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Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes

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Terminal RFLP (T-RFLP) analysis was used to investigate changes in the oral microbiota in saliva and subgingival plaque samples from one patient with aggressive periodontitis (subject A) and two patients with chronic periodontitis (subjects B and C) before and 3 months after periodontal treatment. Substantial changes in the T-RFLP patterns of subgingival plaque samples of subjects B and C were noted after 3 months of improved oral hygiene and full-mouth supra- and subgingival scaling and root planing. However, there was little change in the subgingival microbiota of subject A. Although the proportions of terminal restriction fragments (T-RFs) larger than 1000 bp were notable in the T-RFLP patterns generated after digestion with *Hha*I of the samples from two subjects before treatment (subject B, 35·5 %; subject C, 29·6 %), the proportions of these T-RFs were significantly reduced or not detected after treatment (subject B, none; subject C, 4·1 %). Real-time PCR showed a significant change in the proportions of target bacteria in subgingival plaque samples of subject B. After 3 months, the *Porphyromonas gingivalis* population was markedly reduced ($3{\cdot}1 \times 10^{-3}$ %), whereas the proportion of *Porphyromonas gingivalis* before treatment was 7·6 %. The proportions of *Tannerella forsythensis*, *Treponema denticola* and *Treponema socranskii* were also markedly diminished after treatment. Similarly, the proportion of the T-RF presumed to represent *Porphyromonas gingivalis* was 5·9 % and became undetectable after 3 months. Analysis of 16S rRNA gene clone libraries from subgingival plaque samples of subject B before and after treatment showed a notable change in the subgingival microbiota. These results were in agreement with the T-RFLP analysis data and showed that the T-RFs larger than 1000 bp represent *Peptostreptococcus* species. Our results indicate that T-RFLP analysis is useful for evaluation of the effects of medical treatment of periodontitis.

INTRODUCTION

Periodontal disease, a polymicrobial mixed infection, is one of the major oral diseases. It is caused by several microbial species, such as *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*) (Sakamoto *et al.*, 2002a), *Treponema denticola* and *Treponema socranskii* (Takeuchi *et al.*, 2001). Analysis of the human oral microbiota has been limited by conventional culture-dependent methods; thus, more oral bacteria remain uncultured and uncharacterized. Consequently, studies of causal micro-organisms of oral diseases including periodontal disease are, in general, restricted to cultivable species such as the aforementioned pathogens.

A phylogenetic approach based on 16S rRNA (rDNA) has been applied to investigate the diversity of cultivable and uncultivable species in the human oral cavity, without requiring cultivation (Hutter *et al.*, 2003; Kroes *et al.*, 1999; Paster *et al.*, 2001; Sakamoto *et al.*, 2000). Paster *et al.* (2001) demonstrated that the predominant subgingival bacterial community consisted of 347 species or phylotypes, based on analysis of 2522 16S rRNA clones, and estimated that the total species diversity in the oral cavity is approximately 500 species. However, analysis of individual 16S rRNA clones is an expensive and extremely inefficient approach for comparison of a multitude of bacterial communities.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; SRP, scaling and root planing; T-RF, terminal restriction fragment.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene sequences determined in this study are AB121791–AB121968.

Terminal RFLP (T-RFLP) is an alternative molecular approach that allows the assessment of the diversity of complex bacterial communities and rapid comparison of the community structure and diversity of different ecosystems (Liu *et al.*, 1997). We have already used this technique to characterize the oral microbiota in saliva of healthy subjects and patients with periodontitis and found that T-RFLP analysis is useful for assessment of the diversity of the oral microbiota and rapid comparison of the community structure among individuals with and without periodontitis (Sakamoto *et al.*, 2003a). In addition, it has been demonstrated that T-RFLP analysis is useful for assessment of the diversity of the human faecal microbiota and rapid comparison of the community structure among individuals (Sakamoto *et al.*, 2003b). More recently, two groups (Fujimoto *et al.*, 2003; Zijnge *et al.*, 2003) used denaturing gradient gel electrophoresis (DGGE) analysis to study bacterial community structure in pockets of periodontitis patients. However, it is difficult to create a database from the band profiles obtained by DGGE analysis in comparison with the terminal restriction fragment (T-RF) profiles obtained by T-RFLP analysis. T-RF lengths can be predicted from known 16S rRNA gene sequences.

In the present study, we used T-RFLP analysis to study changes in the oral microbiota in saliva and subgingival plaque samples of patients with periodontitis before and 3 months after periodontal treatment. To our knowledge, this is the first report on characterization of the subgingival microbial community by T-RFLP patterns. In addition, we used real-time PCR and 16S rRNA gene clone library techniques to evaluate the T-RFLP analysis data.

METHODS

Bacterial strains and DNA extraction. *Actinobacillus actinomycetemcomitans* JCM 2434, *Porphyromonas gingivalis* JCM 8525, *Tannerella forsythensis* JCM 10827^T, *Treponema denticola* JCM 8225 and *Treponema socranskii* subsp. *socranskii* JCM 8157^T were used as real-time PCR controls. All bacteria were grown under appropriate culture conditions. Bacterial cell numbers per millilitre were determined using a Petroff–Hauser counting chamber. Bacterial DNA was prepared as described previously (Sakamoto *et al.*, 1999).

Subjects. Three patients (subject A, a 20-year-old male patient with aggressive periodontitis; subject B, a 62-year-old female patient with chronic periodontitis; subject C, a 66-year-old female patient with chronic periodontitis) were enrolled in this study. All patients received oral hygiene instruction and full-mouth supra- and subgingival scaling and root planing (SRP). All patients gave their informed consent prior to participation in the study and the study protocols were approved by the human experimentation ethics committee of our institution.

Sample collection and DNA extraction. Saliva samples were collected in sterile plastic tubes from the above three subjects before and 3 months after treatment. Aliquots (0·5 ml) of saliva samples were diluted 1 : 2 with buffer (10 mM Tris/HCl, 50 mM EDTA, pH 8·0) 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 5 mg ml^{-1}) and *N*-acetylmuramidase (final concentration 1 mg ml^{-1}). After incubation at 37 °C for 1 h, proteinase K and SDS were added to final concentrations of 2 mg ml^{-1} and 1 % (w/v). 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 isopropanol followed by centrifugation. The DNA precipitate was washed with 70 % ethanol and resuspended in 100 µl TE (10 mM Tris/HCl, 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 then precipitated again with isopropanol and pelleted by centrifugation at 17 800 ***g*** for 15 min and the precipitated DNA was then washed with 70 % ethanol, dried under vacuum for 10 min and redissolved in 100 µl TE buffer.

Subgingival plaque samples were collected from the deepest pockets in each quadrant from the same subjects before and 3 months after treatment. The sampling sites were isolated with sterile cotton rolls. Supragingival plaque was removed with a cotton roll and then air-dried. A sterile paper point was inserted into a pocket until resistance was felt. After 30 s, the point was removed and immersed in 0·5 ml sterile distilled water and mixed with a vortex mixer. Bacterial DNA was extracted from the subgingival plaque suspensions as described above.

T-RFLP analysis. T-RFLP analysis was performed as described previously (Sakamoto *et al.*, 2003a). The primers used for PCR amplification of 16S rRNA gene sequences were 27F (5′-AGAGTTTGATC CTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane *et al.*, 1991). Primer 27F was labelled at the 5′ end with 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*, 5 µl 10× Ex *Taq* buffer, 4 µl dNTP mixture (2·5 mM each) and 10 pmol of each primer. 16S rDNAs were amplified in a Biometra TGradient thermocycler using the following program: 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1·5 min, with a final extension at 72 °C for 10 min. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (2 µl) in 1·5 % agarose in 1× TAE buffer. DNA was stained with ethidium bromide and visualized under short-wavelength UV light. PCR products were purified by the PEG precipitation method (Hiraishi *et al.*, 1995) with some modifications. Briefly, a 50-µl aliquot of the 16S rDNA solution was mixed with 30 µl PEG solution (40 % PEG 6000 and 10 mM $MgCl_2$) and 12 µl 3 M sodium acetate, shaken gently for 10 min at room temperature and centrifuged at 17 800 ***g*** for 15 min. The supernatant was removed carefully by pipetting and the precipitated DNA was then washed twice with 70 % ethanol and redissolved in 20 µl sterile distilled water. Purified 16S rDNAs were stored at −20 °C until analysis.

Purified PCR product (2 µl) was digested with 20 U of either *Hha*I or *Msp*I (Takara Shuzo) in a total volume of 10 µl at 37 °C for 3 h. The restriction digest product (1 µl) was mixed with 12 µl 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 and GS 1000 ROX (Applied Biosystems). Each sample was denatured at 95 °C for 2 min and then placed immediately on ice. The length of T-RF was determined on an ABI PRISM 310 Genetic Analyzer (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 of 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 electrophoregrams. Predicted T-RFLP patterns of the 16S rDNAs of known bacterial species were obtained using the GENETYX-MAC program (Software Development Co.).

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; Sakamoto *et al.*, 2003a). 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_t), was used (Iwasaki & Hiraishi, 1998). *D* and MD_t 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).

Real-time PCR. Real-time PCR was performed with the LightCycler system (Roche Diagnostics) and the dsDNA-binding dye SYBR Green I using species-specific primers (Ashimoto *et al.*, 1996; Sakamoto *et al.*, 1999) for the above five periodontopathic bacteria as described previously (Sakamoto *et al.*, 2001).

The total number of bacteria in samples was determined with *Porphyromonas gingivalis* cells as a standard using the universal primers TotalF (5′-TCCTACGGGAGGCAGCAGT-3′) and TotalR (5′-GGAC TACCAGGGTATCTAATCCTGTT-3′) (Nadkarni *et al.*, 2002). Briefly, amplification was performed in a 20-μl final volume containing 2 μl template DNA, 2 μl LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), 0·5 μM of each primer and 3 mM $MgCl_2$. The protocol included an initial denaturation step at 95 °C for 10 min, followed by 45 cycles of heating at 20 °C s^{-1} to 95 °C with a 0-s hold, cooling at 20 °C s^{-1} to 60 °C with a 5-s hold, heating at 20 °C s^{-1} to 72 °C with an 18-s hold and heating at 20 °C s^{-1} to 84 °C with a 1-s hold. Fluorescent products were detected at the last step of each cycle. After amplification, a melting curve was obtained by heating at 20 °C s^{-1} to 95 °C, cooling at 20 °C s^{-1} to 70 °C and heating slowly at 0·1 °C s^{-1} to 95 °C with fluorescence collection at 0·1 °C intervals. Melting peaks were used to determine the specificity of the PCR. Data were analysed using the LightCycler analysis software.

16S rRNA gene clone library analysis. 16S rRNA gene clone library analysis was performed as described previously (Sakamoto *et al.*, 2000). The primers used for PCR amplification of 16S rRNA gene sequences were 27F (without 6-FAM) and 1492R. 16S rDNAs were amplified as described above. The amplified 16S rDNA was purified using an UltraClean PCR Clean-up DNA purification kit (Mo Bio Laboratories). Purified amplicon was ligated into the plasmid vector pCR2.1 and then transformed into One Shot INVαF′ competent cells using the Original TA cloning kit (Invitrogen).

Plasmid DNAs were prepared using the TempliPhi DNA amplification kit (Amersham Biosciences) from randomly selected recombinants and used as templates for sequencing. Sequencing was conducted using primers 27F and 520R (Lane *et al.*, 1991), a Big Dye Terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequences were checked for possible chimaeric artefacts by the CHIMERA CHECK program of the Ribosomal Database Project-II (Cole *et al.*, 2003) and compared with similar sequences of reference organisms by FASTA search (Pearson & Lipman, 1988).

Statistical analysis. All data were expressed as means ± SD. Differences between groups were examined for statistical significance using Student's *t* test. A *P* value less than 0·05 was taken as indicating a statistically significant difference.

RESULTS

Clinical data

Changes in probing depth before and 3 months after periodontal treatment are shown in Table 1. The mean probing depths (mm) for subjects A, B and C were respectively 7·75 ± 1·50, 4·33 ± 1·53, 5·25 ± 0·96 before treatment and 4·25 ± 1·50, 2·00 ± 1·00, 2·25 ± 0·96 after treatment. There was a statistically significant decrease in the probing depths of all three subjects after periodontal treatment (subjects A and C, $P < 0·01$; subject B, $P < 0·05$). However, the probing depth of subject A after treatment remained at an intermediate level (4–6 mm) compared with those of subjects B and C.

Table 1. Changes in probing depth after periodontal treatment

Sample site	Probing depth (mm)	
	Before	After
Patient A		
1	7	3
2	9	6
3	6	3
4	9	5
Patient B		
1	4	2
2	6	3
3	3	1
Patient C		
1	6	3
2	5	3
3	6	2
4	4	1

Reproducibility

The reproducibility of T-RFLP patterns has been investigated previously (Sakamoto *et al.*, 2003a). To assess the reproducibility of the T-RFLP analysis data in this study, the variability generated during two stages of the T-RFLP protocol was investigated: (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 (data not shown). The *D* values obtained for the T-RFLP analysis data generated in the three runs ranged from 0·4 to 2·8 %. This indicates that the data are highly reproducible.

T-RFLP analysis

Fig. 1 shows representative T-RFLP patterns of saliva and subgingival plaque samples of three subjects before and 3 months after treatment. Some T-RFs disappeared or the proportions of these T-RFs were reduced after treatment, and some new T-RFs emerged or the proportions of some known T-RFs were increased after treatment. Among the T-RFLP patterns derived, there was a substantial change in the T-RFLP patterns of subgingival plaque samples of subjects B and C before and after treatment. Although the proportions of T-RFs larger than 1000 bp were notable in the T-RFLP patterns generated after digestion with *Hha*I of the samples

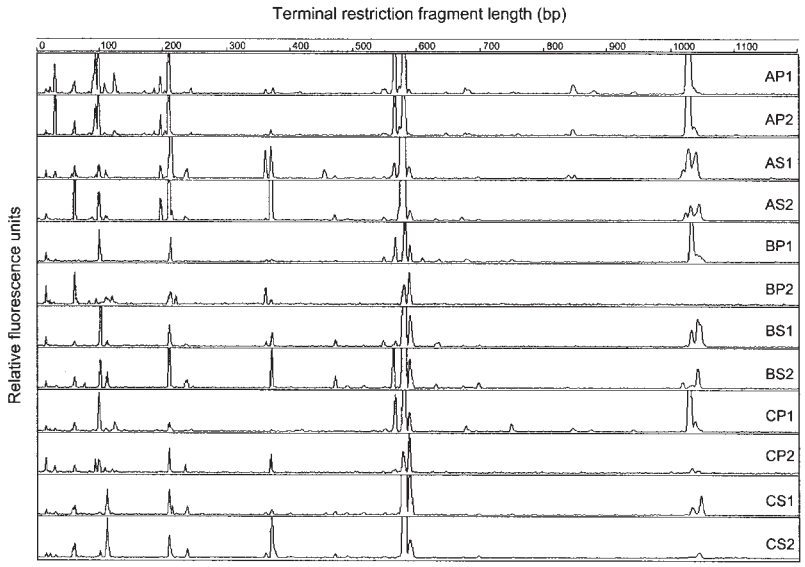

Fig. 1. T-RFLP patterns of 16S rDNAs from plaque (P) and saliva (S) samples of patients A, B and C taken before treatment (1) and after treatment (2) generated after digestion with *Hha*I. 16S rDNAs were amplified with universal primers 27F and 1492R. The minimum and maximum values of the ordinate of each T-RFLP pattern are respectively 0 and 600 fluorescence units.

from subjects B and C before treatment (subject B, 35·5 %; subject C, 29·6 %), the proportions of these T-RFs were significantly reduced or they were not detected after treatment (subject B, none; subject C, 4·1 %).

After 3 months of treatment, the number of T-RFs in saliva and subgingival plaque samples of three subjects tended to reduce, especially in subgingival plaque samples (Table 2). MD_t values for the subgingival plaque samples of subjects A, B and C were respectively 27·0, 16·6, 16·1 before treatment and were 25·3, 11·4, 12·8 after treatment.

A dendrogram was 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 (Fig. 2). Subgingival plaque samples (six samples) were particularly grouped into different clusters. Among the subgingival plaque samples, BP2 and CP2, which are samples taken after treatment, formed a cluster and were separate from the other four subgingival plaque samples. In the analysis of saliva samples, although the samples of subjects A and C before and after treatment were each grouped, the samples of subject B were not grouped. Differences in T-RFLP patterns before and after treatment are shown in Table 3. The difference in T-RFLP patterns of subgingival plaque samples was marked for subjects B and C. This result was in good agreement with the dendrogram. In addition, the dissimilarity value of subject B was the highest in the saliva samples.

Table 2. Numbers of T-RFs detected in plaque and saliva samples taken before and after treatment

Sample names are formed from the patient (A, B or C), source (P, plaque; S, saliva) and whether they were taken before (1) or after (2) treatment.

Sample	T-RFs after digestion with:	
	*Hha*I	*Msp*I
AP1	48 ± 2·0	42 ± 1·5
AP2	36 ± 1·5	40 ± 1·0
AS1	38 ± 1·0	49 ± 0·6
AS2	28 ± 2·0	55 ± 1·5
BP1	23 ± 0·6	23 ± 1·0
BP2	17 ± 1·5	13 ± 0·6
BS1	29 ± 0·6	53 ± 1·5
BS2	30 ± 1·0	31 ± 1·0
CP1	31 ± 2·1	20 ± 1·5
CP2	15 ± 1·5	17 ± 1·5
CS1	33 ± 1·5	26 ± 1·0
CS2	25 ± 1·2	25 ± 1·2

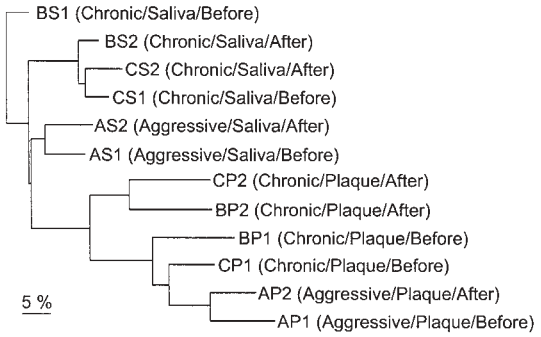

Fig. 2. Dendrogram of oral bacterial community showing the relationships among T-RFLP patterns (see Fig. 1 for explanation of sample names) based on *D*-matrix data calculated from two T-RFLP patterns with different restriction enzymes (*Hha*I and *Msp*I). The scale bar indicates 5 % dissimilarity. Subject type (Aggressive, aggressive periodontitis; Chronic, chronic periodontitis), sample type (Plaque, subgingival plaque sample; Saliva, saliva sample) and sampling time (Before, before periodontal treatment; After, after periodontal treatment) are indicated for each sample.

Real-time PCR

The numbers of target bacteria and total bacteria are shown in Table 4. There was a significant change in the proportions of target bacteria in the subgingival plaque samples of subject B before and after treatment, except for *Actinobacillus actinomycetemcomitans*. After 3 months, the *Porphyromonas gingivalis* population was dramatically reduced ($3{\cdot}1 \times 10^{-3}$ %), compared with before treatment (7·6 %). Like *Porphyromonas gingivalis*, the proportions of *Tannerella forsythensis*, *Treponema denticola* and *Treponema socranskii* after treatment were markedly reduced. On the other hand, changes in the proportions of other target bacteria in the subgingival plaque samples of subjects A and C were not significant, although the proportion of *Porphyromonas gingivalis* after treatment was significantly reduced (subject C, from 4·0 to $2{\cdot}2 \times 10^{-2}$ %). The proportions of target bacteria in saliva samples of the three subjects tended to reduce; these findings supported the data from T-RFLP analysis.

16S rRNA gene clone library analysis

In view of the T-RFLP analysis and real-time PCR data, we constructed 16S rRNA gene clone libraries of the subgingival plaque samples of subject B before and after treatment. Clones detected in the subgingival plaque samples are shown in Table 5. There was a notable change in the subgingival microbiota before and after treatment. Before treatment, putative periodontal pathogens, *Eubacterium* species, especially *Eubacterium nodatum*, *Eubacterium saphenum* and *Eubacterium* sp. clone AP54 (Sakamoto *et al.*, 2000), *Filifactor alocis*, *Fusobacterium nucleatum*, *Mogibacterium timidum*, *Peptostreptococcus* species, including clone AP24, which is significantly associated with periodontitis (Sakamoto *et al.*, 2000, 2002b), *Porphyromonas gingivalis*, *Prevotella intermedia* and *Streptococcus intermedius* were frequently detected. After 3 months, almost none of the above-mentioned pathogens were detected, except for *Fusobacterium nucleatum*. Instead, *Streptococcus* and *Veillonella* species were mainly detected. *Fusobacterium nucleatum* has been detected nearly universally in both healthy and diseased states (Kumar *et al.*, 2003).

Table 3. Differences in T-RFLP patterns from plaque and saliva samples taken before treatment and after treatment

Plaque and saliva samples taken before and after treatment from each patient were compared. Values are dissimilarity indices (*D*).

Comparison	Patient		
	A	B	C
P1–P2	29·3	73·8	63·7
S1–S2	23·0	31·5	15·9

Table 4. Numbers of target bacteria and total bacteria detected in plaque and saliva samples taken before and after treatment

See Table 2 for explanation of sample names. Values are numbers of bacteria (cells per plaque sample or ml saliva). Values in parentheses are percentages of total bacteria. Data were determined from triplicate assays and are means ± SD. ND, Not detected.

Sample	Total bacteria*	*Actinobacillus actinomycetemcomitans*	*Porphyromonas gingivalis*	*Tannerella forsythensis*	*Treponema denticola*	*Treponema socranskii*
AP1	$1{\cdot}0 \pm 0{\cdot}3 \times 10^{8}$	ND	ND	$5{\cdot}7 \pm 0{\cdot}6 \times 10^{5}$ (0·56)	$4{\cdot}6 \pm 1{\cdot}7 \times 10^{5}$ (0·46)	$1{\cdot}0 \pm 1{\cdot}1 \times 10^{6}$ (1·0)
AP2	$5{\cdot}0 \pm 1{\cdot}0 \times 10^{7}$	ND	ND	$3{\cdot}2 \pm 0{\cdot}2 \times 10^{5}$ (0·63)	$4{\cdot}6 \pm 0{\cdot}8 \times 10^{5}$ (0·95)	$3{\cdot}0 \pm 0{\cdot}5 \times 10^{6}$ (6·1)
AS1	$2{\cdot}0 \pm 0{\cdot}1 \times 10^{9}$	ND	ND	$3{\cdot}4 \pm 0{\cdot}4 \times 10^{4}$ ($1{\cdot}7 \times 10^{-3}$)	$1{\cdot}2 \pm 1{\cdot}1 \times 10^{4}$ ($6{\cdot}2 \times 10^{-4}$)	$3{\cdot}6 \pm 2{\cdot}3 \times 10^{5}$ ($1{\cdot}8 \times 10^{-2}$)
AS2	$2{\cdot}8 \pm 2{\cdot}0 \times 10^{10}$	ND	ND	$6{\cdot}9 \pm 1{\cdot}3 \times 10^{3}$ ($2{\cdot}5 \times 10^{-5}$)	$2{\cdot}1 \pm 0{\cdot}3 \times 10^{3}$ ($7{\cdot}5 \times 10^{-6}$)	$2{\cdot}9 \pm 0{\cdot}7 \times 10^{4}$ ($1{\cdot}1 \times 10^{-4}$)
BP1	$1{\cdot}5 \pm 1{\cdot}2 \times 10^{7}$	$2{\cdot}6 \pm 1{\cdot}1 \times 10^{4}$ (0·17)	$1{\cdot}1 \pm 0{\cdot}5 \times 10^{6}$ (7·6)	$2{\cdot}4 \pm 0{\cdot}8 \times 10^{4}$ (0·16)	$6{\cdot}4 \pm 2{\cdot}1 \times 10^{4}$ (0·43)	$5{\cdot}3 \pm 0{\cdot}3 \times 10^{5}$ (3·6)
BP2	$5{\cdot}1 \pm 1{\cdot}0 \times 10^{5}$	$5{\cdot}9 \pm 0{\cdot}5 \times 10^{2}$ (0·12)	$1{\cdot}6 \pm 0{\cdot}3 \times 10^{1}$ ($3{\cdot}1 \times 10^{-3}$)	$5{\cdot}2 \pm 0{\cdot}2 \times 10^{0}$ ($1{\cdot}0 \times 10^{-3}$)	$5{\cdot}8 \pm 0{\cdot}4 \times 10^{-1}$ ($1{\cdot}1 \times 10^{-4}$)	$4{\cdot}8 \pm 0{\cdot}9 \times 10^{1}$ ($9{\cdot}4 \times 10^{-3}$)
BS1	$2{\cdot}0 \pm 0{\cdot}1 \times 10^{9}$	$4{\cdot}3 \pm 0{\cdot}7 \times 10^{4}$ ($2{\cdot}2 \times 10^{-3}$)	$1{\cdot}2 \pm 2{\cdot}3 \times 10^{6}$ ($6{\cdot}2 \times 10^{-2}$)	$7{\cdot}1 \pm 0{\cdot}4 \times 10^{3}$ ($3{\cdot}6 \times 10^{-4}$)	$3{\cdot}9 \pm 1{\cdot}3 \times 10^{4}$ ($2{\cdot}0 \times 10^{-3}$)	$1{\cdot}9 \pm 1{\cdot}6 \times 10^{5}$ ($9{\cdot}5 \times 10^{-3}$)
BS2	$7{\cdot}7 \pm 0{\cdot}5 \times 10^{9}$	$5{\cdot}3 \pm 0{\cdot}4 \times 10^{3}$ ($6{\cdot}8 \times 10^{-5}$)	$2{\cdot}6 \pm 0{\cdot}7 \times 10^{4}$ ($3{\cdot}4 \times 10^{-4}$)	$1{\cdot}2 \pm 1{\cdot}1 \times 10^{4}$ ($1{\cdot}6 \times 10^{-4}$)	$7{\cdot}1 \pm 0{\cdot}6 \times 10^{3}$ ($9{\cdot}2 \times 10^{-5}$)	$1{\cdot}4 \pm 0{\cdot}8 \times 10^{5}$ ($1{\cdot}8 \times 10^{-3}$)
CP1	$2{\cdot}1 \pm 0{\cdot}2 \times 10^{7}$	ND	$8{\cdot}5 \pm 0{\cdot}4 \times 10^{5}$ (4·0)	$1{\cdot}2 \pm 1{\cdot}7 \times 10^{4}$ ($5{\cdot}5 \times 10^{-2}$)	$3{\cdot}9 \pm 0{\cdot}8 \times 10^{4}$ (0·19)	$4{\cdot}1 \pm 0{\cdot}3 \times 10^{5}$ (2·0)
CP2	$8{\cdot}9 \pm 1{\cdot}4 \times 10^{5}$	ND	$2{\cdot}0 \pm 1{\cdot}5 \times 10^{2}$ ($2{\cdot}2 \times 10^{-2}$)	$1{\cdot}2 \pm 1{\cdot}2 \times 10^{4}$ (1·4)	$2{\cdot}0 \pm 0{\cdot}2 \times 10^{3}$ (0·22)	$7{\cdot}8 \pm 0{\cdot}8 \times 10^{4}$ (8·8)
CS1	$1{\cdot}7 \pm 0{\cdot}3 \times 10^{10}$	ND	$6{\cdot}6 \pm 1{\cdot}8 \times 10^{3}$ ($3{\cdot}9 \times 10^{-5}$)	$3{\cdot}0 \pm 0{\cdot}7 \times 10^{4}$ ($1{\cdot}8 \times 10^{-4}$)	$1{\cdot}4 \pm 0{\cdot}4 \times 10^{5}$ ($8{\cdot}0 \times 10^{-4}$)	$5{\cdot}6 \pm 1{\cdot}3 \times 10^{6}$ ($3{\cdot}3 \times 10^{-2}$)
CS2	$2{\cdot}5 \pm 1{\cdot}7 \times 10^{10}$	ND	$1{\cdot}6 \pm 2{\cdot}5 \times 10^{4}$ ($6{\cdot}5 \times 10^{-5}$)	$2{\cdot}9 \pm 1{\cdot}3 \times 10^{4}$ ($1{\cdot}1 \times 10^{-4}$)	$4{\cdot}3 \pm 0{\cdot}8 \times 10^{3}$ ($1{\cdot}7 \times 10^{-5}$)	$8{\cdot}9 \pm 0{\cdot}8 \times 10^{5}$ ($3{\cdot}5 \times 10^{-3}$)

*Determined from triplicate assays with *Porphyromonas gingivalis* cells as a standard.

Table 5. Clones detected in plaque samples from patient B taken before and after treatment

Species or phylotype	Clones (*n*)	
	Before treatment	After treatment
Bulleidia extructa	2	0
Campylobacter concisus	1	0
Capnocytophaga sp. clone BP2-77*	0	1
Desulfomicrobium orale	1	0
Eubacterium nodatum	5	0
Eubacterium saphenum	9	0
Eubacterium sp. clone AP50	0	1
Eubacterium sp. clone AP54	5	0
Eubacterium sp. clone BP1-27*	1	0
Eubacterium sp. clone PUS9.170	1	0
Filifactor alocis	7	0
Fusobacterium nucleatum	4	5
Granulicatella adiacens	1	0
Lachnospiraceae clone MCE9.173	1	0
Lautropia sp. clone AP009	0	1
Leptotrichia sp. clone DA069	1	1
Leptotrichia sp. strain A39FD	0	6
Mogibacterium timidum	3	0
Neisseria pharyngis	0	3
Peptostreptococcus sp. clone AJ062	2	0
Peptostreptococcus sp. clone AP24	3	0
Peptostreptococcus sp. clone BS044	2	0
Peptostreptococcus sp. clone FG014	1	0
Porphyromonas gingivalis	9	0
Prevotella intermedia	5	0
Prevotella melaninogenica	1	0
Prevotella oulorum	0	1
Prevotella sp. clone BP1-4*	1	0
Pseudoramibacter alactolyticus	1	0
Rothia mucilaginosa	1	1
Rothia sp. strain ChDC B202	1	0
Selenomonas sp. BP2-20*	0	1
Streptococcus anginosus	1	6
Streptococcus cristatus	1	2
Streptococcus gordonii	0	4
Streptococcus intermedius	6	2
Streptococcus mitis	3	14
Streptococcus mutans	0	3
Streptococcus salivarius	6	11
Streptococcus sanguinis	1	2
Streptococcus sp. clone RP28	1	1
Streptococcus sp. clone BP1-91*	1	1
'Terrahaemophilus aromaticivorans'	0	5
Veillonella atypica	0	2
Veillonella dispar	1	11
Veillonella parvula	0	2
Veillonella sp. clone 007 (01)	0	1
Total	**90**	**88**

*Identified in this study.

Computer-simulated T-RFLP analysis

On the basis of 16S rRNA gene clone library analysis data, clones were assigned to T-RFs in the T-RFLP patterns of subgingival plaque samples of subject B before and after treatment (Fig. 3). Almost all the T-RFs were presumed to represent species or phylotypes detected by the 16S rRNA gene clone library analysis. From the computer-simulated T-RFLP analysis with *Hha*I, we found that T-RFs larger than 1000 bp represented *Peptostreptococcus* species. This result is in good agreement with that of T-RFLP patterns generated after digestion with *Msp*I. Furthermore, we found that one of the T-RFs larger than 1000 bp represents a certain *Peptostreptococcus* species by cloning and sequencing of target T-RFs (our unpublished data).

DISCUSSION

Changes in the subgingival microbiota in adult Down's syndrome patients with periodontitis (Sakellari *et al.*, 2001) or adult periodontitis patients after SRP (Darby *et al.*, 2001) or antibiotic (amoxicillin or metronidazole) therapy combined with SRP (Feres *et al.*, 2001) have been investigated using chequerboard DNA–DNA hybridization (Socransky *et al.*, 1994) or PCR techniques. However, these studies report changes in only a limited part, representing the cultivable known species, of the subgingival microbiota. Recently, Zijnge *et al.* (2003) used DGGE analysis, which takes into account the presence of unidentified and hard to cultivate species present in subgingival plaque (like T-RFLP analysis), to study shifts in the subgingival microbiota before, 1 day after and 3 months after treatment. They demonstrated that treatment does not result in the complete elimination of bacteria, although there was a change in DGGE profiles. After 1 day, the number of bands decreased, and the number of bands then increased after 3 months. These findings suggested that the microbial population was suppressed by treatment and that, after 3 months, recolonization of the site occurred. In the present study, the number of T-RFs tended to be smaller after 3 months. During 3 months of treatment, the same changes would occur. However, the degree of change in the subgingival microbiota was different among the subjects, although SRP produced a good clinical improvement in all the subjects. In subject B, the composition of the subgingival microbiota was changed drastically after treatment; the subgingival microbiota was shifted from an anaerobic microbiota, composed mainly of putative periodontal pathogens, towards mainly *Streptococcus* and *Veillonella* species. On the other hand, there was little significant change in the subgingival microbiota (T-RFLP patterns) of subject A with aggressive periodontitis. This may be associated with complex interactions between the subgingival microbiota and the host response. Further studies should be aimed at monitoring the microbial succession of the oral biofilm.

In the T-RFLP patterns (*Hha*I and *Msp*I) of subgingival plaque samples from subject B before treatment, the proportion of the T-RF that was presumed to represent *Porphyr-*

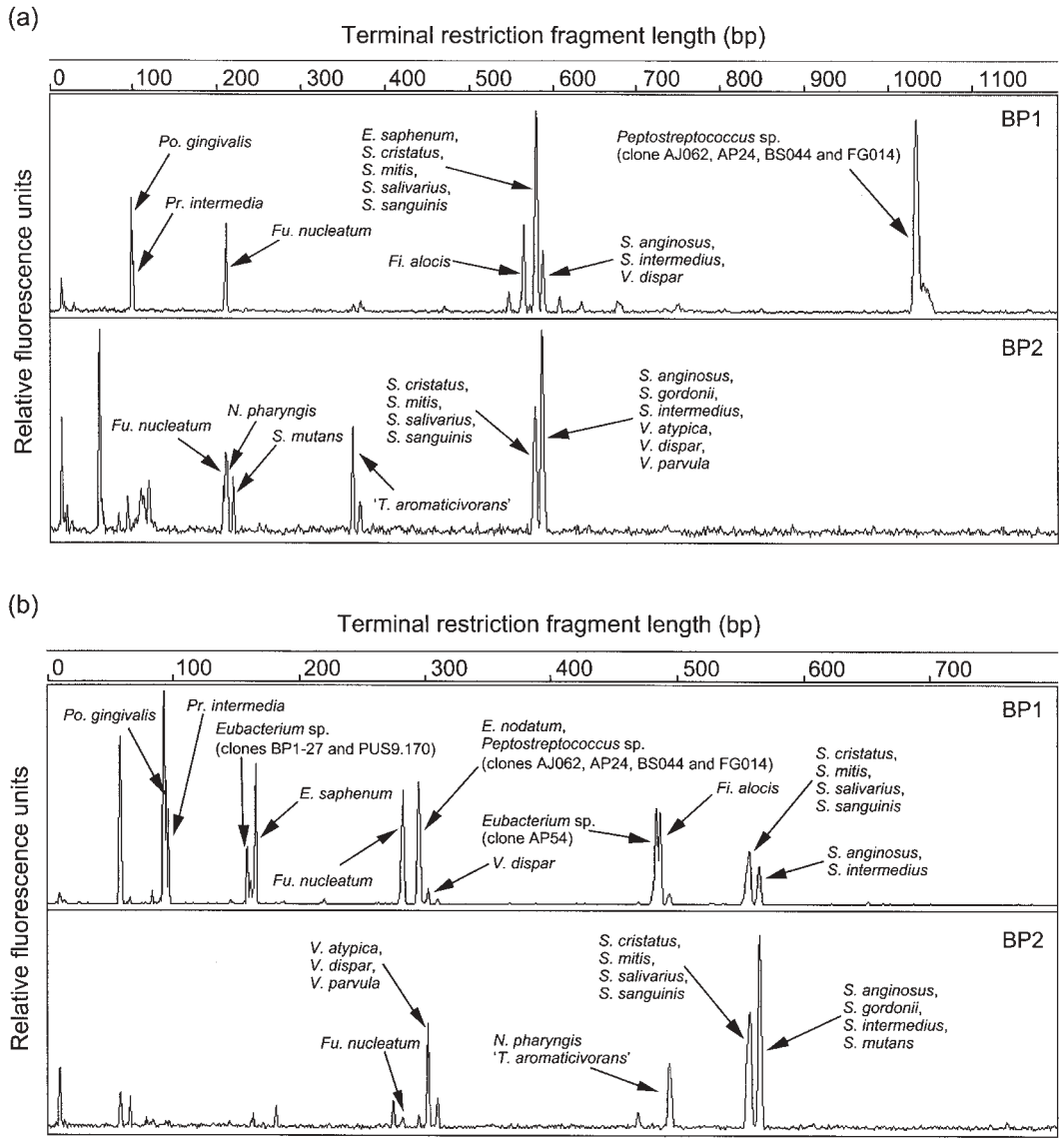

Fig. 3. T-RFLP patterns of 16S rDNAs from subgingival plaque samples of patient B taken before (BP1) and after (BP2) treatment generated after digestion with *Hha*I (a) and *Msp*I (b). 16S rDNAs were amplified with universal primers 27F and 1492R. Almost all the T-RFs were presumed to represent species or phylotypes detected by the 16S rRNA gene clone library analysis. *E.*, *Eubacterium*; *Fi.*, *Filifactor*; *Fu.*, *Fusobacterium*; *N.*, *Neisseria*; *Po.*, *Porphyromonas*; *Pr.*, *Prevotella*; *S.*, *Streptococcus*; *T.*, '*Terrahaemophilus*'; *V.*, *Veillonella*.

omonas gingivalis was 5·9 % (mean of *Hha*I and *Msp*I). After 3 months, this T-RF was not detected in either T-RFLP pattern. Similarly, real-time PCR revealed a change in the proportion of *Porphyromonas gingivalis* in the subgingival plaque sample of subject B after treatment (from 7·6 to $3{\cdot}1 \times 10^{-3}$ %). The T-RF presumed to represent *Prevotella intermedia*, which is an important periodontal pathogen, could be differentiated from the T-RF presumed to represent *Porphyromonas gingivalis* by being 2-bp larger. Before treatment, the proportion of the T-RF presumed to represent *Prevotella intermedia* was 2·8 % (mean of *Hha*I and *Msp*I). After 3 months, this T-RF was not detected in either of the T-RFLP patterns. *Porphyromonas gingivalis* clonal sequences (nine clones) were detected at a level about twofold higher than *Prevotella intermedia* clonal sequences (five clones). This result is in agreement with the T-RFLP data (*Porphyromonas gingivalis*, 5·9 %, compared with *Prevotella intermedia*, 2·8 %). These findings suggest that changes in the proportion of a certain target species in subgingival plaque and saliva samples can be monitored semiquantitatively by T-RFLP analysis, although there was a PCR bias. Interestingly, T-RFs larger than 1000 bp were significantly reduced or not detected in the T-RFLP patterns (*Hha*I) of subgingival plaque samples of subjects B and C after treatment. T-RFs larger than 1000 bp represented several *Peptostreptococcus* species. *Peptostreptococcus* species are members of the normal commensal flora of

humans and animals, but some species are associated with anaerobic infections, including gingivitis and periodontitis. *Peptostreptococcus micros* has been associated with periodontal disease (Rams *et al.*, 1992). Consequently, like the proportions of *Porphyromonas gingivalis* and *Prevotella intermedia*, monitoring of the proportion of T-RFs larger than 1000 bp in the T-RFLP pattern may be useful for the prognosis of periodontal disease.

Recently, Kumar *et al.* (2003) evaluated the association of newly identified bacterial species or phylotypes (Paster *et al.*, 2001) with periodontitis by a PCR technique. They demonstrated the association of several uncultivated phylotypes and the named species *Eubacterium saphenum*, *Porphyromonas endodontalis*, *Prevotella denticola* and *Cryptobacterium curtum* with periodontitis. In the present study, *Eubacterium saphenum* was frequently detected (10 % of the clones analysed) in subgingival plaque samples before treatment. In a previous study (Sakamoto *et al.*, 2002b), we also determined the prevalence of novel phylotypes including clone AP24, which was detected (3 of 90 clones analysed in this study) before treatment in patients with periodontitis and in healthy subjects. Among phylotypes tested, clone AP24 was associated significantly with saliva and subgingival plaque samples from patients with periodontitis (Sakamoto *et al.*, 2002b). This phylotype was one of the T-RFs larger than 1000 bp that were significantly reduced or not detected after treatment. In addition, several other phylotypes (Paster *et al.*, 2001; Sakamoto *et al.*, 2000) were also detected before treatment. These findings suggest that these phylotypes may play an important role in periodontal disease. It is important to identify not only known periodontopathic bacteria but also yet-to-be-cultured organisms in the study of periodontal disease. Therefore, it appears that T-RFLP analysis is one of the potential approaches in this field.

In conclusion, we demonstrated that T-RFLP analysis is useful for evaluation of the effects of medical treatment of periodontitis. Data from T-RFLP analysis were in agreement with data from real-time PCR and 16S rRNA gene clone library analysis. To our knowledge, this is first report on application of T-RFLP analysis to study changes of the oral microbiota in saliva and subgingival plaque samples of patients with periodontitis before and after periodontal treatment. Although the subject population was small, the data reported here should be useful in the future to elucidate the aetiology of periodontal disease.

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