Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis

Mitsuo Sakamoto,¹ Yasuo Takeuchi,² Makoto Umeda,² Isao Ishikawa² and Yoshimi Benno¹

Correspondence

Mitsuo Sakamoto
sakamoto@jcm.riken.go.jp

¹Japan Collection of Microorganisms, RIKEN, Wako, Saitama 351-0198 Japan

²Division of Periodontology, Department of Hard Tissue Engineering, Graduate School, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113-8549, Japan

Terminal restriction fragment-length polymorphism (T-RFLP) analysis was applied to characterize oral bacterial flora in saliva from 18 healthy subjects and 18 patients with periodontitis. The 16S rRNA genes (rDNAs) of oral bacteria and spirochaetes in saliva were amplified by PCR with a 6'-carboxy-fluorescein (6-FAM)-labelled universal forward primer (27F) and a universal reverse primer (1492R) or the *Spirochaeta*-selective reverse primer. The 16S rDNAs were digested with restriction enzymes with 4 bp recognition sites (*HhaI* or *MspI*) and analysed by using an automated DNA sequencer. T-RFLP patterns were numerically analysed using a computer program. From analysis of the oral bacterial community, patterns derived from periodontally healthy subjects and patients with periodontitis were grouped into different clusters, though with some uncertainty. Samples from patients with periodontitis tended to cluster into their respective types (aggressive and chronic periodontitis), although this was not very clear. Analysis of spirochaetal community using T-RFLP showed that the patterns derived from patients with periodontitis were grouped more as compared with the analysis of the oral bacterial community. These results suggest that samples from patients with periodontitis contain an unexpected diversity. T-RFLP patterns of 16S rDNAs from saliva samples of two periodontally healthy subjects over a 5-week period showed host-specific relatively stable oral bacterial flora. Our study indicates that T-RFLP analysis is useful for the assessment of diversity of oral bacterial flora and rapid comparison of the community structure between subjects with and without periodontitis.

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INTRODUCTION

A large number and variety of bacteria exist in the human oral cavity. However, analysis of these bacterial communities has been limited by conventional culture-dependent methods; thus, many oral bacteria remain uncultured and uncharacterized. Consequently, studies of causal microorganisms of oral diseases including periodontal disease are, in general, restricted to cultivable species.

Recently, a phylogenetic approach based on 16S rRNA genes (rDNA) has been applied to investigate the diversity of cultivable and non-cultivable species in the human oral cavity without cultivation (Choi *et al.*, 1994, 1996; Dymock *et al.*, 1996; Kroes *et al.*, 1999; Paster *et al.*, 2001; Sakamoto

et al., 2000; Spratt *et al.*, 1999). The 16S rDNA clone library method can provide direct sequence information. Paster *et al.* (2001) demonstrated that the predominant subgingival microbial community consisted of 347 species or phylotypes, based on analysis of 2522 16S rRNA clones. However, analysis of individual 16S rDNA clones is an expensive and extremely inefficient approach for comparison of a multitude of bacterial communities.

Terminal restriction fragment-length polymorphism (T-RFLP) is a new molecular approach that allows the assessment of a diversity of complex bacterial communities and rapid comparison of the community structure and diversity of different ecosystems (Liu *et al.*, 1997). This technique has been used for assessing the diversity and structure of complex bacterial communities in various environments (Clement *et al.*, 1998; Dunbar *et al.*, 2000; González *et al.*, 2000; Hiraishi *et al.*, 2000; Kaplan *et al.*, 2001; Leser *et al.*, 2000; Lukow *et al.*, 2000; Moeseneder *et al.*, 1999) and evaluated in separate review articles (Kitts, 2001; Marsh, 1999). In addition, the T-

Abbreviations: T-RF, terminal restriction fragment; T-RFLP, terminal restriction fragment length polymorphism.

T-RFLP patterns of 16S rDNAs are available as supplementary data in JMM Online (<http://jmm.sgmjournals.org>).

RFLP analysis program (TAP) has been developed and published on the worldwide web (<http://rdp.cme.msu.edu/html/analyses.html>) (Marsh *et al.*, 2000). Understanding the bacterial flora in the human oral cavity is essential for a full description of the causative bacteria of periodontal diseases. The T-RFLP method allows the recognition of diverse oral bacterial flora and rapid comparison of the community structure among patients with periodontitis. Hence, this method may be also useful for the rapid diagnosis of periodontal disease, although it has not yet been applied for the diagnosis of such diseases.

In the study of the oral bacterial community, saliva seems to be the most suitable sample as it is considered to contain a variety of bacteria from different oral sites (e.g. tongue, subgingival plaque, supragingival plaque), in addition to the ease of sampling. In a 16S rDNA clone library analysis of human saliva, we found the oral bacterial flora in a healthy subject to be different from that in periodontitis patients (Sakamoto *et al.*, 2000). This finding indicated that the use of saliva as well as subgingival plaque in the analysis of the oral bacterial community is effective. In addition, periodontopathic bacteria such as *Actinobacillus actinomycetemcomitans*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*) (Sakamoto *et al.*, 2002), *Porphyromonas gingivalis*, *Treponema denticola* and *Treponema socranskii* were more frequently detected in saliva than in subgingival plaque samples by the real-time PCR (Sakamoto *et al.*, 2001). These results confirmed those of a previous study (Umeda *et al.*, 1998). The levels of target bacteria may vary considerably from pocket to pocket and a pooled sample from sites of periodontitis may occasionally be missing organisms. It may also be that whole saliva simply contains higher concentrations of target bacteria than a subgingival plaque sample. This finding sug-

gested that saliva is equal to or better than subgingival plaque for detecting and quantifying periodontopathic bacteria in the oral cavity.

In the present study, we used T-RFLP analysis to characterize and compare oral bacterial flora present in saliva samples of healthy subjects and patients with periodontitis. As the aim of this study was to apply a novel technique to evaluate the diversity of cultivable and non-cultivable species in the human oral cavity, the cultivation-dependent approach was not used. To our knowledge, this is the first report on characterization of oral bacterial flora based on T-RFLP patterns.

METHODS

Bacterial strains and DNA extraction. The bacterial strains used in the present study are listed in Table 1. All bacteria were grown under appropriate culture conditions. Bacterial DNA was prepared as described previously (Sakamoto *et al.*, 1999).

Subject populations. Patients with periodontitis were divided into three groups based on the amount of probing depth (PD). (i) Group 1 (early periodontitis; P2, P3, P8, P10, P14 and P18) was defined as follows: teeth with PD $\geq$ 4 mm were less than 50 % of non-missing teeth. (ii) Group 2 (moderate periodontitis; P4, P5, P7, P9, P11 and P15) was defined as follows: teeth with PD $\geq$ 4 mm were 50 % or more of non-missing teeth and teeth with PD $\geq$ 6 mm were less than 30 % of non-missing teeth. (iii) Group 3 (severe periodontitis; P1, P6, P12, P13, P16 and P17) was defined as follows: teeth with PD $\geq$ 4 mm were 50 % or more of non-missing teeth and teeth with PD $\geq$ 6 mm were 30 % or more of non-missing teeth.

Sample collection and DNA extraction. Saliva samples were collected in sterile plastic tubes from the above 18 patients with periodontitis [mean age $\pm$ standard deviation (SD) = 45.8 $\pm$ 14.5 years] and from 18 periodontally healthy subjects (33.4 $\pm$ 11.7 years). The 0.5 ml

Table 1. Predicted and observed T-RF lengths (bp) for organisms used in the present study after digestion with *HhaI* and *MspI*

Organism	<i>HhaI</i>		<i>MspI</i> *	
	Predicted	Observed	Predicted	Observed
<i>Actinobacillus actinomycetemcomitans</i> JCM 2434	364	362	309	305
<i>Campylobacter gracilis</i> JCM 8538 ^T	98	95	470	467
<i>Campylobacter rectus</i> JCM 6301 ^T	96	93	468	465
<i>Capnocytophaga gingivalis</i> DSM 3290 ^T	90	86	85	82
<i>Eikenella corrodens</i> DSM 8340 ^T	575	573	89	86
<i>Fusobacterium nucleatum</i> JCM 8532 ^T	212	211	282	292 (283)
<i>Porphyromonas gingivalis</i> JCM 8525	102	98	97	95
<i>Tannerella forsythensis</i> † JCM 10827 ^T	96	92	91	89
<i>Treponema denticola</i> JCM 8225	37	29	284	291 (281)
<i>Treponema socranskii</i> subsp. <i>buccale</i> JCM 8155 ^T	37	29	124	123
<i>Treponema socranskii</i> subsp. <i>socranskii</i> JCM 8157 ^T	37	29	124	123
<i>Treponema vincentii</i> JCM 8227	37	29	289	296 (288)

* Parentheses indicate the T-RF length observed by using only size standard GS 500 ROX.

† *Tannerella forsythensis* is the new name for *Bacteroides forsythus* (Sakamoto *et al.*, 2002).

aliquot of saliva sample was diluted with buffer (10 mM Tris/HCl and 50 mM EDTA, pH 8.0) in a 1:2 ratio (v/v), and washed with the same buffer. The bacterial cell pellet obtained was then resuspended in 0.5 ml of the same buffer containing lysozyme (final concentration of 5 mg ml⁻¹) and *N*-acetylmuramidase (final concentration of 1 mg ml⁻¹). After incubation at 37 °C for 1 h, proteinase K and SDS were added to a final concentration of 2 mg ml⁻¹ and 1% (w/v), respectively. The mixture was incubated at 50 °C for 2 h. Nucleic acids were released by three cycles of freezing in a -80 °C freezer followed by thawing in a 65 °C water bath. The mixture was then extracted with equal volumes of phenol (saturated with 10 mM Tris/HCl, pH 8.0) and phenol/chloroform-isoamyl alcohol (25:24:1). Bulk nucleic acids were precipitated from solution with isopropyl alcohol followed by centrifugation. The DNA precipitate was washed with 70% ethanol and resuspended in 100 µl TE (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). RNase was added to a final concentration of 10 µg ml⁻¹, and the mixture was incubated at 37 °C for 1 h. The DNA was precipitated again with isopropyl alcohol. The DNA was pelleted by centrifugation, washed with 70% ethanol, dried in vacuum for 10 min, and dissolved in 100 µl of TE buffer.

PCR amplification of 16S rDNA. The primers used for the PCR amplification of 16S rDNA sequences were 27F (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTTACG ACTT-3') (Lane, 1991) or the *Spirochaeta*-selective reverse primer (5'-GTTACGACTTCACCCCTCT-3') (Paster *et al.*, 2001; Dewhirst *et al.*, 2000). 27F was labelled at the 5' end with the 6'-carboxyfluorescein (6-FAM), which was synthesized by Applied Biosystems Japan. Amplification reactions were performed in a total volume of 50 µl containing 5 µl dissolved DNA (100 ng), 1.25 U *TaKaRa Ex Taq* (Takara Shuzo), 5 µl 10× *Ex Taq* buffer, 4 µl dNTP mixture (2.5 mM each) and 10 pmol each primer. 16S rDNAs were amplified in a Biometra Thermocycler TGradient using the following programme: 95 °C for 3 min, followed by 30 cycles consisting of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1.5 min, with a final extension period at 72 °C for 10 min. *Spirochaeta*-specific 16S rDNAs were amplified using the following protocol (Paster *et al.*, 2001; Dewhirst *et al.*, 2000): 95 °C for 3 min, followed by 30 cycles consisting of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1.5 min with an additional 5 s for each cycle, with a final extension period at 72 °C for 15 min. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (2 µl) in 1.5% agarose in 1× TAE buffer. PCR products were purified by the PEG precipitation method (Hiraishi *et al.*, 1995) with some modifications. A 50 µl aliquot of the 16S rDNA solution was mixed with 30 µl of a PEG solution (40% PEG 6000 and 10 mM MgCl₂) and 12 µl 3 M sodium acetate, gently shaken for 10 min at room temperature, and centrifuged at 14 000 r.p.m. for 15 min. The supernatant was removed carefully by pipetting, and then the precipitated DNA was washed twice with 70% ethanol and redissolved in 20 µl sterile distilled water. Purified 16S rDNAs were stored at -20 °C until analysis.

T-RFLP analysis. The restriction enzymes were selected according to Moyer *et al.* (1996). Purified PCR product (2 µl) was digested with 20 U of either *HhaI* or *MspI* (Takara Shuzo) in a total volume of 10 µl at 37 °C for 3 h. Preliminary experiments conducted at various digestion times (3, 6 and 12 h) and enzyme concentrations (10, 20 and 30 U) demonstrated that 3 h and 20 U were sufficient for complete digestion of the PCR products. The restriction digest product (1 µl) was mixed with 12 µl of deionized formamide and 1 µl DNA fragment-length standard. The standard size marker was a 1:1 mixture of the size standards GS 500 ROX (including 35, 50, 75, 100, 139, 150, 160, 200, 300, 350, 400, 450, 490 and 500 bp) and GS 1000 ROX (including 29, 33, 37, 64, 67, 75, 81, 108, 118, 244, 275, 299, 421, 539, 674, 677 and 926 bp) (Applied Biosystems). Each sample was denatured at 95 °C for 2 min and then immediately placed on ice. The length of the terminal restriction fragment (T-RF) was determined on an ABI PRISM 310

genetic analyser (Applied Biosystems) in GeneScan mode (15 kV, 8 mA and 60 °C for 48 min for each sample). Fragment sizes were estimated by using the Local Southern Method in GeneScan 3.1 software (Applied Biosystems). T-RFs with a peak height less than 25 fluorescence units were excluded from the analysis. Fragments were resolved to one base pair by manual alignment of the size standard peaks from different electropherograms as described previously (Clement *et al.*, 1998). Predicted T-RFLP patterns of the 16S rDNAs of known bacterial species were obtained using the GENETYX-MAC program (Software Developing Co., Tokyo).

Numerical analysis. The methods developed for numerical analysis of quinone profiles (Hiraishi *et al.*, 1991; Iwasaki & Hiraishi, 1998) have been applied for processing T-RFLP data (Hiraishi *et al.*, 2000). Differences in T-RFLP patterns among samples were evaluated using the dissimilarity (*D*) index (Hiraishi *et al.*, 1991). To evaluate the diversity of microbial communities, another parameter, the microbial divergence index based on T-RFLP patterns (*MD_i*), was used (Iwasaki & Hiraishi, 1998). The *D* and *MD_i* values were calculated using the BioCLUST program (Iwasaki & Hiraishi, 1998). Dendrograms based on *D* matrix data were constructed by the neighbour-joining method (Saitou & Nei, 1987), and drawn using the TreeView program (Page, 1996).

RESULTS AND DISCUSSION

T-RFLP analysis of a single organism

T-RF lengths were determined for all bacteria used. The observed T-RF lengths were different (by 4 bp) from the predicted lengths (Table 1), with one exception: the observed T-RF lengths (29 bp) of *Treponema* species after digestion with *HhaI* were 8 bp smaller than the predicted length (37 bp). Although the predicted length is within the range of standards (29–926 bp), discrepancies were observed. The reason for this difference is not known. In addition, the observed T-RF lengths of *Fusobacterium nucleatum*, *T. denticola* and *Treponema vincentii* after digestion with *MspI* were 10, 7 and 7 bp larger than the predicted length, respectively. However, by using the only size standard GS 500 ROX, the observed T-RF lengths of *F. nucleatum* (283 bp), *T. denticola* (281 bp) and *T. vincentii* (288 bp) were within 3 bp of the predicted T-RF length. These findings emphasize the importance of selecting a suitable standard for accurate determination of the fragment size. These discrepancies between predicted and observed T-RF lengths have been reported previously (Liu *et al.*, 1997; Clement *et al.*, 1998; Kaplan *et al.*, 2001; Kitts, 2001). To compensate for the differences, Kaplan *et al.* (2001) used a matching window that allows an observed T-RF length (from a sample) to be within +1 to -4 of the predicted T-RF length (in the database).

Reproducibility

Reproducibility of T-RFLP patterns was investigated in detail previously (Osborn *et al.*, 2000). To assess the reproducibility of T-RFLP analysis, intragel/polymer variation (same electrophoresis gel/polymer) was investigated by three replicate electrophoresis of a sample (P17) from a patient with periodontitis generated after digestion with *HhaI* on the

same gel/polymer. Intergel/polymer variation (different gels/polymers) was investigated by electrophoresis of the same sample on three different gels/polymers. Seventeen T-RFs were common to all T-RFLP patterns (Table 2). Only one additional T-RF was observed in a T-RFLP pattern. Osborn *et al.* (2000) indicated that the greatest source of variation was the result of uneven sample loading into wells of a gel. They found that the degree of variation between replicate runs of the same sample was lower than that experienced with manual loading of acrylamide gels when they used a capillary-based electrophoresis system (ABI Prism 310) with automated sample loading, which was similar to that used in our study.

In addition, the variability generated during two stages of the T-RFLP protocol was investigated as described previously (Osborn *et al.*, 2000): (i) variation between three different PCRs from a single DNA sample; and (ii) variation between three different digestions of the PCR product from a single PCR from a single DNA sample. Seventeen T-RFs were common to all the T-RFLP patterns as well as the results of intra- and intergel/polymer variation. These results indicate that there is the reproducibility of the data, although minor variation in peak height was observed (data not shown).

Analysis of diversity of oral bacterial community by T-RFLP

The universal primers 27F and 1492R were used to characterize oral bacterial community. Although this primer pair may not amplify all of the 16S rDNAs of organisms in saliva, and not accurately preserve the evenness of the original community DNA template, PCR amplification with primer pair 27F–1492R followed by digestion with *HhaI* or *MspI* is considered to be the simplest approach to classify the largest number of 16S rRNA gene sequences into the largest number of unique T-RFs in the present study.

T-RFLP patterns with two different restriction enzymes were compared –these are available as supplementary data in JMM Online (<http://jmm.sgmjournals.org>). *MspI* generated larger numbers of T-RFs than *HhaI*. Each of the 36 patterns showed 5–35 T-RFs after digestion with *HhaI*. A total of 68 different T-RFs were detected. The size of the majority of *HhaI*-digested T-RFs was less than 600 bp. The major T-RFs of around 578 bp were detected in the T-RFLP patterns of all

samples (31.8–84.3 %). The computer-simulated T-RFLP analysis showed that the T-RFs of around 578 bp were derived from the members of the genus *Streptococcus*. This result is in good agreement with that of the 16S rDNA clone library analysis of human saliva (Sakamoto *et al.*, 2000), in which large numbers of clones were phylogenetically related to the genus *Streptococcus* (i.e. *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus parasanguinis*, *Streptococcus pneumoniae*, *Streptococcus salivarius* and *Streptococcus sanguinis*). In addition, T-RFLP analysis of these clones (streptococci derived) confirmed the above presumption (data not shown).

A 29 bp T-RF was identified in the T-RFLP patterns of only four samples (P1, P7, P15 and P17) from patients with periodontitis generated after digestion with *HhaI* (Fig. 1). According to T-RFLP analysis of a single organism (Table 1) and the computer-simulated T-RFLP analysis, it was considered that the 29 bp T-RF was derived from oral spirochaetes. This presumption was supported by the T-RFLP patterns of the above four samples generated after digestion with *MspI* (data not shown). Furthermore, these four samples produced the specific PCR products for *T. denticola* and *T. socranskii* (our unpublished data). Choi *et al.* (1994) reported that although approximately 20 % of the bacteria in a subgingival plaque sample from a single patient with destructive periodontitis were spirochaetes, treponeme-specific clones represented about 7 and 1 % of clones randomly selected from the DNA and cDNA libraries, respectively. This finding suggested that the proportion of treponeme-specific clones was low for 16S rRNA libraries when all-bacterial primers were used. Thus, the 29 bp T-RF would be detected in only four samples from patients and a small population (0.8–3.4 %). PCR bias has been described previously (Farrelly *et al.*, 1995; Suzuki & Giovannoni, 1996). Liu *et al.* (1997) pointed out that data obtained by using T-RFLPs should be cautiously interpreted. The reasons were as follows. The number of populations represented in the T-RFLP pattern of any given community depends on the rank abundance of each populations. Microbial populations that are not numerically dominant are not represented, because the template DNAs from these populations represent a small fraction of the total community DNA. Hence, the species diversity of the microbial community is underestimated.

The above four samples generated T-RFs larger than 1000 bp (Fig. 1), which was a relatively large population (12.9–26.4 %) compared with the 13 other samples (1.8–11.6 %, those of P6, P10 and P11 were very small) from patients (except for P2 which did not generate T-RFs larger than 1000 bp). T-RFs larger than 1000 bp were detected in T-RFLP patterns of samples from patients with periodontitis rather than periodontally healthy subjects. However, in preliminary experiments with P17, since a decrease in the peak heights of T-RFs larger than 1000 bp was observed with increases in enzyme concentration and digestion time (data not shown), those T-RFs may be composed of partially digested products. The problem of incomplete digestion by the restriction enzyme has been reported in detail by Osborn *et al.* (2000).

Table 2. Reproducibility of T-RFLP pattern

The number of common T-RFs was 17.

Source of variation	No. T-RFs (replicate no.)		
	1	2	3
Intragel/polymer	18	17	17
Intergel/polymer	18	17	17
Digests	18	17	18
PCR	18	17	17

Fig. 1. T-RFLP patterns of 16S rDNAs from saliva digested with *HhaI*. 16S rDNAs were amplified with universal primers 27F and 1492R. Arrows indicate a 29 bp T-RF. Samples: (P) patient with periodontitis. The minimum and maximum values of the ordinate of each T-RFLP pattern are 0 and 700 fluorescence units, respectively.

They mentioned the risk of removing genuine T-RFs that represent different members of the same genus or group when such fragments (partially digested products) are excluded from the analysis, and suggested that such fragments should be retained in the analysis.

Each of the 36 patterns showed 13–40 T-RFs after digestion with *MspI*. A total of 107 different T-RFs were detected. The size of the majority of *MspI*-digested T-RFs was less than 600 bp. The major T-RFs of around 554 bp, presumed to be members of the genus *Streptococcus*, were detected in T-RFLP patterns of all samples (38.6–85.1 %) (see supplementary data in JMM Online; <http://jmm.sgmjournals.org>). This result is in good agreement with that of T-RFLP patterns generated after digestion with *HhaI*.

Analysis of diversity of spirochaetal community by T-RFLP

Bernhard & Field (2000) reported the T-RFLP method using the *Bacteroides-Prevotella* group-specific primers. In our study, we used a *Spirochaeta*-selective primer (Paster *et al.*, 2001; Dewhirst *et al.*, 2000) to characterize the community profiles of oral spirochaetes, although there is a risk that the sequence of this primer is the same as all members of the family *Coriobacteriaceae* (Dewhirst *et al.*, 2001). The problem concerning the specificity of the *Spirochaeta*-selective primer has been discussed by Dewhirst *et al.* (2001). No amplicons were obtained from two samples (H2 and H5) of periodontally healthy subjects with 27F and the *Spirochaeta*-selective reverse primer. In addition, these two samples did

not produce specific PCR products for *T. denticola* and *T. soerensii* (our unpublished data). Each of the 34 patterns showed 2–16 T-RFs after digestion with *HhaI*. A total of 43 different T-RFs were detected. The majority of *HhaI*-digested T-RFs were 29 and 31 bp long – see supplementary data in JMM Online (<http://jmm.sgmjournals.org>). The proportion of the 29 bp T-RF, which is presumed to be oral spirochaetes based on T-RFLP analysis of a single organism, ranged from 21.6 to 78.5 % (49.2 ± 14.9) (Table 3). On the other hand, that of the 31 bp T-RF was unexpectedly 1.4–77.3 % (29.7 ± 21.6). The proportion of 31 bp T-RFs in T-RFLP patterns of samples from periodontally healthy subjects (43.1 ± 19.7 %) was larger than that in T-RFLP patterns of samples from patients with periodontitis (17.1 ± 14.5 %, $P < 0.001$, *t*-test). The 31 bp T-RFs are probably not derived from oral spirochaetes, and further studies are necessary to verify this hypothesis. The proportion of 202 and 203 bp T-RFs in the T-RFLP pattern of P2 was unexpectedly 56.9 % (figure in supplementary data and Table 3), although that of the 29 bp T-RF was small (21.6 %). In addition, the proportion of T-RF larger than 1000 bp in T-RFLP patterns of P17 was relatively larger than that of other samples (27.6 %). The 16S rDNA clone library analysis revealed that at present, there are about 60 oral treponemal species or phylotypes in human subgingival plaque (Paster *et al.*, 2001; Dewhirst *et al.*, 2000). Other as-yet-unidentified species or phylotypes are probably still present in the human oral cavity.

Each of the 34 patterns showed 8–33 T-RFs after digestion with *MspI*. A total of 92 different T-RFs were detected. The

Table 3. Percentage of T-RFs of spirochaetal 16S rDNAs from saliva after digestion with *HhaI* and *MspI*

Subject*	<i>HhaI</i>				<i>MspI</i>		
	29 bp	31 bp	202 bp	203 bp	202 bp	291 bp	296 bp
H1	32.8	67.2	0	0	65.2	0	8.8
H3	44.7	41.6	0	0	47.1	5.4	11.4
H4	34.1	49.5	0	0	58.7	2.1	9.5
H6	38.5	48.8	0	0	70.2	0	2.9
H7	50.4	10.0	0	0	4.6	9.4	15.3
H8	70.9	21.8	1.2	0	15.0	0.6	21.0
H9	29.9	67.8	0	0	75.4	0	1.0
H10	41.9	28.9	0	0	31.5	1.2	18.5
H11	22.7	77.3	0	0	73.2	0	0
H12	41.9	42.5	0	0	57.3	1.1	15.1
H13	36.9	37.3	0	0	68.0	1.9	9.0
H14	37.2	59.9	0	0	51.6	0	1.8
H15	47.5	46.8	0	0	58.3	1.3	13.6
H16	58.1	31.8	1.5	2.6	18.3	7.9	14.4
H17	47.6	14.8	0	0	7.9	2.0	34.5
H18	39.2	57.2	0	0	77.6	0.8	4.5
P1	73.3	5.1	3.0	0	1.1	34.6	30.4
P2	21.6	1.4	36.1	20.8	0	7.9	3.8
P3	46.0	32.6	3.2	0	40.2	9.9	18.9
P4	45.5	19.2	6.6	3.1	30.0	20.6	6.9
P5	55.0	26.6	1.1	1.1	27.1	13.5	20.6
P6	72.4	27.6	0	0	11.3	12.7	36.4
P7	55.1	1.9	1.9	3.0	0	29.1	38.3
P8	39.9	39.4	0	0	20.1	0	5.1
P9	58.4	3.5	6.8	6.2	0	21.5	30.6
P10	48.3	36.0	0	0	27.9	2.1	13.1
P11	78.5	5.3	1.3	0	2.9	0	63.6
P12	55.5	5.9	3.0	1.7	1.5	18.7	32.5
P13	43.6	43.6	0	0	51.9	9.1	5.3
P14	76.6	9.0	2.6	2.9	8.4	26.5	29.9
P15	72.6	3.4	0	0.7	0	28.3	40.0
P16	54.2	13.9	2.2	3.8	8.1	26.4	12.7
P17	42.3	5.5	3.9	2.6	0.4	10.0	47.9
P18	59.9	27.6	1.0	0	26.2	13.5	25.5

*(H) periodontally healthy subject, (P) patient with periodontitis.

size of the majority of *MspI*-digested T-RFs was less than 600 bp (see supplementary data in JMM Online; <http://jmm.sgmjournals.org>). In T-RFLP patterns of samples from periodontally healthy subjects, T-RFs of around 202 bp were predominant (figure in supplementary data and Table 3). On the other hand, T-RFs of 202 bp plus approximately 291 and 296 bp were predominant in T-RFLP patterns of samples from patients with periodontitis. Using a 16S rRNA universal forward primer and a *Spirochaeta*-selective primer, Dewhirst *et al.* (2001) reported that 75 % of the clones obtained were *Treponema* species, and the majority of non-spirochaete clones were closely related to the genus *Atopobium* in the family *Coriobacteriaceae*. The predicted T-RF lengths of

Atopobium species after digestion with *HhaI* were 37 bp, which were identical to those of spirochaetes. However, the predicted T-RF lengths of *Atopobium* species after digestion with *MspI* were different from those of spirochaetes. Consequently, the majority of 16S rDNAs amplified from samples of periodontally healthy subjects may be closely related to the genus *Atopobium* but not spirochaetes. Further studies are necessary.

Numerical analysis

In the analysis of diversity of oral bacterial community, the number of T-RFs in patients with periodontitis (*MspI*:

27.9 ± 5.8) was relatively larger than in periodontally healthy subjects (*MspI*: 22.3 ± 5.6) ($P < 0.05$, *t*-test). Although the number of *HhaI*-digested T-RFs in patients with periodontitis (17.1 ± 7.4) was slightly larger than in periodontally healthy subjects (14.4 ± 5.7), the difference was not statistically significant ($P > 0.05$, *t*-test). In addition, in the analysis of diversity of spirochaetal community, the number of T-RFs in patients with periodontitis (*HhaI*: 10.1 ± 3.7 and *MspI*: 22.4 ± 5.7) was larger than in periodontally healthy subjects (*HhaI*: 6.3 ± 3.7 and *MspI*: 15.9 ± 5.9) ($P < 0.01$, *t*-test). Dewhirst *et al.* (2000) reported that the species diversity of spirochaetes in subgingival plaque was greatest in patients with acute necrotizing ulcerative gingivitis and periodontitis and least in periodontally healthy and patients with HIV-periodontitis. Our results support their report.

Dendrograms were constructed by the neighbour-joining method based on the *D* matrix data calculated by the combination of two T-RFLP patterns with two different restriction enzymes (Figs 2 and 3). Based on the analysis of the oral bacterial community, samples from periodontally healthy and patients with periodontitis were grouped into different clusters, though with some uncertainty (Fig. 2).

Fig. 2. Dendrogram of oral bacterial community showing the relationships among T-RFLP patterns of saliva based on *D* matrix data calculated by the combination of two T-RFLP patterns with two different restriction enzymes (*HhaI* and *MspI*). The scale bar indicates 10 % dissimilarity. Samples: (H) periodontally healthy subjects and (P) patients with periodontitis. Data presented in Figs 2 and 3 include group (1, group 1; 2, group 2; 3, group 3), type (Agg, aggressive periodontitis; Chr, chronic periodontitis), age and sex (F, female; M, male).

Fig. 3. Dendrogram of spirochaetal community showing the relationships among T-RFLP patterns of saliva based on *D* matrix data calculated by the combination of two T-RFLP patterns with two different restriction enzymes (*HhaI* and *MspI*). The scale bar indicates 10 % dissimilarity. Samples: (H) periodontally healthy subjects and (P) patients with periodontitis.

Samples from patients with periodontitis tended to cluster with their respective types (aggressive and chronic periodontitis), although this was not very clear. Interestingly, P8 and P14, which are in the maintenance stage, formed a cluster with the samples from periodontally healthy subject and were relatively separate from the samples of other patients. P8 did not produce specific PCR products for *A. actinomycetemcomitans*, *T. forsythensis*, *P. gingivalis*, *T. denticola* or *T. socranskii*, and P14 did not produce specific PCR products for *A. actinomycetemcomitans* or *T. denticola* (our unpublished data). MD_t values ranged from 4.3 to 20.6. In the analysis of the spirochaetal community (Fig. 3), the patterns derived from patients with periodontitis showed a better grouping compared with the analysis of oral bacterial community. These results suggest that samples from patients with periodontitis contain an unexpected diversity. MD_t values ranged from 3.5 to 13.0.

Computer-simulated T-RFLP analysis

Spirochaetes exist in several environments (Choi *et al.*, 1997; Iida *et al.*, 2000; Lilburn *et al.*, 1999; Paster *et al.*, 1996) in addition to the human oral cavity. Furthermore, a number of

Fig. 4. Computer-simulated analysis of T-RFs in the genus *Treponema*. The 194 sequences were selected for analysis.

spirochaetes are present in termite guts (Lilburn *et al.*, 1999). The 16S rDNA clone library analysis revealed that all termite-gut spirochaetal clones were affiliated with the genus *Treponema* (Iida *et al.*, 2000; Lilburn *et al.*, 1999; Paster *et al.*, 1996). Lilburn *et al.* (1999) reported that at least 21 new species of *Treponema* were recognized within one termite species. In our study, 194 treponemal sequences (including termite-gut spirochaetal clones) retrieved from the DDBJ/EMBL/GenBank were selected for the T-RFLP analysis. The size distribution of T-RFs predicted for treponemes is shown in Fig. 4. The number of T-RFs after digestion with *MspI* was larger than that after digestion with *HhaI*. These results are in agreement with the analysis of diversity of the spirochaetal community. From the computer-simulated T-RFLP analysis with *HhaI*, although the proportion of the 37 bp T-RF, which corresponds to the 29 bp T-RF observed in this study, was about 90%, that of the 29 bp T-RF ranged from 21.6 to 78.5%. Unexpectedly, the proportion of the 31 bp T-RF was large (1.4–77.3%). Furthermore, T-RFs of around 202 bp plus approximately 291 and 296 bp (from oral spirochaetes) were predominant in the analysis with *MspI*. In the computer-simulated T-RFLP analysis, the proportion of T-RFs of around 202 bp was not large. These findings indicate that the 31 bp T-RF (with *HhaI*) and T-RFs of around 202 bp (with *MspI*) are probably not derived from oral spirochaetes. In the future, we will design and use a more specific primer for oral spirochaetes.

Host-specific and stable T-RFLP patterns of oral bacterial community in human saliva

Finally, we also investigated the stability of the oral bacterial

community over a 5-week period (Fig. 5). Although a minor change in T-RFLP pattern was observed, saliva samples from the same subject produced similar T-RFLP patterns irrespective of the sampling time. These results suggested that the change in oral bacterial flora was relatively small in daily life and demonstrates the usefulness of this method for monitoring the oral bacterial flora of an individual over a period of time.

Conclusion

Although the subject population was small, our study demonstrated that T-RFLP analysis is useful for assessment of the diversity of the oral bacterial flora and rapid comparison of the community structure among individuals with and without periodontitis. The approach reported here should be useful in the future to elucidate the aetiology of periodontal disease and to develop new diagnostic tools, disease-prevention programmes and treatment modalities. Studies are under way in our laboratories to examine the effects of medical treatment of periodontitis on T-RFLP patterns.

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Fig. 5. T-RFLP patterns of 16S rDNAs from saliva samples of two periodontally healthy subjects (H10 and H17) from a 5-week period (24 July 2001, 31 July 2001, 7 August 2001, 14 August 2001 and 21 August 2001) generated after digestion with *HhaI* (a) and *MspI* (b). 16S rDNAs were amplified with universal primers 27F and 1492R. The minimum and maximum values of the ordinate of each T-RFLP pattern are 0 and 300 fluorescence units, respectively.

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