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Improved accuracy in terminal restriction fragment length polymorphism phylogenetic analysis using a novel internal size standard definition

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Background: Terminal restriction fragment length polymorphism (T-RFLP) analysis is commonly used to analyze microbial communities, including oral microflora. However, accurate identification of terminal restriction fragment (T-RF) origins is prevented by unpredictable errors in sizing, thus necessitating the clone library analysis. To minimize sizing errors, we proposed optimizing the size definition of internal standards.
Methods: GeneScan-1000 ROX was regenerated as an internal standard by redefining the fragment sizes in terms of molecular weight (MW) based on their mobility relative to 6-carboxyfluorescein (FAM) -labeled restriction fragments derived from the 16S recombinant RNA gene of *Porphyromonas gingivalis*. Using the new size definition, the average sizing error among eight oral bacteria from six phyla was estimated and compared with that of the conventional method. Microbial communities isolated from saliva were analyzed using the new MW size definition. Bacterial species were assigned to peaks using TRFMA, a Web-based tool for T-RFLP analysis, and compared with those identified in a clone library analysis.
Results: Using the new size definition, the average sizing error for 40 T-RFs was drastically reduced from 2.42 to 0.62 bases, and large sizing errors (more than two bases) were eliminated. More than 90% of the total bacterial clones detected by the clone library analysis were assigned by T-RFLP.
Conclusion: The size definition of the newly constructed internal standards reduced fragment sizing errors and allowed for accurate assignment of bacteria to peaks by the T-RFLP analysis. This provided a more effective means for studying microbial communities, including the oral microflora.

Key words: 16S recombinant RNA gene; oral microflora; terminal restriction fragment length polymorphism

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Conventional methods for the analysis of microbial flora rely on the cultivation of bacteria under anaerobic conditions; they are time-consuming and laborious, and can miss bacteria that are difficult or impossible to culture. Consequently, culture-independent molecular tools based on the detection of 16S recombinant RNAs (rRNAs) or the corresponding genes have recently been developed for analyzing bacterial communities. Using this approach, it was found that the human oral cavity is inhabited by 500–700 species of bacteria (1, 11, 23) and that 50% of oral bacteria are difficult or impossible to culture (23). Currently, the best technique for exploring microbial diversity involves isolating DNA from the target environment, amplifying the

16S rRNA by polymerase chain reaction (PCR), cloning the amplicons into *Escherichia coli*, and sequencing the cloned 16S rRNA gene inserts (1, 3, 11, 23). However, analyzing individual 16S rRNA clones is a laborious, expensive, and inefficient method for comparing several bacterial communities.

Community analysis by terminal restriction fragment length polymorphism (T-RFLP) allows a compromise between sample throughput and phylogenetic resolution (13, 16). A gene of interest is amplified from a community of DNA templates using PCR with a fluorescently labeled primer. Terminal restriction fragments (T-RFs) with lengths that are specific for different species of the bacterial community are then obtained by restriction enzyme digestion and are compared with T-RFs of known sequence to identify the species. Known sequences of T-RFs obtained by DNA digestion with specific endonucleases are archived in databases that can be accessed via the Internet (12, 18). Although the T-RFs generated by digestion with one endonuclease often have the same length in multiple species of bacteria, the specificity can be increased by digesting DNA with multiple enzymes (4, 9, 16, 19).

One difficulty commonly associated with species identification using T-RFLP involves fragment-sizing errors owing to a discrepancy between the fragment size observed after electrophoresis and that calculated from the nucleotide composition (9, 10, 17, 26). Marsh (17) indicated that the fragment size difference could be as great as ±7 bases, and this size range would include fragments representing far too many species to allow a specific identification. To compensate for the apparent size discrepancy, many studies have analyzed the lengths of T-RFs in conjunction with sequence analysis of library clones to assign the candidates manually to a peak (21, 25). However, rapid and affordable analysis of a large number of environmental samples is impossible using manual compensation. A new method for reducing sizing errors is therefore needed to achieve the full potential of T-RFLP analysis.

The limitations of precise and accurate sizing of DNA fragments based on their mobility during electrophoresis are well documented (5, 14). With capillary electrophoresis, the observed mobility of an oligonucleotide depends primarily on mass but other factors can also affect mobility. To minimize variability and obtain reproducibility, an internal standard can be included in the same lane, and the T-RF size can be estimated by comparison with the mobility of the internal standard fragments. However, additional sizing errors can be produced at this step in T-RFLP analysis. Marsh (17) showed that differences in the fluorescent dyes used for labeling can also cause differential mobility, and Kaplan and Kitts (10) indicated that sequence differences might result in differential electrophoretic mobility. Therefore, it is proposed that sizing errors could be reduced by preparing size markers from structures similar to the T-RF samples and by using the same fluorescent dye for the standard as for the T-RFs.

In this study, we prepared a size marker that included 6-carboxyfluorescein (6-FAM) -labeled T-RFs derived from 16S rRNA genes. The apparent size of each fragment in a commercially available internal standard, GeneScan-1000 ROX, was calculated in relation to the relative mobility of the FAM-labeled T-RFs, and a new size definition was established for GeneScan-1000 ROX. We confirmed the accuracy of our method by determining the sizes of T-RFs derived from various species. In addition, we used the newly defined standard to assign the identity of bacteria from two saliva samples and compared the assignments to those based on 16S rRNA clone library analyses to evaluate the efficiency of this novel analysis system.

Material and methods

Bacterial strains and DNA extraction

This study used eight bacterial strains representing the six main phyla present in the oral microflora, as determined by 16S rRNA gene clone library analysis (1, 11, 23). These included two members of the phylum Firmicutes (*Streptococcus mutans* UA159 and *Veillonella parvula* ATCC 10790), two Bacteroidetes (*Porphyromonas gingivalis* W83 and *Porphyromonas endodontalis* ATCC35406), one member each of the Proteobacteria (*Neisseria mucosa* ATCC19695), Spirochaetes (*Treponema denticola* ATCC35405) and Fusobacteria (*Fusobacterium nucleatum* ATCC10953), and a clinical isolate of *Rothia dentocariosa* belonging to the Actinobacteria.

The bacteria were grown using previously described culture conditions (6, 8, 22, 24). Chromosomal DNA was prepared from the gram-negative bacteria using IsoQuick (Orca Research, Inc., Bothell, WA), according to the manufacturer's instructions, and from the gram-positive bacteria as previously described (22).

PCR amplification

PCR amplification of the bacterial 16S rDNA was carried out using 6-FAM-labeled or unlabeled universal forward primer D88 and unlabeled universal reverse primer E96 (23). Amplification was performed in a 50-μl reaction mixture containing 5 μl extracted DNA, 5 U Ex*Taq* polymerase (Takara Bio Inc., Shiga, Japan), an appropriate dilution of the manufacturer's buffer, 250 μM dNTPs, and 1 μM of each primer. The samples were heated at 95°C for 2 min and then amplified by 30 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and elongation 72°C for 1.5 min in a Biometra T3 thermocycler (Biometra, Göttingen, Germany). Aliquots (1 μl) of the 16S rDNA amplicons were analyzed by gel electrophoresis in 0.8% agarose gels and visualized by staining with ethidium bromide. The PCR products were purified by Labopass (COSMO Genetech, Seoul, Korea), and the DNA was stored at –20°C until analysis.

T-RFLP analysis

The amplified DNA fragments (3 μl) were digested with 2.5 U restriction enzyme in a total volume of 10 μl for 2 h at 37°C for *Alu*I, *Hae*III, and *Msp*I, or at 60°C for *Bst*UI. These restriction enzymes were selected because they produce several short T-RFs (<300 bases) from most bacteria. The restriction digest products (2 μl) were mixed with 10 μl deionized formamide and 0.5 μl GeneScan-1000 ROX standard (Applied Biosystems, Foster City, CA). The samples were denatured at 95°C for 2 min, followed by rapid chilling on ice. The fluorescently labeled T-RFs were separated by size on an ABI 3130 genetic analyzer (Applied Biosystems), with electrophoresis at 60°C and 15 kV, for 30 min with the POP-7 polymer. The electropherograms were analyzed with GeneMapper version 4.0 software (Applied Biosystems), and the fragment sizes were estimated using the Local Southern method. The FAM-MW definition (described below) was used for sizing, and T-RFs with peak heights <100 fluorescence units were excluded from the analysis.

Preparation of the FAM size marker

We used *P. gingivalis* W83 to define the relationship between the mobility and the size of T-RFs derived from the 16S rRNA gene of bacteria. The *P. gingivalis* chromosomal DNA was prepared as described

above. To create FAM-labeled size markers with sequences identical to those of the T-RFs from *P. gingivalis*, the 16S rRNA gene of *P. gingivalis* was amplified by PCR using a FAM-labeled primer, and 18 fragments were prepared.

Twelve DNA fragments were prepared using universal forward primer D88 (23) labeled with 6-FAM (D88FAM) and the non-labeled reverse primers described in Table 1. The amplification was performed in a 50-μl reaction mixture containing 5 μl extracted DNA, 5 U KOD DNA polymerase (TOYOBO, Osaka, Japan), an appropriate dilution of the manufacturer's buffer, 250 μM dNTPs, 1 mM $MgCl_2$, and 1 μM of each primer. The samples were heated at 95°C for 2 min and amplified by 30 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 2 s, and elongation at 72°C for 30 s in a Biometra T3 thermocycler (Biometra). After amplification, the products were purified through agarose gel electrophoresis using a QIAEX II gel extraction kit (Qiagen, Hilden, Germany).

Amplification of D88FAM and 800 m was performed in the same manner. After purification, 5 μl of the product was digested separately with each of four restriction enzymes (*Taq*I, *Alu*I, *Afl*III, and *Aci*I) at 37°C for 2 h. The enzymes were inactivated by heating to 65 or 80°C for 20 min. A 5-μl aliquot of each digestion, 0.05 pmol D88FAM (5 μl), and the original full-length PCR product (2 μl) were mixed and combined with the twelve DNA fragments described in the paragraph above to produce the FAM size marker for 16S rRNA. Thus the FAM size marker for 16S rRNA contained eighteen 6-FAM-labeled fragments with base lengths of 21, 56, 77, 116, 172, 237, 294, 361, 414, 475, 542, 601, 663, 730, 800, 861, 919, and 977.

The FAM-base definition of the internal standard (GeneScan-1000 ROX)

First, we redefined the base length of the fragments in the GeneScan-1000 ROX standard based on the results of capillary electrophoresis with the FAM size marker. Briefly, GeneScan-1000 ROX (1 μl) was mixed with 10 μl deionized formamide and 2 μl of the FAM size marker described above, and the mixture was subjected to capillary electrophoresis. Using the FAM size marker as an internal standard, the apparent length corresponding to the mobility of each GeneScan-1000 ROX fragment was estimated relative to that of the internal standard. After four repetitions, the average lengths of the GeneScan-1000 ROX fragments were determined as the FAM-base definition.

Sizing accuracy using the FAM size marker and the FAM-base definition

The sizes of T-RFs generated from *P. gingivalis* were estimated using the FAM size marker or the GeneScan-1000 ROX internal standard with the FAM-base definition, and the results were compared. The *P. gingivalis* 16S rRNA gene was amplified by PCR using D88FAM and E96 (23) and purified using Labopass. The purified PCR product (3 μl) was digested with 2.5 U restriction enzymes in a total reaction volume of 10 μl for 2 h at 37°C for *Hae*III, *Msp*I, *Rsa*I, and *Hha*I, or at 60°C for *Tsp*509I and *Bst*UI. The restriction digest products (2 μl) were mixed with 10 μl deionized formamide and 1 μl of the FAM size marker or GeneScan-1000 ROX, and the fragment sizes were estimated by T-RFLP analysis performed in triplicate. As a negative control, the FAM size marker was electrophoresed with distilled water (2 μl) replacing the digest product. The GeneScan-1000 ROX fragments were sized according to the FAM-base definition.

T-RF MW calculation and the FAM-MW definition of the internal standard

The mobility of a DNA fragment in electrophoresis is largely affected by its mass. However, the mass of a T-RF does not always correspond to its sequence length because of differences in sequence composition. This discrepancy can complicate the analysis of DNA fragments of various sequences. For more accurate size estimations, we used molecular weight (MW), instead of the number of nucleotides, as the unit of size. The MWs of the FAM-labeled T-RFs were calculated from the nucleotide sequence as follows: MW = (#A × 313.21) + (#C × 289.21) + (#G × 329.21) + (#T × 304.19) − 62 + 535.47, where the MW value of the fluorescent dye (6-FAM) is 535.47 and the subtraction of 62 takes into account the removal of HPO_2 and the addition of two hydrogens in synthesized oligonucleotides.

Substituting the calculated MWs for the base lengths of the fragments in the FAM size marker, the value of each peak in the GeneScan-1000 ROX standard was calculated based on the results obtained in the procedure for constructing the FAM-base definition. The MWs of the 18 fragments included in the FAM size marker were 7032.24, 17,887.97, 24,461.30, 36,628.33, 54,005.89, 74,480.33, 92,561.25, 113,046.91, 131,725.31, 151,326.16, 169,363.16, 187,830.15, 207,333.12, 228,246.89, 249,637.12, 268,513.67, 286,589.65, and 304,319.4. These values were established as the FAM-MW definition.

Accuracy of the size estimation of T-RFs derived from various bacteria

We performed sizing analyses of various T-RFs from seven bacteria using three different size definitions of the GeneScan-1000 ROX standard and compared the accuracy of the results. The 16S rRNA genes of the seven bacteria named above were amplified by PCR using D88FAM and E96 (23). The purified PCR products (3 μl) were digested with 2.5 U of a restriction enzyme in a total volume of 10 μl for 2 h, at 37°C for *Alu*I, *Hae*III, *Msp*I, and *Hha*I, or at 60°C for *Bst*UI. These tetrameric restriction enzymes are frequently used in T-RFLP analyses (5, 17). The restriction digest products (2 μl) were mixed with 10 μl deionized formamide and 1 μl GeneScan-1000 ROX, and

Table 1. PCR primers used in this study

Primer	Orientation	Sequence	Fragment size[1]
D88 [23]	Forward	5′ GAGAGTTTGATYMTGGCTCAG 3′	
E94 [23]	Reverse	5′ GAAGGAGGTGWTCCARCCGCA 3′	
237 m	Reverse	5′ CGCATGCCTATCTTACAGCT 3′	237 bp
295 m	Reverse	5′ AGTTCCCCTACCCATCGTCG 3′	295 bp
361 m	Reverse	5′CCTCACTGCTGCCTCCCGTA 3′	361 bp
414 m	Reverse	5′GTCTTCCTTCACGCGACTTG 3′	414 bp
475 m	Reverse	5′ CAATGCAATACTCGTATCGC 3′	475 bp
542 m	Reverse	5′ CTCGCATCCTCCGTATTACC 3′	542 bp
601 m	Reverse	5′TCACCGCTGACTTACCGAAC 3′	601 bp
664 m	Reverse	5′ CTGCCGCCACTGAACTCAAG 3′	664 bp
731 m	Reverse	5′ AAGCTGCCTTCGCAATCGGA 3′	731 bp
800 m	Reverse	5′ GGACTACCRGGGTATCTAA 3′	800 bp
861 m	Reverse	5′GCTTTCGCTGTGGAAGCTTG 3′	861 bp
F17 [23]	Reverse	5′ CCGTCWATTCMTTTGAGTTT 3′	919 bp
977 m	Reverse	5′ GTAAGGTTCCTCGCGTATCA 3′	977 bp

[1]The fragment sizes are obtained by using D88 as forward primer.

the sizes were estimated following capillary electrophoresis. Three size definitions were used for this analysis: the ROX-base definition, the FAM-base definition, and the FAM-MW definition. The ROX-base definition is the conventional definition representing the lengths of the ROX-labeled fragments as reported by Applied Biosystems. The sizing error was calculated as the difference between the estimated size following capillary electrophoresis and the size predicted from the sequence content. With the FAM-MW definition, the sizing error was calculated using MW but was presented as both MW units and base units, assuming one base to be 308.95 MW units (the average MW of adenine, thymine, cytosine, and guanine), to facilitate the comparison among the three definitions.

Sample collection and DNA extraction

Saliva samples (0.5 ml) from two volunteers were collected into sterile plastic tubes. The bacteria were harvested by centrifugation (20,400 ***g***, 15 min, 4°C), and the pellet was resuspended in 150 µl buffer containing 50 mM Tris-HCl, 1 mM EDTA, and 1% sodium dodecyl sulfate (pH 7.6). The suspension was added to a plastic tube containing 0.3 g zirconia–silica beads (bead size 0.1 mm; Biospec Products, Bartlesville, OK) and one tungsten–carbide bead (bead size 3 mm; Qiagen). The sample was heated at 90°C for 10 min, followed by violent agitation for 3 min in a cell disruptor (Disruptor Genie; Scientific Industries, Inc., Bohemia, NY). After centrifugation at 6000 ***g*** for a few seconds, 200 µl of 1% sodium dodecyl sulfate were added, and the sample was heated at 70°C for 10 min. The mixture was extracted using 400 µl phenol–chloroform–isoamyl alcohol (25 : 24 : 1), and the nucleic acids were precipitated with 100% ethanol. Following centrifugation, the DNA was washed with 70% ethanol, resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and frozen for later analysis.

Software tools

TRFMA, a Web-based tool for T-RFLP analysis based on MW (20), was used to perform the phylogenetic analysis. The T-RFLP data, including the names of the restriction enzymes and the MWs of the fragment sizes, were entered, and the results were sorted by number of matches. The matching window was set at ± 330 for T-RFs with a MW <66,000 and at ± 0.5% for T-RFs >66,000. A small database containing 667 strains (including 442 species or phylotypes) of oral bacteria (light version) was used in this study. TRFMA is available at http://myamagu.dent.kyushu-u.ac.jp/bioinformatics/trfma/index.html

Cloning, sequencing, and data analysis

DNA fragments were amplified using unlabeled primers and were cloned into the vector pGEM-T Easy (Promega, Madison, WI). The recombinant plasmids were purified from a culture derived from a single colony, and the nucleotide sequences of the inserts were determined using M13 (–40) forward and reverse primers. The sequence data were extended to at least 500 base pairs from the forward primer. The sequences were checked for chimeric properties using CHIMERA_CHECK of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) (15). After eliminating

Table 2. Redefinition of the ROX standard based on FAM size marker fragments

Peak No.[1]	Actual size of each fragment in GeneScan-1000 ROX (base) (ROX-base definition)	Experimental size based on FAM size marker in base unit (base)		Experimental size based on FAM size marker in MW unit (MW)	
		Mean (FAM-base definition)	SD	Mean (FAM-MW definition)	SD
1	29	36.53	0.05	11,805.97	13.39
		38.04	0.02	12,276.09	6.91
2	33	40.51	0.07	13,043.12	23.04
		43.24	0.05	13,891.78	13.92
3	37	44.62	0.02	14,323.21	6.01
		67.65	0.03	21,516.81	10.59
4	64	68.93	0.04	21,916.79	12.12
		70.46	0.05	22,398.12	16.30
5	67	71.30	0.06	22,658.99	18.37
		78.88	0.07	25,032.38	22.11
6	75	84.02	0.06	26,635.32	20.03
		85.00	0.07	26,941.21	21.55
7	81	109.49	0.08	34,567.23	26.13
		111.30	0.05	35,129.80	15.32
8	108	119.34	0.06	37,631.43	17.91
		121.51	0.02	38,306.85	6.45
9	118	243.59	0.05	76,202.72	15.42
		246.52	0.05	77,118.45	15.85
10	244	275.49	0.03	86,147.22	9.94
		276.72	0.02	86,529.49	7.06
11	275	293.81	0.25	91,858.68	77.85
		298.76	0.04	93,394.93	11.62
12	299	417.55	0.13	130,354.25	42.51
		421.59	0.08	131,612.75	25.96
13	421	540.14	0.08	168,453.41	23.66
14	539	542.00	0.05	169,033.49	15.53
15	674	677.74	0.15	211,289.27	45.17
16	677	679.69	0.05	211,899.03	15.54
17	926	927.07	0.44	288,770.09	135.87

Mean and SD of experimental size was estimated for four replications.
[1]Some fragments in GeneScan-1000 ROX are observed as split peaks. Left side Peak from each split peak pair was defined in Rox-base definition.

Table 3. Estimated length of T-RFs from *Porphyromonas gingivalis* after capillary electrophoresis using two different size markers

Restriction enzyme (predicted size)	FAM size marker[1]	GeanScan-1000[2]
*Msp*I (98)	97.97 ± 0.04	98.03 ± 0.21
*Hha*I (103)	103.01 ± 0.05	102.82 ± 0.16
*Bst*UI (118)	117.78 ± 0.04	117.80 ± 0.07
*Rsa*I (3l9)	318.85 ± 0.01	318.90 ± 0.23
*Tsp*5091 (668)	667.87 ± 0.07	668.76 ± 0.12
*Hae*III (922)	922.64 ± 0.19	923.38 ± 0.33

The data are represented as mean ± SD for triplicate experiments.
[1]Fragment length was estimated by using the FAM size marker.
[2]Fragment length was estimated by using GeneScan-1000 ROX internal standard with the FAM-base definition.

chimeric sequences, the partial 16S rRNA sequences were compared with the sequences in the Ribosomal Database Project and GenBank, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (2). Cloned sequences were identified as representing the species or phylotype of the sequence with the highest matching score. Clone sequences with 99–100% identity with a GenBank sequence were considered to be derived from the species with the highest matching score. Sequences with less than 99% identity with a GenBank sequence were defined as a new phylotype. In addition, we checked whether the sequenced clones had the correct T-RFs as compared with the sequence information.

Statistical analysis

The data are presented as means ± SD. Accuracy improvement was examined using *t*-tests and statistical significance was taken as $P < 0.05$.

Table 4. Sizing error for various bacteria after digestion with *Hae*III, *Hha*I, *Alu*I, *Bsf*UI, and *Msp*I

Organism[1]	Digestion	Predict size (base)	ROX-base definition (base)	FAM-base definition (base)	FAM-MW definition count as base[2]	MW
Pg	*Alu*I	77	**−3.98**	−0.08	−0.13	−39.26
	*Msp*I	98	0.39	0.03	−0.06	−18.83
	*Hha*I	103	−0.30	−0.17	−0.29	−88.42
	*Bsf*UI	118	**−1.57**	−0.20	−0.37	−114.91
	*Hae*III	922	**1.99**	**1.38**	**1.13**	347.63
Pe	*Alu*I	77	**−3.86**	0.24	0.17	51.69
	*Msp*I	98	0.25	−0.20	−0.22	−69.14
	*Hha*I	103	−0.44	−0.63	−0.53	−162.81
	*Bsf*UI	118	**−1.71**	−0.36	−0.47	−144.47
	*Hae*III	921	**3.42**	**3.85**	**2.98**	921.84
Nm	*Alu*I	76	**−4.11**	−0.19	−0.19	−59.95
	*Msp*I	497	**1.10**	0.51	−0.34	−103.60
	*Hha*I	214	**1.02**	0.82	0.38	116.32
	*Bsf*UI	396	**3.38**	0.43	−0.03	−9.73
	*Hae*III	207	**1.31**	**1.56**	0.80	246.97
Sm	*Alu*I	169	0.06	0.23	−0.13	−41.57
	*Msp*I	564	**−4.74**	0.64	0.52	161.27
	*Hha*I	223	**1.04**	0.48	0.54	167.39
	*Bsf*UI	105	**−1.05**	−0.55	−0.24	−74.90
	*Hae*III	318	**1.03**	**1.09**	0.66	205.10
Vp	*Alu*I	76	**−4.10**	−0.05	−0.36	−112.34
	*Msp*I	303	0.31	0.30	0.32	97.41
	*Hha*I	591	**−8.49**	0.36	−0.08	−24.87
	*Bsf*UI	116	**−1.53**	−0.10	−0.16	−50.43
	*Hae*III	213	**1.09**	0.95	0.78	239.92
Rd	*Alu*I	73	**−4.65**	**−1.02**	**−1.02**	−314.61
	*Msp*I	164	−0.93	−0.74	−0.93	−286.36
	*Hha*I	375	**4.06**	0.70	−0.17	−52.39
	*Bsf*UI	397	**3.32**	0.04	−0.65	−200.26
	*Hae*III	*234*	**1.21**	0.58	−0.05	−14.00
Fn	*Alu*I	190	**1.44**	**1.02**	0.99	306.52
	*Msp*I	269	**1.14**	**1.37**	**1.22**	376.54
	*Hha*I	199	**1.39**	0.82	0.85	263.65
	*Bsf*UI	97	0.88	0.43	0.34	104.91
	*Hae*III	272	1.00	**1.44**	**1.26**	390.18
Td	*Alu*I	73	**−3.86**	−0.23	−0.22	−68.31
	*Msp*I	285	**−1.39**	−0.94	**−1.28**	−396.28
	*Hha*I	38	**−9.47**	**−1.48**	**−1.50**	−464.24
	*Bsf*UI	38	**−9.47**	**−1.85**	**−1.70**	−524.84
	*Hae*III	227	−0.25	−0.96	−0.55	−170.54
	Average[3] ± SD		2.42 ± 2.39	0.72 ± 0.70	0.62 ± 0.58	

[1]*Pg, Porphyromonas gingivalis; Pe, Porphyromonas endodontalis; Nm, Neisseria mucosa Sm, Streptococcus mutans; Vp, Veillonella parvula; Rd, Rothia dentocariosa', Fn, Fusobacterium nucleatum; Td, Treponema denticola.*
[2]Using the FAM-MW definition, 308.95 (MW) equals 1 base.
[3]Average indicates the average of absolute errors in sizing. The sizing error represents the difference between estimated size after capillary electrophoresis and the size predicted from the sequence. Sizing errors greater than one base are shown in bold.

Results

Novel size definition of the internal standard GeneScan-1000 ROX

DNA fragments of the GeneScan-1000 ROX standard were subjected to capillary electrophoresis together with the FAM size marker, which was composed of 18 6-FAM-labeled T-RFs derived from the *P. gingivalis* 16S rRNA gene. The relationship between the mobility and the actual fragment size was different between the GeneScan-1000 ROX standard and the 6-FAM-labeled DNA fragments derived from the 16S rRNA gene of *P. gingivalis*. Therefore, the sizes of the GeneScan-1000 ROX fragments were newly defined in terms of base units and MW units, based on their mobility relative to that of the FAM size marker fragments, as shown in Table 2. Although the small standard deviations indicated that the new size definition was reproducible, there was a maximum size difference of 7.53 bases between the defined size and actual size for each GeneScan-1000 ROX fragment. To further investigate this size discrepancy, the sizes of the GeneScan-1000 ROX fragments were defined by three different criteria. The actual sizes of the fragments were established as the ROX-base definition of the GeneScan-1000 ROX standard sizes. The apparent sizes of the fragments, estimated in base units relative to the FAM size marker, were established as the FAM-base definition, and the sizes estimated in MW units were established as the FAM-MW definition.

The sizes of the T-RFs generated by the endonuclease digestions (*Msp*I, *Hha*I, *Bst*UI, *Rsa*I, *Tsp*509I, or *Hae*III) of FAM-labeled *P. gingivalis* 16S rRNA were estimated using the FAM size marker or the GeneScan-1000 ROX internal size standard with the FAM-based definition. The results reflected closely the actual size of each fragment and were comparable to those obtained using the FAM size markers as an internal standard (Table 3). In addition, the average size error estimated by electrophoresis was within one base for T-RF lengths of less than 668 bases (Table 3).

Improved accuracy in sizing oral bacterial 16S rRNA T-RFs

Next we compared the accuracy in sizing T-RFs from the 16S rRNA of eight different oral bacterial species representing six phyla. Using five different restriction enzymes (*Hae*III, *Alu*I, *Bst*UI, *Hha*I, and *Msp*I), we prepared 40 T-RFs. The sizes of these T-RFs were estimated following capillary electrophoresis with GeneScan-1000 ROX as an internal standard. The results were determined using each of the three different definitions (ROX-base, FAM-base, and FAM-MW) for the sizes of the GeneScan-1000 ROX fragments. With the ROX-base definition, the average sizing error was 2.42 ± 2.39 bases, and the maximum error was −9.47 bases for the *Hha*I and *Bst*UI digest fragments (38 bases) from *T. denticola*. Also using the ROX-base definition, the sizing errors for the *Alu*I-digest fragments were relatively high (−9.47 bases in *T. denticola*, −3.98 bases in *P. gingivalis*, −3.86 in *P. endodontalis*, −4.11 in *N. mucosa*, −4.10 in *V. parvula*, and −4.65 in *R. dentocariosa*), especially considering the small size of these fragments (38, 73, 76, and 77 bases) in many species. Using the other two definitions significantly decreased the average sizing error, reducing the error from 2.42 bases to 0.72 bases with the FAM-based definition and to 0.62 bases with the FAM-MW definition (Table 4). A small but significant difference in the average error was found between the FAM-based definition and the FAM-MW definition ($P < 0.05$). Furthermore, the maximum error for the 591 bases estimated was 1.85 bases with the FAM-base definition and 1.70 bases with the FAM-MW.

Phylogenetic analysis using TRFMA

The T-RFLP profile resulting from the *Hae*III digestions of a sample prepared from the saliva of a volunteer (case 1) is shown in Fig. 1. The bacterial species and phylotypes were predicted using four endonucleases, as described in the *Materials and methods* section. Ninety-nine species or phylotypes were assigned to the 17 peaks in the electropherogram generated after digestion by *Hae*III (Fig. 1). Twenty-four species or phylotypes were identified by clone library analysis (53 clones), and 21 of these were assigned by T-RFLP analysis in combination with TRFMA (Table 5).

The T-RFLP profile produced by digestion of a sample prepared from the saliva

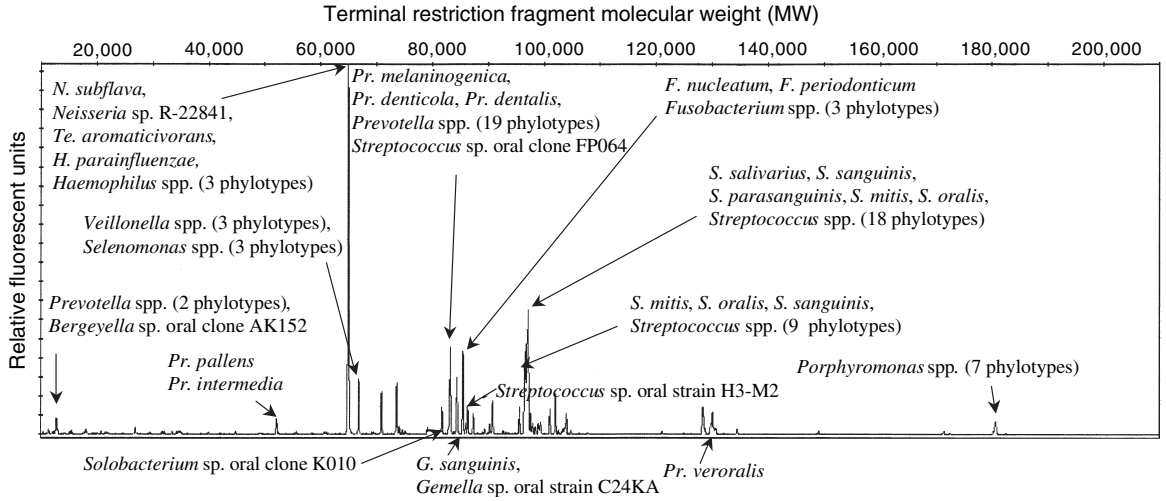

Fig. 1. T-RFLP profiles resulting from *Hae*III digestions of 16S rDNA from the saliva of case 1. Bacterial species and phylotypes were assigned by T-RFLP analysis of the fragments produced by *Hae*III, *Alu*I, *Bst*UI, and *Msp*I restriction enzyme digestions. Peaks with a peak area of less than 1% of total area were omitted from the designation. *N.*, *Neisseria*; *Te.*, *Terrahaemophilus*; *Pr.*, *Prevotella*; *F.*, *Fusobacterium*; *G.*, *Gemella*; *S.*, *Streptococcus*; *H.*, *Haemophilus.*

Table 5. Comparison of the clone library and T-RFLP results from case 1

%	No. of clones	Detected in clone library analysis	Peaks in *Hae*III digestion	Assigned in T-RFLP analysis	Peak area proportion (%)
0.00			12,656.25	*Prevotella* spp. (2 phylotypes) *Bergeyella* sp. oral clone AK152	1.47
1.88	1	***Prevotella pallens;* 9423; Y13106**	52,171.92	***Prevotella pallens*** *Prevotella intermedia*	1.05
39.62	14	***Neisseria subflava* U37; AJ239291**	64,942.81	***Neisseria subflava***	28.07
	4	***Neisseria* sp. R-22841; AJ786809**		***Neisseria* sp. R-22841**	
	1	***Terrahaemophilus aromaticivorans* 127W; AB098612**		***Terrahaemophilus aromaticivorans*** *Haemophilus parainfluenzae*	
	2	**Uncultured bacterium clone Y167; AY975728**		***Haemophilus* spp. (3 phylotypes)**	
1.88	1	***Veillonella* sp. clone SC004B06; AY807839**	66,821.04	***Veillonella* spp. (3 phylotypes)** *Selenomonas* **spp. (3 phylotypes)**	3.93
0.00			81,751.24	*Solobacterium* **sp. oral clone K010**	1.98
5.66	3	***Prevotella melaninogenica ATCC* 25845; L16469**	83,222	***Prevotella melaninogenica*** *Prevotella denticola* *Prevotella dentalis* *Prevotella* **spp. (19 phylotypes)** ***Streptococcus* sp. oral clone FP064; AF432139**	*7.55*
1.88	1	***Gemella sanguinis* 2045-94; Y13364**	84,452.26	***Gemella sanguinis*** *Gemella* **sp. oral strain C24KA**	5.45
3.77	2	***Fusobacterium periodonticum;* KP-F10; AJ810271**	85,504.44	***Fusobacterium periodonticum*** *Fusobacterium nucleatum* *Fusobacterium* **spp. (3 phylotypes)**	
0.00			85,972.09	*Eubacterium* **sp. oral clone HU029** ***Peptostreptococcus* spp. (5 phylotypes)**	0.75
1.88	1	***Streptococcus* sp. oral strain H3-M2; AF385523**	86,328.8	***Streptococcus* sp. oral strain H3-M2**	2.16
5.66	3	***Abiotrophia paraadiacens* TKT1; AB022027**	87,365.14[1]		1.53
7.54	1	***Streptococcus oralis;* ATCC 700233; AY281080**	96,657.67	***Streptococcus oralis***	9.22
	1	***Streptococcus* sp. oral clone BM035; AY005043**		*Streptococcus mitis*	
	1	***Streptococcus* sp. oral clone P2PA_41 P2; AY207051**		*Streptococcus sanguinis*	
	1	***Streptococcus* sp. oral clone BW009; AY005042**		***Streptococcus* spp. (9 phylotypes)**	
26.42	5	***Streptococcus salivarius* (T); ATCC 7073; AY188352**	97,160.6 l[2]	***Streptococcus salivarius***	20.77
	2	***Streptococcus* sp. oral strain T1-E5; AF385525**		***Streptococcus parasanguinis***	
	1	***Streptococcus parasanguinis* GIFU7994; AB006124**		*Streptococcus mitis*	
	1	***Streptococcus* sp. oral clone DP009; AF432132**		*Streptococcus oralis*	
	1	**KUD11 *(Streptococcus* AY207062 98%)**[3]		*Streptococcus sanguinis*	
	2	***Streptococcus* sp. oral strain T4-E3; AF385526**		***Streptococcus* spp. (18 phylotypes)**	
	2	**Uncultured bacterium clone NS03; AY981757**			
1.88	1	*Firmicutes sp.* **oral clone F058; AF287779**	97,580.04[1]		1.70
0.00			98,027.75	*Gemella* **sp. oral strain A31SC**	1.01
0.00			103,990.71	*Peptostreptococcus* **sp. oral clone CK035**	1.87
0.00			129,828.4	*Prevotella* **sp. oral clone BI027**	0.93
0.00			130,065.25	*Prevotella veroralis*	2.24
1.88	1	***Porphyromonas* sp. oral clone CW034; AY008310**	180,693.69	***Porphyromonas* spp. (7 phylotypes)**	1.68
	53				

Bacterial species and phylotypes detected by both T-RFLP and the 16S rRNA gene clone library are shown in bold.
[1]No species or phylotypes was assigned to these peaks by using TRFMA in this analysis.
[2]The value represented the highest peak was used in case of incompletely separated peaks.
[3]For sequences with less than 99% similarity to known 16S rDNA sequences, the per cent similarity to the closest match from each bacterial 16S rDNA phylotype or species is displayed.

of a second volunteer (case 2) is shown in Fig. 2. Forty-four species or phylotypes were assigned to the seven peaks in the electropherogram generated after digestion with *Hae*III (Fig. 2). In this case, 15 species or phylotypes (56 clones) were identified by clone library analysis, and 13 of these were assigned by T-RFLP analysis using TRFMA (Table 6).

Discussion

The comparison between fragments generated in T-RFLP analysis and reference sequences in a phylogenetic study is frequently complicated by a discrepancy between the observed T-RF sizes and the predicted sizes based on the T-RF sequence composition. The extent of the discrepancy is reproducible for each T-RF but is initially unpredictable. To improve the accuracy of size estimates, we used the 16S rRNA gene of a bacterium to optimize the internal size standard commonly used for T-RFLP analysis of bacterial communities.

Estimates of fragment size in T-RFLP analyses are made by comparison with internal size standards that are incorporated into every capillary. However, fragments in the commercially available size markers commonly used in conventional T-RFLP methods are composed of nucleotide sequences and fluorescent dyes that differ from those of fragments prepared from environmental samples. We hypothesized that the sizing error could be reduced by the use of an internal standard containing fragments composed of nucleotide sequences similar to those of the target T-RFs and labeled with the same fluorescent dye. As shown in Table 3, the error in

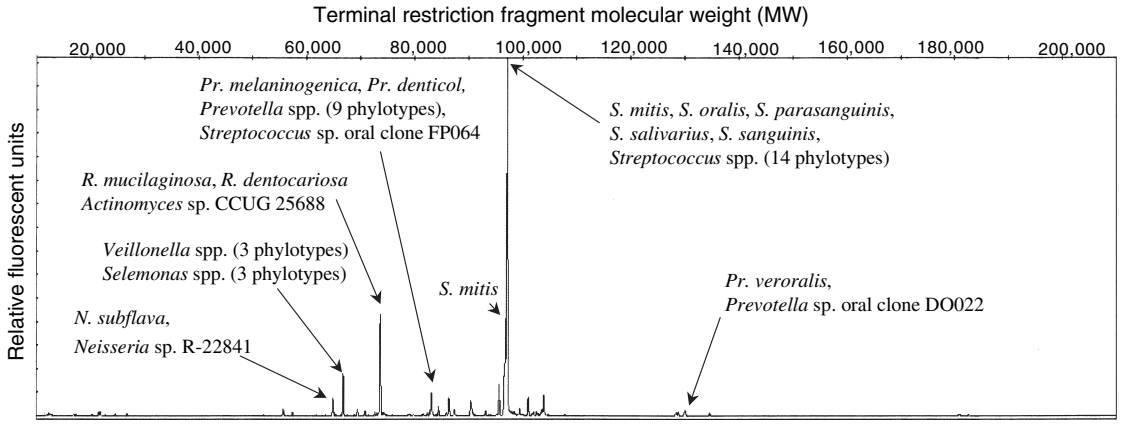

Fig. 2. T-RFLP profiles resulting from *Hae*III digestions of 16S rDNA from the saliva of case 2. Bacterial species and phylotypes indicated were assigned by T-RFLP analysis of the fragments produced by *Hae*III, *Alu*I, *Bst*UI, and *Msp*I restriction enzyme digestions. *N.*, *Neisseria*; *R.*, *Rothia*; *Pr.*, *Prevotella*; *S.*, *Streptococcus*.

Table 6. Comparison of the results of clone library and T-RFLP in case 2.

%	No. of clones	Detected in clone library analysis	Peaks in *Hae*III digestion	Assigned in T-RFLP analysis	Peak area proportion (%)
5.35	1	***Neisseria subftava* U37; AJ239291**	64,860.54	***Neisseria subflava***	2.88
	2	***Neisseria* sp. R-22841; AJ786809**		***Neisseria* sp. R-22841**	
0.00			66,746.91	*Veillonella* **spp. (3 phylotypes)**	6.35
				***Selenomonas* spp. (3 phylotypes)**	
3.57	2	***Rothia mucilaginosa DSM;* X87758**	73,663.63	***Rothia mucilaginosa***	16.44
				Rothia dentocariosa	
				Actinomyces **sp. CCUG 25688**	
1.79	1	***Prevotella sp.* oral clone FM005; AF432133**	83,173.06	*Prevotella melaninogenica*	4.49
				Prevotella denticola	
				***Prevotella* spp. (9 phylotypes)**	
				Streptococcus **sp. oral clone FP064**	
3.57	2	*AbiotrophiaparaadiacensTKII;ABQ22Q27*	87,398.51[1]		1.08
8.77	4	***Streptococcus mitis* Sm91; AYS18677**	96,917.3	***Streptococcus mitis***	4.27
	1	*Streptococcus cristatus* **ATCC 51100; AY584476**			
76.78	1	***Streptococcus sanguinis* ATCC 10556; AF003928**	97,293.92	*Streptococcus mitis*	63.01
	7	***Streptococcus salivarius* ATCC 13419; M58839**		*Streptococcus oralis*	
	24	***Streptococcus salivarius* ATCC 7073; AY188352**		***Streptococcus sanguinis***	
	1	***Streptococcus parasanguinis* ATCC 15909; AY281087**		***Streptococcus parasanguinis***	
	4	**uncultured bacterium NS03; AY981757**		***Streptococcus salivarius***	
	4	***Streptococcus* sp. oral clone DN025; AF432131**		***Streptococcus* spp. (14 phylotypes)**	
	1	***Streptococcus* sp. oral clone FO042; AF432136**			
	1	***Streptococcus* genomosp. C6; AY278634**			
0.00			130,118.2	*Prevotella verordis*	1.47
				Prevotella sp. oral clone DO022	
100.00	56				100.00

Bacterial species and phylotypes detected by both T-RFLP and the 16S rRNA gene clone library are shown in bold.
[1]No species or phylotype was assigned to this peak by using TRFMA in this analysis.

the size estimation of T-RFs from *P. gingivalis* was reduced to 0.3 bases for T-RF lengths of less than 668 bases by using the FAM size marker, which contained sequences and fluorescent dyes identical to those of the *P. gingivalis* T-RFs. Although accurate size estimation was possible using internal size standards appropriate for the target fragments, it is difficult to distinguish between the standards and fragments in an electrophoretic pattern composed of multiple peaks when the sample and internal standard are labeled with the same fluorescent dye. Labeling the internal standard and target fragments with different fluorescent dyes could facilitate their distinction. Thus, in the present study, we changed the size values of the fragments of GeneScan-1000 ROX, based on the relative mobility to the FAM size marker. Even though the GeneScan-1000 ROX fragments and target T-RFs were labeled with different fluorescent dyes, the use of the FAM-base definition resulted in accuracy almost equal to that achieved with the FAM size marker as an internal standard (Table 3). This approach could be expanded to define differently labeled internal standards by calibrating the standard fragments using another size marker labeled with the fluorescent dye of a sample.

The FAM-base definition is optimized for estimating the sizes of 6-FAM-labeled T-RFs prepared from the *P. gingivalis* 16S rRNA gene. Kaplan and Kitts demonstrated that the trend in the degree of sizing error was similar among related bacteria (10). The phylogenetic differences

compared with *P. gingivalis* could be a cause of sizing error when using the FAM-base definition. To investigate the sizing error attributable to phylogenetic differences, the sizes of T-RFs from various species were estimated using the FAM-base definition. With the FAM-base definition, the average of the sizing errors in eight species from six different phyla found in the oral cavity (0.72 bases) was much lower than that with the conventional ROX-base definition, and large sizing errors (>2 bases) were eliminated for T-RF lengths of <668 bases (Table 4). Thus, the 16S rRNA gene of *P. gingivalis* can function as a representative internal standard for gene sizing of oral bacterial 16S rRNAs.

Kaplan and Kitts (10) demonstrated that the purine content of T-RFs correlated with the extent of the sizing error and indicated that phylogenetic differences were presumably caused by differences in MW owing to sequence differences. Indeed, the analyses of DNA fragments of various sequences indicated that fragment base-length did not always reflect mass. For example, after digestion with *Rsa*I, the MW of the T-RF of *Delftia acidovorans* ACM 489 (MW: 147,378.40) was less than that of *Eubacterium yurii* subsp. *yurii* ATCC 43713 (MW: 147,451.95), even though the *D. acidovorans* T-RF was longer (472 vs. 471 bases). Here, the use of the MW unit instead of nucleotide number overcame this discrepancy, and the use of the FAM-MW definition significantly improved the sizing accuracy ($P < 0.05$).

A phylogenetic analysis of bacteria in the saliva from two volunteers was performed with the new size definition, and the results were compared with those from a 16S rRNA clone library analysis (Tables 5 and 6). Of 109 clones identified by clone library analysis, 101 (>90%) were assigned to peaks with T-RFLP analysis using the new size definition in combination with TRFMA. Most of the species identified by the clone library analysis were assigned using TRFMA, and only two or three species were missed. In addition, the proportion of each species was mostly consistent with the peak area in the T-RFLP profile. These results indicate that the optimization of the size standard allowed more accurate identification of peaks.

Sizing errors in the T-RFLP analysis of fragments derived from a specific region of the 16S rRNA genes of bacteria are mainly the result of differences in the sequences and fluorescent dyes between the T-RFs and the fragments of the internal standard. We used *P. gingivalis* as a model size marker in this study, but it might not be the most appropriate marker for every analysis. More research is needed to further improve the accuracy of the phylogenetic identification of microbial community structures by T-RFLP analysis. However, a major decrease in sizing errors was achieved by applying the new size definition of the GeneScan-1000 ROX standard in estimating the sizes of 40 T-RFs derived from six phyla. Although bacteria are classified into 23 phyla mainly based on the nucleotide sequence of the 16S rRNA gene in the second edition of Bergey's *Manual of Systematic Bacteriology* (7), most oral bacterial species are included in the six phyla tested in this study (1, 11, 23). This suggests that choosing the eight strains from the six phyla that dominantly occupy the oral cavity was reasonable when evaluating the validity of applying our T-RFLP to the oral flora.

The new size definition reported in the present study provided a more precise method of phylogenetic identification of oral bacteria using T-RFLP analysis. This system could also be applied to the analysis of other microbial communities that have suitable databases. In addition, T-RFLP profiles obtained previously with GeneScan-1000 ROX could be reanalyzed using our definition. T-RFLP analysis using this technique may be useful in examining oral microflora for combinations of bacteria correlated with disease or health.

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References

1. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. J Clin Microbiol 2005: **43**: 5721–5732.
2. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990: **215**: 403–410.
3. Becker MR, Paster BJ, Leys EJ et al. Molecular analysis of bacterial species associated with childhood caries. J Clin Microbiol 2002: **40**: 1001–1009.
4. Dunbar J, Ticknor LO, Kuske CR. Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Environ Microbiol 2001: **67**: 190–197.
5. Engebretson JJ, Moyer CL. Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. Appl Environ Microbiol 2003: **69**: 4823–4829.
6. Fukamachi H, Nakano Y, Okano S, Shibata Y, Abiko Y, Yamashita Y. High production of methyl mercaptan by L-methionine-α-deamino-γ-mercaptomethane lyase from *Treponema denticola*. Biochem Biophys Res Commun 2005: **331**: 127–131.
7. Guerrero R. Bergey's manuals and the classification of prokaryotes. Int Microbiol 2001: **4**: 103–109.
8. Harmsen D, Singer C, Rothganger J et al. Diagnostics of Neisseriaceae and Moraxellaceae by ribosomal DNA sequencing: ribosomal differentiation of medical microorganisms. J Clin Microbiol 2001: **39**: 936–942.
9. Kaplan CW, Astaire JC, Sanders ME, Reddy BS, Kitts CL. 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. Appl Environ Microbiol 2001: **67**: 1935–1939.
10. Kaplan CW, Kitts CL. Variation between observed and true Terminal Restriction Fragment length is dependent on true TRF length and purine content. J Microbiol Methods 2003: **54**: 121–125.
11. Kazor CE, Mitchell PM, Lee AM et al. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J Clin Microbiol 2003: **41**: 558–563.
12. Kent AD, Smith DJ, Benson BJ, Triplett EW. Web-based phylogenetic assignment tool for analysis of terminal restriction fragment length polymorphism profiles of microbial communities. Appl Environ Microbiol 2003: **69**: 6768–6776.
13. Liu WT, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 1997: **63**: 4516–4522.
14. Luckey JA, Smith LM. A model for the mobility of single-stranded DNA in capillary gel electrophoresis. Electrophoresis 1993: **14**: 492–501.
15. Maidak BL, Cole JR, Lilburn TG et al. The RDP-II (Ribosomal Database Project). Nucleic Acids Res 2001: **29**: 173–174.
16. Marsh TL. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr Opin Microbiol 1999: **2**: 323–327.
17. Marsh TL. Culture-independent microbial community analysis with terminal restriction fragment length polymorphism. Methods Enzymol 2005: **397**: 308–329.
18. Marsh TL, Saxman P, Cole J, Tiedje J. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community

analysis. Appl Environ Microbiol 2000: **66**: 3616–3620.
19. Matsumoto M, Sakamoto M, Hayashi H, Benno Y. Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota. J Microbiol Methods 2005: **61**: 305–319.
20. Nakano Y, Takeshita T, Yamashita Y. TRFMA: a web-based tool for terminal restriction fragment length polymorphism analysis based on molecular weight. Bioinformatics 2006: **22**: 1788–1789.
21. Nyman JL, Marsh TL, Ginder-Vogel MA, Gentile M, Fendorf S, Criddle C. Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms. Biodegradation 2006: **17**: 303–316.
22. Oho T, Yamashita Y, Shimazaki Y, Kushiyama M, Koga T. Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral Microbiol Immunol 2000: **15**: 258–262.
23. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
24. Persson S, Edlund MB, Claesson R, Carlsson J. The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. Oral Microbiol Immunol 1990: **5**: 195–201.
25. Sakamoto M, Rocas IN, Siqueira JF Jr, Benno Y. Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. Oral Microbiol Immunol 2006: **21**: 112–122.
26. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. J Med Microbiol 2003: **52**: 79–89.