tree was constructed from 16S rDNA sequences of 30 bifidobacterial species and 13 related bacteria with accordance as described by Miyake *et al.*, (1998) (Figure 1). *Bifidobacterium* is a relatively coherent genus and was composed of 30 bifidobacterial species and *Gardnerella vaginalis*. Jian *et al.* have recently proposed that *Scardovia inopinatum* and *Parascardovia denticolens* should be transferred from *Bifidobacterium* to two new genera, since these two species have distinct phenotypic characteristics and low G+C contents compared with other bifidobacteria (Jian *et al.*, 2002). There are five groups that were characterized by high bootstrap values (>97%) and 16S rDNA sequence similarity (>98.9%). The closely related groups of species are (1) *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum* (16S rDNA similarity 99.5%), (2) *Bifidobacterium indicum* and *Bifidobacterium coryneforme* (similarity 99.1%), (3) *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium suis* (similarities 99.1-99.2%), (4) *Bifidobacterium gallinarum*, *Bifidobacterium pullorum*, and *Bifidobacterium saeculare* (similarities 99.3-99.9%), and (5) *Bifidobacterium animalis* and *Bifidobacterium lactis* (similarity 98.9%) (Miyake *et al.*, 1998). These groups also show moderate (40 to 60%) to high (>70%) relatedness in DNA-DNA hybridization tests (Scardovi, 1984; Wayne *et al.*, 1987). Cai *et al.* (2000) reported that the levels of DNA-DNA hybridization between the type strains of *B. lactis* and *B. animalis* ranged from 85.5 to 92.3%, indicating that *B. lactis* should be included in *B. animalis*.

When 16S rDNA is used as an index, it is difficult to differentiate *Gardnerella* and the genus *Bifidobacterium*. However, it is reported that the G+C% of *G. vaginalis* (42%) is significantly lower than that of bifidobacteria (55-67%) (Scardovi, 1984). In the phylogenetic analysis based on HSP60 sequences, *G. vaginalis* and the bifidobacteria formed another cluster (Jian *et al.*, 2001). Thus, the phylogenetic position of *G. vaginalis* is still under discussion, and further studies are expected.

### Currently Used Genus-and Species-specific Primers

It has been reported that *Bifidobacterium adolescentis*, *B. catenulatum*, *B. pseudocatenulatum*, *B. longum*, and *B. bifidum* are major bifidobacterial species of the adult intestinal microflora (Biavati *et al.*, 1986; Mutai and Tanaka, 1987), and that *B. infantis* and *Bifidobacterium breve* are predominant species in the intestinal tract of human infants

(Benno *et al.*, 1984; Biavati *et al.*, 1984). In addition, *Bifidobacterium angulatum*, *Bifidobacterium dentium*, and *Bifidobacterium gallicum* have been also reported as human intestinal bifidobacteria (Scardovi and Crociani, 1974; Lauer, 1990). *B. longum*, *B. breve*, *Bifidobacterium bifidum*, *B. infantis*, and *B. lactis* are frequently used in probiotic products (Lee *et al.*, 1999).

For the identification and detection on these species, genus- and species-specific primers have been developed. Table 1 shows the currently used genus- and species-specific primers for *Bifidobacterium*. Most of them are designed on the basis of 16S rDNA sequences. Depending on the target sequence chosen, 16S rDNA-targeted specific primers can be used to detect bacteria on different phylogenetic levels. Since *B. catenulatum* and *B. pseudocatenulatum* are closely related in their 16S rDNA similarity (Miyake *et al.*, 1998) and DNA-DNA relatedness (Lauer and Kandler, 1983), these two species are treated as the members of the *B. catenulatum* group (Matsuki *et al.*, 1998). In addition to the 16S rDNA-targeted specific primers, species- and strain-specific primers have been derived on the basis of 16S to 23S internal transcribed spacer (ITS) sequences (Brigidi *et al.*, 2000; Ventura *et al.*, 2001).

### Identification

Until recently, isolates of *Bifidobacterium* have been identified to the genus level by using Gram-staining, morphological observations, end-product analysis of glucose metabolism, and fructose-6-phosphate conversion ability as indices. Species identification has also been made on the basis of phenotypic traits (Scardovi, 1984). However, these identification methods require a large amount of time and labour, and do not always provide accurate results. On the other hand, the identification methods using the 16S rRNA sequence are less subjective, being nucleic acid sequence-based. For the 16S rDNA-based identification, three methods have been suggested; (1) sequence analysis, (2) species-specific hybridization method (Yamamoto *et al.*, 1992), and (3) the specific PCR techniques. When these approaches are compared, PCR provides identification results with the least effort and in the shortest time. For the precise identification of bacterial species, the DNA-DNA homology should be determined to confirm the existence of more than 70% homology with the type strain, but the species-specific primers are capable of accurate identification of bifidobacteria (Matsuki *et al.*, 1998). This section describes the isolation of bifidobacteria and their identification using species-specific primers.

### Isolation of *Bifidobacterium* Strains

Feces or dairy products are sampled, and serial 10-fold dilutions are prepared anaerobically (Holdman *et al.*, 1977). Suitable aliquots of dilutions are smeared onto bifidobacteria-selective media (Tanaka and Mutai, 1980; Nebra and Blanch, 1999), and are anaerobically incubated in a glove box or in anaerobic jars. Colonies formed on the media are picked with a sterile toothpick, and suspended in 50 µl of TE (10 mM Tris-HCl, 1mM EDTA; pH 8.0). For

Table 1. Currently used genus- and species-specific primers for *Bifidobacterium*

| Target bifidobacteria       | Name of primers               | Sequence   | Product size (bp) | References                        |
|-----------------------------|-------------------------------|--|-------------------|-----------------------------------|
| <i>Bifidobacterium</i>      | g-Bifid-F<br>g-Bifid-R        | CTCCTGGAACGGGTGG<br>GGTGTCTTCCCGATATCTACA        | 549-563           | Matsuki <i>et al.</i> , 2002      |
| <i>Bifidobacterium</i>      | Bif164-PCR<br>Bif662-PCR      | GGGTGGTAATGCCGGATG<br>CCACCGTTACACCGGAA          | 523               | Kok <i>et al.</i> , 1996          |
| <i>Bifidobacterium</i>      | Im26<br>Lm3r                  | GATTCTGGCTCAGGATGAACG<br>CGGGTGCTICCCACTTTCATG   | 1.35k             | Kaufmann <i>et al.</i> , 1997     |
| <i>B. adolescentis</i>      | BiADO-1<br>BiADO-2            | CTCCAGTTGGATGCATGTC<br>CGAAGGCTTGCTCCAGT         | 279               | Matsuki <i>et al.</i> , 1998      |
| <i>B. angulatum</i>         | BiANG-1<br>BiANG-2            | CAGTCCATCGCATGGTGGT<br>GAAGGCTTGCTCCCAAC         | 275               | Matsuki <i>et al.</i> , 1998      |
| <i>B. bifidum</i>           | BiBIF-1<br>BiBIF-2            | CCACATGATCGCATGTGATTG<br>CCGAAGGCTTGCTCCCAAA     | 278               | Matsuki <i>et al.</i> , 1998      |
| <i>B. breve</i>             | BiBRE-1<br>BiBRE-2            | CCGGATGCTCCATCACAC<br>ACAAAGTGCCCTTGCTCCCT       | 288               | Matsuki <i>et al.</i> , 1998      |
| <i>B. catenulatum</i> group | BiCATg-1<br>BiCATg-2          | CGGATGCTCCGACTCCT<br>CGAAGGCTTGCTCCCGAT          | 285               | Matsuki <i>et al.</i> , 1998      |
| <i>B. longum</i> group      | BiLON-1<br>BiLON-2            | TTCCAGTTGATCGCATGGTC<br>TCSCGCTTGCTCCCGAT        | 277               | Matsuki <i>et al.</i> , 1998      |
| <i>B. longum</i>            | BiLON-1<br>BiLON-2            | TTCCAGTTGATCGCATGGTC<br>GGGAAGCCGTATCTCTACGA     | 831               | Matsuki <i>et al.</i> , 1999      |
| <i>B. infantis</i>          | BiINF-1<br>BiINF-2            | TTCCAGTTGATCGCATGGTC<br>GGAAACCCCATCTCTGGGAT     | 828               | Matsuki <i>et al.</i> , 1999      |
| <i>B. dentium</i>           | BiDEN-1<br>BiDEN-2            | ATCCCGGGGGTTGCGCT<br>GAAGGCTTGCTCCCGA            | 387               | Matsuki <i>et al.</i> , 1999      |
| <i>B. gallicum</i>          | BiGAL-1<br>BiGAL-2            | TAATACCGGATGTTCCGCTC<br>ACATCCCCGAAAGGACGC       | 303               | Matsuki <i>et al.</i> , 1999      |
| <i>B. lactis</i>            | Bflac2<br>Bflac5 <sup>a</sup> | GTGGAGACACGGTTTTCCC<br>CACACCACACAATCCAATAC      | 680               | Ventura <i>et al.</i> , 2001      |
| <i>B. breve</i>             | BreU3<br>BreL4                | CTCCAGCTCGACTGTGCGC<br>GCACTTTGTGTTGAGTGACCTTTCG | 811               | Roy <i>et al.</i> , 1996          |
| <i>B. infantis</i>          | InfU5<br>InfL6                | CCATCTCTGGGATCGTCGG<br>TATCGGGGAGCAAGCGTGA       | 565               | Roy <i>et al.</i> , 1996          |
| <i>B. longum</i>            | LonU7<br>LonL8                | GCCGTATCTCTACGACCGTCG<br>TATCGGGGAGCAAGCGAGAG    | 567               | Roy <i>et al.</i> , 1996          |
| <i>B. adolescentis</i>      | BIA-1<br>BIA-2                | GGAAAGATTCTATCGGTATGG<br>CTCCAGTCAAAAGCGTT       | 244               | Wang <i>et al.</i> , 1996         |
| <i>B. longum</i>            | BIL-1<br>BIL-2                | GTTCCCGACGGTCGTAGAG<br>GTGAGTTCCCGGCATAATCC      | 153               | Wang <i>et al.</i> , 1996         |
| <i>B. breve</i>             | L<br>Bre-BV.R <sup>a</sup>    | GCTGGATCACCTCCTTCT<br>GCAAGAACGAGGAATCAAAGG      | 340               | Brigidi <i>et al.</i> , 2000      |
| <i>B. bifidum</i>           | PBI245f <sup>b</sup>          | GCTTGTTGGTGAGGTAACGGCT                           | 1180              | Dong <i>et al.</i> , 2000a        |
| <i>B. breve</i>             | PBR442f <sup>b</sup>          | AGGGAGCAAGGCACTTTGTGT                            | 991               | Dong <i>et al.</i> , 2000a        |
| <i>B. infantis</i>          | PIN710f <sup>b</sup>          | CTGTTACTGACGCTGAGGAGCT                           | 723               | Dong <i>et al.</i> , 2000a        |
| <i>B. adolescentis</i>      | PAD805f <sup>b</sup>          | GTGGGGACCAATCCACGGTC                             | 628               | Dong <i>et al.</i> , 2000a        |
| <i>B. longum</i>            | PLO965f <sup>b</sup>          | TCCCGACGGTCGTAGAGATAC                            | 467               | Dong <i>et al.</i> , 2000a        |
|                             | Lm3r-m                        | CGGGGTGCTGCCCACTTTCATG                           | –                 | Kaufmann <i>et al.</i> , modified |

<sup>a</sup> Specific primers based on 16S to 23S internal transcribed spacer sequences

<sup>b</sup> These specific primers were used in the multiplex PCR system with genus-specific primer Lm3r-m.

verification, 1 µl of the suspension is smeared onto a slide glass and Gram-stained. The bacterial suspension is heated at 95°C for 10 minutes, and 1 µl of the supernatant is subjected to PCR.

### Identification of the Isolates with Species-specific Primers

For the identification of the isolates, species-specific primers designed by Matsuki *et al.* (1999) should basically be used for the following reasons: the primers cover all of the bifidobacterial species that have been isolated from, and detected in, the human intestinal tract; specificity has been confirmed for all of the *Bifidobacterium* species; the PCR reactions can be conducted using the same thermal cycling conditions. PCR is carried out in a total volume of 25 µl of reaction mixture containing 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 2.5 mM of MgCl<sub>2</sub>, 200 µM of dNTP mixture, 25 µM of each primer, 0.45 U Taq DNA polymerase, and 1 µl of template DNA. The PCR amplification program consisted of one cycle of 94°C for 5 minutes, then 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, and finally one cycle of 72°C for 5 minutes. The products are separated by electrophoresis in 1% agarose gels, and ethidium bromide staining is performed to observe the presence of bands under UV transillumination. The use of Real-Time PCR equipment, which is discussed later, makes it possible to identify bacterial species from the melting curve analysis without conducting electrophoresis (Ririe *et al.*, 1997), thus making identification simpler in our laboratory.

It is useful to use genus-specific primers together with the species-specific primers because the bifidobacteria media are not completely selective. For the identification of bifidobacteria isolated from human adult feces, it is recommended to use primers for *B. adolescentis*, *B. longum*, the *B. catenulatum* group, and *B. bifidum* at first. In our study, more than 90% of the isolates were identified as one of these four species. Unidentified isolates were then examined with the primers targeting the remaining five species. For isolates from infant feces, it is efficient to apply the primers for *B. breve*, *B. infantis*, *B. longum*, and *B. bifidum* at first, and then test with the remaining primers. More than 80% of the bifidobacterial isolates from infants are identified as one of these four species. Specific primers for *B. breve*, *B. bifidum*, *B. longum*, and *B. infantis* are also capable of identifying isolates from dairy products. But it is necessary to use them in combination with the *B. lactis*-specific primers prepared by Ventura *et al.* (2001).

In addition to the primers described here, the multiplex PCR method reported by Dong *et al.* (2000a) is effective in the identification of cultured bifidobacteria. The reaction mixture has a total volume of 25 µl and contains 50-100 ng of template DNA, 100 pmol of dNTP, 2.5 mM of MgCl<sub>2</sub>, 10 pmol each of PBR442f and PAD805f, 20 pmol each of PBI245f and PLO965f, 30 pmol of PIN, 50 pmol of Im3r, and 7.5U Taq of polymerase. The condition of the thermal cycler is as follows: one cycle consisting of 95°C for 5 minutes, followed by 30 cycles consisting of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 60 seconds. Amplification products are detected by electrophoresis on

1% agarose, followed by ethidium bromide staining. This method can simultaneously identify the five species - *B. adolescentis*, *B. bifidum*, *B. breve*, *B. longum*, and *B. infantis* - based on the differences in PCR product sizes. The reported protocol needed a larger amount of Taq polymerase than is used in conventional PCR.

The specific primers for *B. infantis*, *B. breve*, and *B. longum*, which were prepared by Roy *et al.* (1996), and the *B. breve*-specific primers prepared by Bridgidi *et al.* (2000) are also available. These primers should be used in combination with the primers described by Matsuki *et al.* (1999). The primers prepared by Wang *et al.* (1997) have not been sufficiently tested for specificity, and their conditions were optimized by the capillary PCR method. Experiments using the primers under general thermal-cycling conditions require some caution. Brigidi *et al.* (2000) reported that the *B. longum*-specific primers BIL-1 and BIL-2 cross-react with some strains of *B. breve*.

### Specific PCR Detection of *Bifidobacterium*

The species-specific detection of bifidobacteria without cultivation is possible using the DNA extracted from feces as a PCR template. This section describes the DNA preparation method, specific PCR detection using fecal DNA, the distribution of bifidobacterial species in the human intestinal microflora.

### DNA Preparation Method

Fecal samples are difficult specimens with which to perform PCR analysis because (1) some bacterial cells are difficult to lyse (Zoetendal *et al.*, 1998) and, (2) the presence of multiple substances that inhibit the polymerase enzyme (Wilson, 1997; Satake *et al.*, 1997). Although various procedures have been employed in recent molecular studies to overcome these problems (Wang *et al.*, 1996; Kageyama *et al.*, 2000), it is necessary to standardize the DNA extraction procedure for future analyses.

Mechanical procedures, which shake the sample vigorously in the presence of glass beads, have been shown to be effective in disrupting bacterial cells from a variety of ecosystems (Zoetendal *et al.*, 1998). This method has been employed in an increasing number of studies (Zoetendal *et al.*, 1998; Ventura *et al.*, 2001). However, in our experience, PCR inhibitors are not completely removed by this procedure. The DNA solution can be diluted for use as the template, but the detection sensitivity is influenced by the degree of dilution. In order to remove PCR inhibitors, fecal samples should be washed in buffers before breaking the bacterial cells. The combination of the washing step and the mechanical disruption can provide DNA that is suitable for use in PCR analysis. The protocol of our DNA extraction method is described below.

A portion of a fecal sample is taken and uniformly suspended in nine parts by volume of PBS buffer. Then, 200 µl of the one-tenth feces dilution is placed in a 2-ml tube and stirred together with 1 ml of PBS and several glass beads (2-3 mm diameter). After centrifugation at 15,000 rpm for five minutes, the supernatant is removed. The procedure is repeated two additional times in the same

manner. These steps will produce a pellet that is the equivalent of 20 mg of feces. At this point the pellet can be cryopreserved, if necessary. The pellet is suspended in 500  $\mu$ l of lysate buffer (100 mM of Tris-HCl, 40 mM of EDTA, and 1% of SDS, pH 9.0), 0.3 g of small glass beads (0.1 mm diameter), and 500  $\mu$ l of buffer-saturated phenol. The mixture is vigorously shaken for 30 seconds with FastPrepFP120 at a power level of 5.0 m/s to physically break the bacterial cells. The suspension is then centrifuged, and 400  $\mu$ l of the supernatant is transferred to another tube. Then, 400  $\mu$ l of phenol-chloroform-isoamyl alcohol (25:24:1) is added and the mixture is shaken again, and centrifuged. Next, 250  $\mu$ l of the supernatant is transferred to another tube, and subjected to ethanol precipitation. If these steps are followed, 50% of the DNA fraction, or DNA equivalent to 10 mg of feces, will be collected. After being air-dried, the DNA is dissolved in 1 ml of TE, and 1  $\mu$ l of the solution is subjected to PCR. Instead of fecal homogenate, bacterial cultures or dairy products can also be used as the sample.

### Specific PCR Detection From Fecal DNA

The use of species-specific primers prepared by Matsuki *et al.* (1999) is recommended for the reasons given in the section on bacterial-species identification. In our protocol, PCR is conducted by repeating 35 cycles of three steps each, as described above. It is also recommended that the cycle number be increased to 40, as this will improve the detection limit by yet another order of magnitude. Under these conditions, the target bifidobacterial species were detected at a concentration of 10 cells per PCR assay (equivalent to  $10^6$  cells per g of feces) (Matsuki *et al.*, 1999).

It has been reported by Dong *et al.* (2000) that, with the multiplex PCR method, it is difficult to detect multiple species simultaneously. It would therefore be difficult to detect bifidobacteria in fecal samples by the multiplex PCR

method. The specific primers prepared by Roy *et al.* (1996) and Bridgidi *et al.* (2000) can be used in combination with our primers.

The advantage of this specific PCR technique is its high detection sensitivity. The conventional method, which identifies bacterial strains isolated from bifidobacteria-selective media, can only analyze the more numerous bacteria in the sample. As the predominant *Bifidobacterium* species are usually present at the level of  $10^9$  to  $10^{10}$  cells per gram in human feces (Mitsuoka *et al.*, 1974; Moore and Moore, 1995; Mutai and Tanaka., 1987), the detection limit of the culture method for minor bifidobacterial species is about  $10^8$  cells per g. In FISH analysis, the lower detection limit for reliable detection is  $10^8$  cells per gram (Franks *et al.*, 1998). Therefore, the specific PCR method has a sensitivity approximately 100 times greater than that of the culture and FISH methods. Other advantages include easy sample handling and simple operations. With the culture method, it is necessary to use fresh samples and perform operations after sampling, such as preparation of dilutions and culture, under strict anaerobic conditions. On the other hand, the PCR method does not require anaerobic conditions, and DNA can be preserved in the freezer, and it is possible to transport the DNA internationally. The scope of studies using PCR will increase in the future because of these factors. Establishment of the procedure for quantitative detection of bacteria is a future task and current progress will be described later in the review.

Satokari *et al.* (2001) combined the genus-specific primers with DGGE (denaturing gradient gel electrophoresis) to achieve species-specific detection of bifidobacteria in human feces. With the DGGE method, the PCR products were electrophoresed in polyacrylamide gels with a concentration gradient of DNA denaturant (urea and formamide) gradient to separate PCR products based on differences in their base sequences. When a fecal sample is analyzed with the bifidobacteria genus-specific primers, the 16S rRNA gene of each species can be detected as a separate band. This method has the advantage of being effective in the analysis of bifidobacterial species that are not targeted by the species-specific primers and it is considered effective in monitoring individual microfloras over time. The method has several problems, however, such as the fact that the formation of heteroduplexes may exhibit false bands, DNA fragments with different sequences may migrate to the same position, the copy number of 16S rDNA varies, and numerically subdominant species cannot be detected.

### Distribution of Bifidobacterial Species in the Human Gut Microflora

Examination of the bifidobacterial species distribution in the human intestinal tract was accomplished with the species-specific PCR method (Table 1)(Matsuki *et al.*, 1999). In adult intestinal tracts, the *B. catenulatum* group was the most common taxon, followed by *B. longum*, *B. adolescentis*, and *B. bifidum* (Table 2). In breast-fed infants, *B. breve* was the most frequently found species, followed by *B. infantis*, *B. longum* and *B. bifidum*. It was a notable finding that the *B. catenulatum* group inhabited human

Table 2. Distribution of the species of *Bifidobacterium* in human adults and infants

|                             | No. of positive samples<br>(% of total) |                   |
|-----------------------------|---|-------------------|
|                             | Adults<br>(n=48)                        | Infants<br>(n=27) |
| <i>B. adolescentis</i>      | 29 (60%)                                | 2 (7.4%)          |
| <i>B. angulatum</i>         | 2 (4.2%)                                | 1 (3.7%)          |
| <i>B. bifidum</i>           | 18 (38%)                                | 6 (22%)           |
| <i>B. breve</i>             | 6 (13%)                                 | 19 (70%)          |
| <i>B. catenulatum</i> group | 44 (92%)                                | 5 (19%)           |
| <i>B. longum</i>            | 31 (65%)                                | 10 (37%)          |
| <i>B. infantis</i>          | 0 (0%)                                  | 11 (41%)          |
| <i>B. dentium</i>           | 3 (6.3%)                                | 3 (11%)           |
| <i>B. gallicum</i>          | 0 (0%)                                  | 0 (0%)            |

The adults were  $38.8 \pm 8.9$  years old (mean  $\pm$  standard deviation), and the infants were  $31.2 \pm 4.5$  days old (Matsuki *et al.*, 1999).

adults with the highest frequency, although it has often been reported that *B. adolescentis* is the most common species (Mutai and Tanaka., 1987; Finegold *et al.*, 1974; Mitsuoka *et al.*, 1974). This contradiction may be explained by the difference in the identification methods used. It has been reported that *B. adolescentis*, *B. catenulatum*, and *B. pseudocatenulatum* are difficult to differentiate based on the usual carbohydrate fermentation pattern (Matsuki *et al.*, 1998). Therefore, the *B. catenulatum* group may have been confused with *B. adolescentis* in some studies. It is also interesting to note that *B. breve* was detected in adult fecal samples, even though it has been recognized as a typical infantile bifidobacterial species. This may be explained by the difference between the detection limits of the culture method and the specific PCR method. It will be interesting to determine the source of the bifidobacteria detected in infants. The highly sensitive specific PCR detection method stands a good chance of elucidating the route of transmission of bifidobacteria to infants.

### Quantitative PCR Detection

In the analysis of a microbial community structure, it is important to detect the target bacteria quantitatively. Although the PCR analyses have primarily been conducted qualitatively, the quantitative PCR analysis of some bacterial communities is already underway. There are two strategies for quantitative PCR detection: (1) the competitive PCR method using internal standards, and (2) real-time PCR detection using external standards. This section briefly explains these principles, and describes the quantitative PCR method using external standards that we have investigated.

### Competitive PCR Method

Until recently, quantitative PCR methods have been conducted primarily by the competitive PCR method using internal standards. With the competitive PCR method, competitive DNA fragments against the same primers are added at different concentrations to PCR solutions. Following PCR, the quantities of the PCR products from the target DNA and the competitive DNA fragments are compared to determine the quantity of the target DNA. In the case of the gastrointestinal microflora, the competitive PCR method has been used to count the number of *Clostridium proteoclasticum* and its related species present in rumen contents (Reilly and Attwood, 1998). This report suggested that the method was applicable to human fecal microflora studies. However, the method requires the preliminary preparation of competitive DNA, and the amplification efficiency of the competitive DNA and target DNA must be the same. There are many other measurement limits and thus establishment of the method requires considerable effort.

### Quantitative PCR With External Standards

In recent years, real-time PCR equipment has become commercially available. This equipment provides a quantification method using external standards; the quantity

of PCR products is measured in every cycle using fluorescent substances such as SYBR Green I and TaqMan probe, and is quantified based on an amplification curve. There have already been some reports on quantitative PCR detection using bacterial species-specific primers (Morrison *et al.*, 1999; Pahl *et al.*, 1999; Tajima *et al.*, 2001; Kageyama *et al.*, 2000). We studied a quantitative PCR detection method for the genus *Bifidobacterium* using a Light Cycler, which is described below.

### Quantitative PCR Detection of the Genus *Bifidobacterium*

This method analyzes DNA extracted from feces, using DNA extracted from pure cultures and enumerated bifidobacteria as an external standard. In our study, SYBR Green I was used as the fluorescent substance, and a Light Cycler were used as the detection equipment for PCR. The reaction solution had a total volume of 10  $\mu$ l and contained 10 mM of Tris-HCl, pH 8.3; 50 mM of KCl; 1.5 mM of MgCl<sub>2</sub>; 500 ng/ $\mu$ l of bovine serum albumin; 200  $\mu$ M of each dNTP; template DNA; 1:30,000 dilution of SYBR Green I (Molecular Probes, Eugene, OR); 11 ng/ $\mu$ l of TaqStart™ antibody (ClonTech, Palo Alto, CA); 0.05 U/ $\mu$ l of Taq DNA polymerase (Takara, Tokyo, Japan); and 0.25  $\mu$ M of the *Bifidobacterium* group-specific primers (g-Bifid-F [5'-CTC CTG GAA ACG GGT GG-3'] and g-Bifid-R [5'-GGT GTT CTT CCC GAT ATC TAC A-3']). The PCR was conducted as follows: 40 cycles of three steps each, comprised of heating at 20°C/sec to 95°C with a 0-second hold, cooling at 20°C/sec to 55°C with a 10-second hold, and then heating at 20°C /sec to 72°C with a 20-second hold. Fluorescent product was detected in the last step of each cycle. After amplification, a melting curve was obtained by heating at 20°C/sec to 96°C, cooling at 20°C/sec to 60°C, and slowly heating at 0.2°C/sec to 96°C, with fluorescence collection at intervals of 0.2°C. Melting curves were used to determine the specificity of the PCR. Comparison of bifidobacterial count obtained by culture and real-time PCR showed that there was good correlation (Requena *et al.*, 2002).

The approach to detect the target bacteria with quantitative PCR described here will contribute to future studies of the composition and dynamics of the intestinal microflora. In addition, automation of DNA extraction and distribution of PCR solutions will be necessary to reduce the labor required.

### Conclusion

This review describes the advantages of PCR methods in the analysis of the gut ecosystem with emphasis on the species-specific primers for bifidobacteria. The PCR methods allow the highly sensitive detection of specific bacteria and will have a significant effect on the analysis of gut community structure. The next significant challenges for researchers are the selection of bacterial genera and species for the preparation of further primers, and the selection of appropriate subjects for analysis.

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