Application of New Primer-Enzyme Combinations to Terminal Restriction Fragment Length Polymorphism Profiling of Bacterial Populations in Human Feces

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New primer-enzyme combinations for terminal restriction fragment length polymorphism (T-RFLP) targeting of the 16S rRNA gene were constructed by using the T-RFLP analysis program (designated TAP T-RFLP) located at the Ribosomal Database Project website, and their performance was examined empirically. By using the fluorescently labeled 516f primer (Escherichia coli positions 516 to 532) and 1510r primer (positions 1510 to 1492), the 16S rRNA gene was amplified from human fecal DNA. The resulting amplified product was digested with RsaI plus BfaI or with BstI. When the T-RFLP was carried out with fecal DNAs from eight individuals, eight predominant operational taxonomic units (OTUs) were detected with RsaI and BfaI digestion and 14 predominant OTUs were detected with BstI digestion. The distribution of the OTUs was consistent with the results of the computer simulations with TAP T-RFLP. The T-RFLP analyses of the fecal DNA from individuals gave characteristic profiles, while the variability of the T-RFLP profiles between duplicate DNA preparations from the same samples was minimal. This new T-RFLP method made it easy to predict what kind of intestinal bacterial group corresponded to each OTU on the basis of the terminal restriction fragment length compared with the conventional T-RFLP and, moreover, made it possible to identify the bacterial species that an OTU represents by cloning and sequencing.

A large number of bacteria exist in the human intestinal tract, which form a complex community referred to as the intestinal microflora. The microflora is affected by various factors, such as food and medicine, and the microflora itself can affects human health. Therefore, to assess the effects of the microflora, a method that is able to easily and rapidly monitor the changes of the flora is required. Conventional methods for flora analysis that rely on the cultivation of bacteria under anaerobic conditions are time-consuming and laborious and miss the large population of bacteria that are difficult to culture or are unculturable (6). Consequently, culture-independent molecular tools, based on the detection of 16S rRNAs or the corresponding genes, have been used recently for the analysis of bacterial communities; these include fluorescent in situ hybridization (FISH) (4, 7, 9), temperature gradient gel electrophoresis (TGGE) (21), denaturing gradient gel electrophoresis (DGGE) (13, 18, 21), the random cloning approach (19), and terminal restriction fragment polymorphism (T-RFLP) (1, 5, 10, 11). These methods have various advantages and disadvantages and therefore complement each other.

T-RFLP, and particularly T-RFLP using a capillary electrophoresis sequencer, seems to have the advantages of higher throughput and reproducibility in monitoring the bacterial community (16). Therefore, we intended to apply T-RFLP to monitoring the state of human intestinal microflora during a particular diet or a medical treatment and for assessing the effect of health foods or medicines on the microflora. However, to date, the primer-enzyme combination typically used in T-RFLP analysis, namely, the combination of the 5f PCR primer (Escherichia coli positions 8 to 27) and HhaI, MspI, or others (10, 11, 14), has had a number of problems. First, the primer is not suitable for amplification of 16S rRNA genes from some species of Bifidobacterium (11, 19), which are some of the predominant bacteria in the human intestinal tract and are considered to play an important role in the maintenance of the intestinal environment under healthy conditions. Second, since many sequences in the Ribosomal Database Project (RDP) lack the 5f primer sequence and the primer-enzyme combination yields a relatively complex T-RFLP profile, it is difficult to fully and easily assign bacterial species to the terminal restriction fragments (T-RFs) (10).

To overcome these problems, we searched for new primer-enzyme combinations for T-RFLP analysis that have the capacity to distinguish a bacterial community in human feces at the genus level by using the T-RFLP analysis program (TAP T-RFLP) located at the RDP website (12) and empirically examined the performance of the adopted combinations. The size distribution of the T-RFs generated by the new primer-enzyme combination was consistent with that of the T-RFs derived from an in silico digestion of the sequences of the intestinal bacteria in the RDP. This T-RFLP method reduced the complexity of the profile so that data analyses could readily be made. In addition, this method made it possible to recover the T-RFs by agarose gel electrophoresis, so that they could then be cloned and sequenced.

MATERIALS AND METHODS

TAP T-RFLP analysis. TAP T-RFLP was performed for every combination of five different 16S rRNA gene universal primers, where two base mismatches were

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allowed within the sequence providing that they did not occur within 3 bases from the 3' end of the primer, and all restriction enzymes with a 4-bp recognition site that are entered into the program. The five primers used were 341f (5'-CC AGGCGGACGCACTGAGG-3'; E. coli positions 341 to 360), 518f (5'-TGC CAGCGTGCCCTGTTAC-3'; E. coli positions 518 to 532), 926f (5'-GCATCGCT 5'-TTCTTTAGTTT-3'; E. coli positions 926 to 970), 1406r (5'-ACGGCAAGCG TGTGATC-3'; E. coli positions 1406 to 1392), and 1510r (5'-GTTGATTCC TTACGACT-3'; E. coli positions 1510 to 1540). The lengths of T-RFs produced by in silico digestion of the 16S RNA gene sequences from bifidobacteria (53 sequences), Bacteroides (46 sequences), Prevotella (48 sequences), clindamycin-resistant streptococci (38 sequences), enterococci (84 sequences), lactobacilli (106 sequences), and E. coli (39 sequences) were summed.

DNA isolation from fecal samples. Two hundred to 500 milligrams of fresh fecal samples from eight healthy individuals (A to H), differing in age (2 weeks to 49 years old) and sex (five males and three females), were collected and stored at 4°C until use (not more than several hours). The fecal samples were suspended in 9 volumes of sterile distilled water by vigorous shaking. After the samples were allowed to stand for 10 min, aliquots of 0.1 ml were transferred into a 1.5-ml tube containing 0.9 ml of distilled water and the tube was centrifuged at 18,000 g for 5 min. The pellet was washed twice by centrifugation in 1 ml of distilled water followed by centrifugation and was finally suspended in 250 μl of a solution containing 100 mM Tris- HCl (pH 9.0) and 40 mM EDTA. The suspension was transferred into a 0.5-ml tube containing about 0.6 g of glass beads (diameter, 0.1 mm), treated at 5,000 rpm for 3 min in a mini-bead beater (BioSpec Products, Bartlesville, Okla.) (21, 22), and recovered according to the manufacturer's instructions. DNA was extracted from the bead-treated suspension by using benzy1 chloride as described by Zhu et al. (20). Briefly, 150 μl of benzyl chloride and 50 μl of 10% sodium dodecyl sulfate were added to the suspension, and the mixture was vigorously shaken at 50°C for 30 min. Thereafter, 150 μl of 3 M sodium acetate was added, and the mixture was set on ice for 15 min and then centrifuged at 18,000 × g for 10 min. The upper layer was recovered, and DNA was precipitated by addition of an equal volume of isopropanol. After the pellet was washed twice by centrifugation, it was dissolved in 10 μl of TE (pH 8.0, 0.1 mM EDTA) containing 50 μg of RNase ml \(-1\) and incubated at 37°C for 30 min. Next, the DNA preparation was purified by using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, N.J.) and quantified and analyzed by measurement at 260, 280, and 320 nm with a DU 7000 spectrophotometer (Beckman, Fullerton, Calif.). The concentration of DNA was calculated by the following equation: micrograms milliliter \(^{-1}\) = \[360 × (A_{260} - A_{230}) + 62.9 × (A_{280} - A_{260})\] (see the manual for the Beckman spectrophotometer). Finally, the DNA preparation was adjusted to a final concentration of 10 μg ml \(^{-1}\) in TE and checked by 1% agarose gel electrophoresis.

Enzyme (lysozyme and achromopeptidase) treatment of the washed fecal sample was performed as described by Liu et al. (11).

PCR conditions. PCR was performed with a model 2400 thermal cycler (Applied Biosystems, Foster City, Calif.) in a reaction mixture (20 μl) containing 0.05 μl of fourfold-diluted GeneScan-2500 size standard (Applied Biosystems) and 12 μl of deionized formamide, followed by denaturation at 90°C for 2 min and immediate chilling on ice. The incubation time was 20 s for analysis of T-RFs from the digestion with RsaI plus BglII and 40 s for that of T-RFs from the digestion with BseI or BclI. The run time was 40 min.

Construction and analysis of clone library. Using the fecal DNA preparations from individuals C and G, 16S rRNA genes were amplified by the above-described PCR condition except that nonlabeled 516f primer was used. The resulting PCR products were purified through S-400HR MicroSpin columns (Amersham) and cloned into E. coli TOP10 by using the TOP10 TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, Calif.). Insert DNAs (about 1,000 bp long) were recovered by the colony direct PCR method with T3 and T7 primers. The DNA sequences were purified by using the MultiScreen BF filter plate (Millipore) and were sequenced by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Chimeric sequences were detected with the RDP CHECK CHIMERA program. Homology searches of the obtained sequences were performed with the BLAST and FASTA programs at the web site of the DNA Data Bank of Japan (DDBJ).

Cloning and sequencing of T-RFs. PCR was performed as described above except that 0.4 μm concentrations of biotin-labeled NolI-516f primer (5'-TAG ACAGGCGGACGCACTGAGG-3') and 1510r primer were used. The resulting PCR products were purified through S-400HR MicroSpin columns (Amersham) and cloned into E. coli TOP10 by using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, Calif.). Insert DNAs (about 1,000 bp long) were recovered by the colony direct PCR method with T3 and T7 primers. The DNA sequences were purified by using the MultiScreen BF filter plate (Millipore) and were sequenced by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Chimeric sequences were detected with the RDP CHECK CHIMERA program. Homology searches of the obtained sequences were performed with the BLAST and FASTA programs at the web site of the DNA Data Bank of Japan (DDBJ).

T-RFLP data analysis. The lengths and peak areas of T-RFs were determined with the GeneScan software. A standard curve was drawn by using DNA fragments with lengths of 94, 109, 116, 172, 186, 222, 233, 238, 269, 286, 361, 470, 490, 536, 827, and 1,115 bp, and 1,100 bp in order to match the real size (995 bp) of the fragment of 536, 827, and 1,115 bp by the local Southern method, in which the fragment of 995 bp was treated as 94, 109, 116, 172, 186, 222, 233, 238, 269, 286, 361, 470, 490, 536, 827, and 1,115 bp.

To compare the T-RFLP patterns among samples, the dissimilarity \((x_kj)\) index, where

\[
x_{ki} = \frac{\text{area of T-RF} \times \text{peak area of fragment} \times \text{percentage of peak area} \\times 100}{\text{area of T-RF} \times \text{peak area of fragment} \\times \text{percentage of peak area} \\times 100}
\]

To determine which T-RFs provided meaningful differences among the samples, we used the above-described T-RFLP patterns.
RESULTS AND DISCUSSION

New primer-enzyme combinations for T-RFLP analysis. Based on computer simulations, Liu et al. (11) concluded that amplification with the primer pair 8f-926r followed by digestion with HhaI or MspI was the simplest way to classify the largest number of 16S rRNA gene sequences into the largest related species to the sequences and the OTUs accompanied by individual names.

The representative sequences of 111- and 124-bp T-RF clones derived from individual G and those of 376-and 377-bp T-RF clones derived from individual H have been deposited in the DDBJ database under accession numbers AB086439, AB086440, AB086441, and AB086442, respectively. The DDBJ accession numbers of representative sequences from the clone library from individuals C and G, with the most closely related species to the sequences and the OTUs accompanied by individual names and restriction enzyme names as subscripts in parentheses, are as follows: AB094149 (Bifidobacterium adolescentis, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,C}), AB094150 (Bifidobacterium longum, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,C}), AB094151 (Bifidobacterium psudocatenulatum, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,C}), AB094152 (Clostridium indolis, 130-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094153 (Clostridium nEc, 130-bp OTU\textsubscript{R,C}/944-bp OTU\textsubscript{Bsl,C}), AB094154 (Eubacterium eligens, 130-bp OTU\textsubscript{R,C}/919-bp OTU\textsubscript{Bsl,C}), AB094155 (Roseburia intestinals, 114-bp OTU\textsubscript{R,B,G}/749-bp OTU\textsubscript{Bsl,C}), AB094156 (Ruminococcus gravis, 130-bp OTU\textsubscript{R,C}/940-bp OTU\textsubscript{Bsl,C}), AB094157 (Ruminococcus obeum, 130-bp OTU\textsubscript{R,C}/749-bp OTU\textsubscript{Bsl,C}), AB094158 (Ruminococcus orbiEc, 130-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094159 (Ruminococcus orbiEc, 138-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094160 (Clostridium indolis, 158-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094161 (Clostridium saccharolyticum, 158-bp OTU\textsubscript{R,C}/494-bp OTU\textsubscript{Bsl,C}), AB094162 (Clostridium saccharolyticum, 158-bp OTU\textsubscript{R,C}/494-bp OTU\textsubscript{Bsl,C}), AB094163 (Eubacterium eligens, 158-bp OTU\textsubscript{R,C}/494-bp OTU\textsubscript{Bsl,C}), AB094164 (Ruminococcus gravis, 158-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094165 (Ruminococcus orbiEc, 158-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB095023 (Ruminococcus obeum, 158-bp OTU\textsubscript{R,C}/956-bp OTU\textsubscript{Bsl,C}), AB094166 (Ruminococcus obrEc, 158-bp OTU\textsubscript{R,C}/944-bp OTU\textsubscript{Bsl,C}), AB094167 (Ruminococcus lacticum, 158-bp OTU\textsubscript{R,C}/939-bp OTU\textsubscript{Bsl,C}), AB094168 (Ruminococcus Schinkii, 158-bp OTU\textsubscript{R,C}/956-bp OTU\textsubscript{Bsl,C}), AB094169 (Bacteroides distasonis, 308-bp OTU\textsubscript{R,C}/469-bp OTU\textsubscript{Bsl,C}), AB094170 (Clostridium orbiscindens, 308-bp OTU\textsubscript{R,C}/370-bp OTU\textsubscript{Bsl,C}), AB094171 (Fusobacterium prausnitzii, 308-bp OTU\textsubscript{R,C}/749-bp OTU\textsubscript{Bsl,C}), AB094172 (Ruminococcus bromii, 308-bp OTU\textsubscript{R,C}), AB094173 (Ruminococcus Schinkii, 308-bp OTU\textsubscript{R,C}/494-bp OTU\textsubscript{Bsl,C}), AB094174 (Bacteroides acidofaciens, 377-bp OTU\textsubscript{R,C}/370-bp OTU\textsubscript{Bsl,C}), AB094175 (Bacteroides eggerthii, 377-bp OTU\textsubscript{R,C}/370-bp OTU\textsubscript{Bsl,C}), AB094176 (Bacteroides thetaiotaomicron, 377-bp OTU\textsubscript{R,C}/370-bp OTU\textsubscript{Bsl,C}), AB094177 (Bacteroides vulgatus, 377-bp OTU\textsubscript{R,C}/969-bp OTU\textsubscript{Bsl,C}), AB094178 (Ruminococcus obeum, 377-bp OTU\textsubscript{R,C}/944-bp OTU\textsubscript{Bsl,C}), AB094179 (Bifidobacterium breve, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,G}), AB094180 (Bifidobacterium infantis, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,G}), AB094181 (Bifidobacterium longum, 123-bp OTU\textsubscript{R,C}/124-bp OTU\textsubscript{Bsl,G}), AB094182 (Ruminococcus obeum, 136-bp OTU\textsubscript{R,C}/754-bp OTU\textsubscript{Bsl,G}), AB094183 (Bacteroides thetaiotaomicron, 142-bp OTU\textsubscript{R,C}/967-bp OTU\textsubscript{Bsl,G}), AB094184 (Bacteroides thetaiotaomicron, 142-bp OTU\textsubscript{R,C}/947-bp OTU\textsubscript{Bsl,G}), AB094855 (Veillonella ratti, 159-bp OTU\textsubscript{R,C}/110-bp OTU\textsubscript{Bsl,G}), AB094186 (Streptococcus pasteurii, 308-bp OTU\textsubscript{R,G}/332-bp OTU\textsubscript{Bsl,G}), AB094187 (E. coli, 377-bp OTU\textsubscript{R,C}/940-bp OTU\textsubscript{Bsl,C}), and AB094188 (Enterococcus malodoratus, 377-bp OTU\textsubscript{R,C}/520-bp OTU\textsubscript{Bsl,C}).
number of unique 5’ T-RFs. Since then, the combination of the labeled 8f primer and these enzymes has been used in almost all cases of T-RFLP analysis. However, the T-RFLP pattern produced by this method is so complex that data analysis followed by comparison of the patterns among samples cannot readily be done. To resolve this problem and to apply T-RFLP to monitoring changes of bacterial populations in the human gut, we have examined new primer-enzyme combinations and

FIG. 2. Effect of annealing temperature in PCR on T-RFLP profile. (A) Electropherograms of the T-RFs produced by BsuI digestion of 16S rRNA gene amplicons from PCR at the indicated annealing temperatures. Fecal DNA from individual G was used as the PCR template. Arrows indicate the positions of OTUs: 110, 124, 160, 332, 370, 469, 494, 520, 657, 749, 853, 919, 940, 995, and 990 bp from the left side. Vertical bars indicate the positions of size markers: 94, 109, 116, 172, 186, 222, 233, 238, 269, 286, 361, 470, 490, 516, and 827 bp from the left side. (B) Comparison of relative abundances of OTUs among the electropherograms. Data are expressed as means ± standard deviations from three independent experiments with the same DNA preparation. The numbers in the key indicate the annealing temperature (°C). D values are presented in the inset table.
have identified those that provided the best resolution for major genera known to be present in the gut, including the *Bifidobacterium*, *Bacteroides*, *Prevotella*, *Clostridium*, *Ruminococcus*, *Eubacterium*, *Enterococcus*, *Streptococcus*, and *Lactobacillus*, and for *E. coli* by using TAP T-RFLP. TAP T-RFLP was run as described in Materials and Methods with every combination of five different universal primers (341f, 516f, 926r, 1406r, and 1510r) and 4-bp cutters of restriction enzymes. As a result, two combinations of 516f-*Rsa*I plus *Bfa*I and 516f-*Bsl*I were selected. Frequency distribution profiles of terminal fragments derived from TAP T-RFLP with each of these combinations are shown in Fig. 1. The profiles indicate that either of the two combinations distinguishes 16S rRNA gene sequences derived from the fecal bacterial community at the genus level or higher comparatively well, although the latter combination had a higher resolution than the former one. T-RFLP profiling of the same sample with these two combinations seemed to provide more information about

FIG. 3. Effect of cycle number in PCR on T-RFLP profile. (A) Electropherograms of the T-RFs produced by *Bsa*I digestion of 16S rRNA gene amplicons from PCR with the indicated cycle number. Fecal DNA from individual G was used as the PCR template. Arrows and bars are as described for Fig. 2. (B) Comparison of relative abundances of OTUs among the electropherograms. Data are expressed as means ± standard deviations from three independent experiments with the same DNA preparation. The numbers in the key indicate the cycle number of the PCR. D values are presented in the inset table.
the constituents of a bacterial community in feces. Many of the T-RFs shown in Fig. 1 were detected in T-RFLP analysis with fecal samples, as described in detail below.

Conditions for T-RFLP analysis with new primer-enzyme combinations. It was predicted that T-RFs of over 900 bp would be produced in the case of BslI digestion, as shown in Fig. 1. Therefore, the 1510r universal primer was selected as a reverse primer. Since the melting temperatures of 516f and 1510r are 72.8 and 53.1°C, respectively, PCR was performed at a different annealing temperature of approximately 50°C (48, 49, 50, 51, and 52°C over 30 amplification cycles) with the fecal DNA and then the PCR products were subjected to T-RFLP analysis. It was found that there are only minor differences among the T-RFLP profiles derived from different annealing temperatures (Fig. 2). The larger the difference in the annealing temperature was, the larger was the D value between the T-RFLP profiles (Fig. 2B); the D values among the T-RFLP profiles derived from different annealing temperatures ranged from 2.7 to 11.6, while those between the T-RFLP profiles derived from the experiments at 50°C and at the other temperatures were less than 7. A similar result was observed in the T-RFLP analysis of 16S rRNA gene amplicons obtained after 20, 25, 30, or 35 cycles of amplification at an annealing temperature of 50°C (Fig. 3); the D values among the T-RFLP
TABLE 1. Correspondence of OTUs to bacteria

<table>
<thead>
<tr>
<th>Restriction enzyme(s) and OTU</th>
<th>Bacteria from which sequences in RDP were derived (predicted T-RF length [bp]:no. of sequences)</th>
<th>Most closely related species (predicted T-RF length [bp]:no. of clones:similarity [%]) predicted from sequences obtained from a clone library from:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsal-Bfla</td>
<td>Eubacteria (112:1, 114:1), Prevotella (111:1, 114:1), streptococci (113:1, 114:102)</td>
<td>Roseburia intestinalis (114:1:97)  None</td>
</tr>
<tr>
<td>109</td>
<td>Bifidobacteria (125:1, 126:45), clostridia (125:1, 129:1)</td>
<td>Bifidobacterium adolescentis (126:2:99), Bifidobacterium longum (126:3:99–100), Bifidobacterium pseudocatenulatum (126:1:99)</td>
</tr>
<tr>
<td>138</td>
<td>Eubacteria (254:1)</td>
<td>None</td>
</tr>
<tr>
<td>377</td>
<td>Clostridia (114:3), eubacteria (114:3), lactobacilli (114:3), Veillonella (114:5)</td>
<td>None</td>
</tr>
<tr>
<td>110</td>
<td>Bifidobacteria (127:40, 126:1)</td>
<td>None</td>
</tr>
<tr>
<td>124</td>
<td>B. lactis (317:12, 318:3)</td>
<td>None</td>
</tr>
<tr>
<td>317</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>332</td>
<td>Bifidobacteria (329:1), clostridia (330:1, 336:1, 337:1), eubacteria (330:1), lactobacilli (335:1), Prevotella (328:1, 329:1), streptococci (327:8, 328:71)</td>
<td>None</td>
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profiles derived from different amplification cycles ranged from 3.5 to 12.5, while those between the T-RFLP profiles derived from the experiments with 30 cycles of amplification and those with the other amplification cycles were less than 10 (Fig. 3B). Finally, we adopted 50°C as an annealing temperature and 30 amplification cycles.

One of the most critical factors for bacterial community analysis using molecular tools is the ability to efficiently extract DNA from every variety of bacteria, especially gram-positive bacteria. Therefore, we tried three methods for DNA extraction: method I, treatments with bacteriolytic enzymes, bead beating, and benzyl chloride (in that order); method II, treatments with bead beating and benzyl chloride (in that order); and method III, treatment with benzyl chloride. There was little difference between methods I and II in terms of the amount and integrity of the extracted DNA (data not shown) and the appearance of the T-RFLP profiles (Fig. 4). Compared with methods I and II, the treatment with only benzyl chloride (method III) resulted in the smallest amount of extracted DNA (half as much as in methods I and II), an extreme decrease in the relative quantity of the 159-bp OTU that seems to mainly represent gram-positive bacteria, and a large increase in the relative quantity of the 138-bp OTU that seems to mainly represent gram-negative bacteria (see Table 1). The D

<table>
<thead>
<tr>
<th>Restriction enzyme(s) and OTU</th>
<th>Bacteria from which sequences in RDP were derived</th>
<th>Most closely related species (predicted T-RF length [bp]:no. of clones:similarity [%])</th>
<th>Individual C</th>
<th>Individual G</th>
</tr>
</thead>
<tbody>
<tr>
<td>469 Bacteroides (466:6, 467:37), clostridia (465:1, 466:7, 468:17, 469:1, 472:1), eubacteria (466:1, 468:1, 469:2, 472:3), Prevotella (467:2, 468:1)</td>
<td>Bacteroides distasonis (467:1:98), Bacteroides vulgatus (467:1:98)</td>
<td>None</td>
<td></td>
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<tr>
<td>853 Bacteroides (849:1)</td>
<td>None</td>
<td>Bacteroides thetaiotaomicron (847:1:99)</td>
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<tr>
<td>919 Enterococci (918:2, 920:3), eubacteria (912:1, 914:2), ruminoococci (913:3, 914:1, 915:8, 916:1)</td>
<td>None</td>
<td>Eubacterium eligens (919:1:93)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>990 Bacteroides (NA:4), clostridia (NA:10), eubacteria (NA:7), E. coli (NA:17), Prevotella (NA:4), fusobacteria (NA:7), ruminoococci (NA:6)</td>
<td>None</td>
<td>None</td>
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</table>

*a A 172-bp T-RF is produced from each sequence of these clones by in silico digestion with BslI.

*b A 69-bp T-RF is produced from the sequence of one of these clones by in silico digestion with BslI.

*c NA, not applicable.
value between T-RFLP profiles derived from methods I and II was 11.9, and that between profiles derived from methods II and III was 32.4 (Fig. 4B). Moreover, when *Staphylococcus aureus* cells were added to a fecal sample and the DNA was extracted by method I, II, or III, followed by PCR with *S. aureus*-specific primers (2, 15), *S. aureus* DNA amplicons were detected at a fold-higher dilution in the cases of DNA extracts from methods I and II compared to that from method III (data not shown). Therefore, we decided to extract the DNA by using bead beating and benzyl chloride as described in Materials and Methods. The amounts of DNA extracted from fecal samples (about 10 mg [wet weight]) from eight individuals by using method II ranged from 1.1 to 6.3 μg.

**FIG. 5.** T-RFLP profiling of fecal samples from different individuals with *Rsa*I-plus- *Bfa*I digestion. (A) Electropherograms of the T-RFs produced by *Rsa*I-plus- *Bfa*I digestion of 16S rRNA gene amplicons from fecal DNAs of eight individuals (individual A, 48-year-old male; individual B, 49-year-old female; individual C, 38-year-old male; individual D, 38-year-old male; individual E, 2-year-old female; individual F, 37-year-old male; individual G, 2-month-old female; individual H, 2-week-old female). Arrows and bars are as described for Fig. 4. (B) Relative abundances of OTUs in each electropherogram. Peak area is expressed as an average from experiments with two different DNA preparations from the same fecal sample. The numbers in the key indicate OTUs.

**T-RFLP analysis of fecal samples from different individuals.**
The T-RFLP analysis was performed with fecal DNAs from eight individuals. Overall, eight dominant OTUs with lengths of 109, 123, 130, 138, 159, 251, 308, and 377 bp in the case of digestion with Rsal plus BfaI (designated OTU_{Rsal}) (Fig. 5A) and 14 dominant OTUs with lengths of 110, 124, 160, 317, 332, 370, 469, 494, 520, 657, 749, 853, 919, 940, 990, and 995 bp in the case of digestion with BslI (designated OTU_{BslI}) (Fig. 6A) were detected. The size distribution of these OTUs in the T-RFLP profile derived from each enzyme digestion showed good agreement with that of the T-RFs produced by in silico digestion of 16S rRNA gene sequences from the intestinal bacteria with the same enzyme, although there is a minor difference in size due to the accuracy limitations of electrophoresis. The T-RFLP profiles from the individuals were highly distinctive from one another. The $D$ values...
between the profiles from different individuals ranged from 8.8 to 76.0 for Rsal-plus-BfaI digestion (Fig. 5B) and from 23.8 to 88.0 for BsiI digestion (Fig. 6B), while the D values between the profiles from T-RFLP analyses with two different DNA preparations extracted from the same fecal sample ranged from 1.7 to 10.2 (average of 5.5) for Rsal-plus-BfaI digestion and from 1.5 to 11.0 (average of 4.5) for BsiI digestion. This indicates that our T-RFLP method has the capacity to distinguish between the variation in the fecal bacterial communities observed among individuals.

**Assignment of bacterial groups to OTUs.** By comparing the results of the computer simulation shown in Fig. 1, we attempted to assign bacterial groups to some OTUs in Fig. 5 and Fig. 6 (Table 1). Since the in silico digestion of 16S rRNA gene sequences from many strains belonging to the genus *Veillonella*, the 332-bp OTU was to streptococci, the 370-bp OTU to *Bacteroides*, the 469-bp OTU to *Bacteroides*, and the 520-bp OTU to enterococci.

The 377-bp OTU is roughly divided into the 373-bp T-RF and the T-RFs of other lengths. These T-RFs were detected as different peaks in T-RFLP profiles derived from different individuals; in this case, the 373-bp T-RF may represent the *Bacteroides* group (Table 1).

**Identification of OTU-representing bacterial species by cloning of T-RFs.** In the DGGE-TGGE analysis, characteristic bands are cloned and sequenced after their purification by repetition of PCR followed by further DGGE-TGGE to identify bacterial species from which the bands are derived. However, in T-RFLP analysis, cloning and sequencing of the T-RFs has not been tried so far, which is probably due to the high complexity of T-RF patterns. Our T-RFLP method provides moderately complex profiles with comparatively evenly distributed OTUs. Therefore, this method enables relatively accessible cloning of the OTUs followed by sequencing without further purification of T-RFs.

As examples, we separated T-RFs that composed the 124-bp OTU from individual G (2 months old) and the 377-bp OTU from individual H (2 weeks old) (Fig. 7) and cloned them into *E. coli* DH5α as described in Materials and Methods. In the first case, of the insert DNA sequences from 12 transformants, 11 sequences (124 bp long, including the 516f sequence) were determined, within which there were four sequence variants with one to three base substitutions. The sequences showed over 99% similarity to the corresponding sequences of some kinds of bifidobacteria (for example, *B. catenulatum* [accession no. AF432082] and *B. breve* [AF491836]). This result together with the above-mentioned results indicates that the 124-bp OTU was derived from bifidobacteria and that our T-RFLP method makes it possible to readily detect and monitor bifidobacteria in the fecal community. The remaining sequence (111 bp long), which is presumably derived from the 110-bp OTU in individual G, showed 100% similarity to the corresponding sequence of *Alisonella histimoniformans* (AF548373). In the second case, of the insert DNA sequences from 12 transformants, nine sequences (376 bp long) were determined, within which there were three sequence variants with one or two base substitutions. The sequences showed over 99% similarity to the corresponding sequence of *E. coli* (AE005607) or related bacterial species (for example, *Shigella sonnei* [X96964]). The remaining three sequences (377 bp long), which contained two sequence variants with one substitution, showed over 99% similarity to the corresponding sequences of some kinds of enterococci (for example, *E. casseliflavus* [AF367978] and *E. gallinarum* [AJ420805]). This result for individual H shows good agreement with the report of Favier et al. (3), who detected DGGE
bands derived from E. coli and Enterococcus spp. in the feces during the first few days of life.

In conclusion, we have developed a new T-RFLP analysis method in which new primer-enzyme combinations were used. This method had high reproducibility and throughput and low cost of performance. Therefore, this system is considered to be useful for monitoring or mass screening of fecal samples prior to detailed analysis using FISH or cultivation of bacteria. In addition, the procedure allows the cloning and sequencing of the T-RFs characteristic of either a healthy condition or some types of diseases and allows identification of the bacterial species from which the T-RFs were derived. Since the nucleotide sequence data from the 16S rRNA gene clone library can be directly related to the OTUs detected by T-RFLP analysis, collection of such types of data from some individuals will increase the utility of our T-RFLP method. We have been acquiring more sequencing data from the clone library. It is expected that the accumulation of the T-RFLP profile data derived from different individuals will provide us with new insights into the relationships between a host and its microflora.

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