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Profiling of subgingival plaque biofilm microflora from periodontally healthy subjects and from subjects with periodontitis using quantitative real-time PCR

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Background and Objective: Qualitative and quantitative changes of the subgingival plaque biofilm microflora in periodontal pockets are thought to be associated with the development and progression of periodontitis. The aims of the present study were to quantify the proportions of nine periodontitis-associated bacterial species and four *Streptococcus* species in subgingival plaque, and to evaluate their relationship with periodontitis quantitatively.

Material and Methods: Subgingival plaque samples were obtained from 12 periodontally healthy subjects and from 28 patients with periodontitis. The amounts of total and target bacteria were measured by quantitative real-time PCR using universal and species-specific primers, respectively.

Results: The proportion of total obligate anaerobes was found to be higher in subjects with periodontitis than in periodontally healthy subjects ($p < 0.05$). Among obligate anaerobes, *Tannerella forsythia* (2.04 ± 5.27%, $p < 0.05$), *Porphyromonas gingivalis* (0.54 ± 1.41%) and *Eubacterium saphenum* (0.30 ± 0.96%) were detected at high proportions in subjects with periodontitis, but not in periodontally healthy subjects. By contrast, the proportion of total streptococci was lower in subjects with periodontitis ($p < 0.05$). Specifically, the proportion of *T. forsythia*, *P. gingivalis* or *E. saphenum* increased (≥ 2.78%) and the proportion of *Streptococcus* species decreased to virtually undetectable levels, in subjects with periodontitis.

Conclusion: Obligate anaerobes, including *T. forthysia*, *P. gingivalis* and *E. saphenum*, were identified predominantly in microflora from subjects with periodontitis, whereas *Streptococcus* species were identified predominantly in microflora from periodontally healthy subjects, suggesting a change in the subgingival environment that resulted in conditions more suitable for the survival of obligate anaerobes. The proportion of these obligate anaerobes in the subgingival plaque of subjects with periodontitis appears to be associated with the status of human periodontitis.

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The human oral cavity is considered healthy when the oral microflora is composed of indigenous bacteria and is balanced properly (1). Numerous environmental changes in the oral microflora, including pH, anaerobiosis and nutrition, may lead to an accumulation of periodontitis-associated bacteria in the subgingival sulcus, resulting in the initiation of human periodontitis (2–6). Both qualitative and quantitative changes in subgingival plaque biofilms in periodontal pockets are thought to be highly associated with both the initiation and progression of periodontitis (6,7). The establishment of anaerobic bacterial culture and molecular biological methods has enabled researchers to isolate and detect various species of periodontitis-associated bacteria from subgingival plaque biofilms (8–11). It has been estimated that more than 600 species of bacteria are present in the human subgingival plaque biofilm (9–11). Of these, *Porphyromonas gingivalis* and *Mogibacterium timidum* are detected more frequently in the subgingival microflora of subjects with periodontitis than in the subgingival microflora of periodontally healthy subjects (8,9,11,12).

However, few studies have specifically investigated the proportions of periodontitis-associated bacteria as well as early colonizers on the teeth (namely *Streptococcus* species) in the subgingival plaque biofilm. The aims of the present study were to quantify the proportions of *P. gingivalis* and *M. timidum*, in addition to seven other periodontitis-associated species of bacteria (*Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eubacterium saphenum*, *Prevotella tannerae*, *Prevotella intermedia*, *Slackia exigua* and *Tannerella forsythia*) and four *Streptococcus* species (*Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus salivarius*) in the subgingival plaque of periodontitis-affected subjects and periodontally healthy subjects by quantitative real-time PCR, and to evaluate the relationship between periodontitis and the quantity of these bacteria.

Material and methods

Subjects and sample collection

Twenty-eight subjects with periodontitis (mean age 62 ± 9.9 years; range 41–80 years; mean numbers of residual teeth 24.5 ± 4.1) and 12 periodontally healthy subjects (mean age 26 ± 1.8 years; range 22–29 years; mean numbers of residual teeth 27.9 ± 1.5) were included in the present study. The subjects had not received periodontal treatment or antimicrobial therapy for at least 6 mo before sampling and were free of systemic diseases. Informed consent was obtained from each subject before the collection of samples. This study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan. Probing depths were measured in all teeth, at six sites per tooth for each subject, and the teeth with the deepest probing depths were chosen as the target sites for sampling. The deepest probing depths were < 4 mm (range 2.0–3.0 mm; mean 2.4 ± 0.5 mm) in periodontally healthy subjects (H1–H12, $n = 12$) and ≥ 4 mm (range 4.0–10.0 mm; mean 6.7 ± 1.9 mm) in subjects with periodontitis (P1–P28, $n = 28$). The samples were then collected using sterile periodontal pocket probes. All samples were immediately suspended in 1 mL of sterile distilled water and stored at −20°C before extraction of genomic DNA.

Bacterial strains

Nine strains of anaerobic bacteria, including the microaerophilic bacterial strains *A. actinomycetemcomitans* JCM 8578, *C. rectus* JCM 6301, *E. saphenum* ATCC 49989, *M. timidum* ATCC 33093, *P. gingivalis* W83, *P. intermedia* JCM 12248, *P. tannerae* ATCC 51259, *S. exigua* JCM 11022 and *T. forsythia* JCM 10827, and three mitis groups of *Streptococcus*, including *S. gordonii* JCM 12995, *S. oralis* JCM 12997 and *S. sanguinis* JCM 5708, and the salivarius group of *Streptococcus*, *S. salivarius* JCM 5707, were cultured in an anaerobic glove box (Model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2 at 37°C for 2–7 d. One loopful of a colony of each strain was then suspended in 1 mL of sterile distilled water and used for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted using the GFX Genomic Blood DNA Purification kit (GE Health Care Bio-Science Corp., Piscataway, NJ, USA) and the InstaGene Matrix kit (Bio-Rad Laboratories, Richmond, CA, USA), in accordance with the manufacturer's instructions.

Quantification of total bacterial levels using quantitative real-time PCR

In order to quantify the total amount of bacteria in the samples, quantitative real-time PCR was undertaken using the 16S ribosomal RNA gene universal primers 357F and 907R (13,14) and iQ SYBR Green Supermix (Bio-Rad Laboratories), according to the manufacturer's instructions. The primer sequences were: 357F, 5′-CTC CTA CGG GAG GCA GCA G-3′; and 907R, 5′-CCG TCA ATT CMT TTR AGT TT-3′. Quantitative real-time PCR cycling conditions have been described previously (14) and were undertaken using an iCycler (Bio-Rad Laboratories) programmed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 55°C for primer annealing and 30 s at 72°C for extension. During the extension step, fluorescence emissions were monitored and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). Standard curves were analyzed by comparing the universal primer set against a serial dilution of *P. gingivalis* W83 genomic DNA.

Quantification of specific bacterial species by quantitative real-time PCR

In order to quantify populations of specific bacteria in the samples, quan-

titative real-time PCR was performed as described above, using bacterial species-specific primers (Table 1). Quantitative real-time PCR amplification protocols for each bacterium were as follows. (i) *C. rectus*, *E. saphenum*, *P. gingivalis*, *S. exigua*, *T. forsythia*, *S. gordonii*, *S. salivarius*, *S. sanguinis* and *S. oralis*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. (ii) *A. actinomycetemcomitans*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 83°C for 10 s. (iii) *M. timidum* and *P. tannerae*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 82°C for 10 s. (iv) *P. intermedia*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 81°C for 10 s.

Detection frequency of each bacterial species

Based on the results obtained from the quantitative real-time PCR, the detection frequency of each bacterial species in subgingival plaque was calculated (i.e. the prevalence, in per cent, of each bacterial species was determined).

Data analysis

Fisher's exact probability test and Tukey tests were used to determine statistical significance. A p-value of < 0.05 was considered statistically significant.

Results

The quantitative real-time PCR analysis results demonstrated that the total amounts of bacteria per probing depth in periodontitis subjects (4.10 ± 0.85 Log fg DNA/μL per probing depth mm) were similar to those in periodontally healthy subjects (3.86 ± 0.53 Log fg DNA/μL per probing depth mm) (Fig. 1). By contrast, the proportion of total obligate anaerobes (*T. forsythia*, *P. gingivalis*, *E. saphenum*, *M. timidum*, *P. intermedia*, *S. exigua* and *C. rectus*) in subgingival plaque biofilm was higher in the subjects with periodontitis than in the periodontally healthy subjects ($p < 0.05$, Fig. 2). *T. forsythia* (2.04 ± 5.27%, $p < 0.05$), *P. gingivalis* (0.54 ± 1.41%) and *E. saphenum* (0.30 ± 0.96%) were detected at high proportions in subjects with periodontitis (Fig. 3) and were not detected at all in periodontally healthy subjects, with the exception of *P. gingivalis*, which was detected in one sample (H12, Fig. 4).

The proportions of *S. exigua*, *C. rectus* and *P. intermedia* were slightly higher in subjects with periodontitis than in periodontally healthy subjects (Fig. 3). *M. timidum* was only detected in subjects with periodontitis, but at a relatively low proportion (0.04 ± 0.21%). *A. actinomycetemcomitans* and *P. tannerae* were not detected in either group of subjects in the present study.

Table 1. Target bacteria and their species-specific primers used in the present study

Species	Target genes	Sequence (5′–3′)	Product size (bp)	References
Aggregatibacter actinomycetemcomitans	16S rRNA	CTC AGA GAT GGG TTT GTG CC AGA TTC ACT CCC CAT CGC TG	273	(15)
Campylobacter rectus	16S rRNA	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598	(16)
Eubacterium saphenum	16S rRNA	TCT ACT AAG CGC GGG GTG A ATA CCC GAT TAA GGG TAC	430	(17)
Mogibacterium timidum	16S rRNA	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524	(17)
Porphyromonas gingivalis	16S rRNA	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518	(15)
Prevotella intermedia	16S rRNA	TCC ACC GAT GAA TCT TTG GTC ATC CAA CCT TCC CTC CAC TC	98	(18)
Prevotella tannerae	16S rRNA	CTT AGC TTG CTA AGT ATG CCG CAG CTG ACT TAT ACT CCC G	550	(19)
Slackia exigua	16S rRNA	GCC AAG CGG CCT CGT CGA AG GCC GGC TTT AAG GGA TTC GCT CG	697	(17)
Tannerella forsythia	16S rRNA	AGC GAT GGT AGC AAT ACC TGT C TTC GCC GGG TTA TCC CTC	88	(18)
Streptococcus gordonii	*gtf*G	CTA TGC GGA TGA TGC TAA TCA AGT G GGA GTC GCT ATA ATC TTG TCA GAA A	440	(20)
Streptococcus oralis	*gtf*R	TCC CGG TCA GCA AAC TCC AGC C GCA ACC TTT GGA TTT GCA AC	374	(20)
Streptococcus salivarius	*gtf*K	GTG TTG CCA CAT CTT CAC TCG CTT CGG CGT TGA TGT GCT TGA AAG GGC ACC ATT	544	(20)
Streptococcus sanguinis	*gtf*P	GGA TAG TGG CTC AGG GCA GCC AGT T GAA CAG TTG CTG GAC TTG CTT GTC	313	(20)

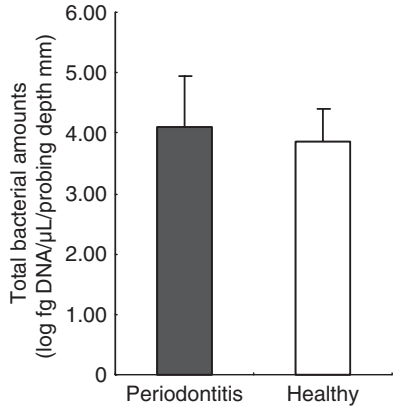

Fig. 1. Comparison of total bacterial amounts per probing depth between subgingival plaque biofilm obtained from subjects with periodontitis and that from periodontally healthy subjects.

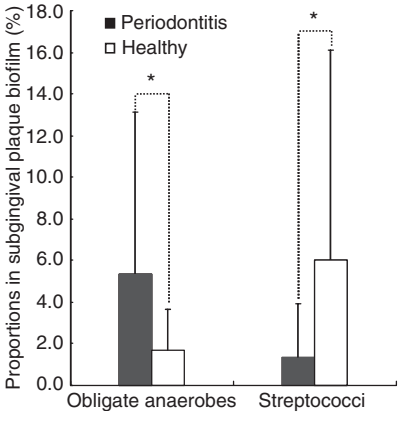

Fig. 2. Proportions of total obligate anaerobes and total streptococci in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects. $*p < 0.05$.

The proportion of total streptococci was significantly higher in periodontally healthy subjects than in periodontitis subjects ($p < 0.05$, Fig. 2). In particular, the proportions of *S. oralis* (5.53 ± 9.81%) and *S. sanguinis* (0.33 ± 0.57%) were higher in periodontally healthy subjects than in periodontitis subjects (Fig. 3), while the proportions of *S. salivarius* (0.32 ± 1.68%) and *S. gordonii* (0.16 ± 0.42%) were slightly higher in periodontitis subjects than in periodontally healthy subjects (Fig. 3).

The detection frequencies of *T. forsythia*, *P. gingivalis*, *E. saphenum*, *M. timidum*, *S. exigua*, *C. rectus*, *P. intermedia* and *S. gordonii* were higher in subjects with periodontitis than in periodontally healthy subjects (Fig. 5).

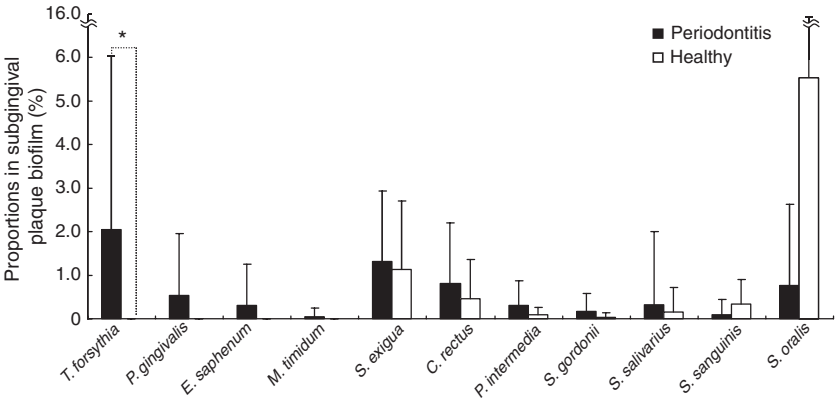

Fig. 3. Proportions of *Tannerella forsythia*, *Porphyromonas gingivalis*, *Eubacterium saphenum*, *Mogibacterium timidum*, *Slackia exigua*, *Campylobacter rectus*, *Prevotella intermedia*, *Streptococcus salivarius*, *Streptococcus gordonii*, *Streptococcus sanguinis* and *Streptococcus oralis* in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects. $*p < 0.05$.

The proportions of target bacteria for each sample and its probing depth are shown in Fig. 4. Among obligate anaerobes, *T. forsythia*, *P. gingivalis* and *E. saphenum* were found in 43% of subjects with periodontitis (samples P17–P28), while *Streptococcus* species were detected in small amounts ($\leq$ 8.91%) in 25% of subjects with periodontitis (samples P17–P22, P26). However, *Streptococcus* species disappeared as any of *T. forsythia*, *P. gingivalis* or *E. saphenum* increased (2.78–27.04%) in subjects with periodontitis (samples P23–P25, P27 and P28).

In the subjects with periodontitis, there was no apparent relationship between probing depths and total numbers of bacterial species present or between probing depths and the proportions of the above-mentioned three species (*T. forsythia*, *P. gingivalis* and *E. saphenum*) (Fig. 4).

Discussion

In the present study, the total bacterial amounts per probing depth in subjects with periodontitis were similar to those in periodontally healthy subjects. However, the predominant bacterial species identified in subgingival plaque biofilm microflora from subjects with periodontitis differed from those identified in periodontally healthy subjects. The proportions of obligate anaerobes, in particular *T. forsythia*, *P. gingivalis* and *E. saphenum*, were higher in subgingival plaque biofilm microflora from subjects with periodontitis. However, the proportions of *Streptococcus* species, especially *S. oralis* and *S. sanguinis*, were higher in subgingival plaque biofilm microflora from periodontally healthy subjects, indicating that *Streptococcus* species are one of the major components of periodontally healthy subgingival plaque biofilm. Furthermore, the findings of the present study led to an understanding of total subgingival plaque biofilm and its significant difference in health and disease.

Focusing attention on the proportion of *T. forsythia*, *P. gingivalis* and *E. saphenum* that are frequently isolated and detected in human periodontal pockets (8,9,11,21,22), periodontitis subjects were divided into three groups; these were termed the no-detection group (samples P1–P16), the low-level group (0.01–1.38%, samples P17–P22) and the high-level group (2.78–27.04%, samples P23–P28). In order to investigate the relationship between the three obligate anaerobes (*T. forsythia*, *P. gingivalis* and *E. saphenum*) and *Streptococcus*, the proportions of the three obligate

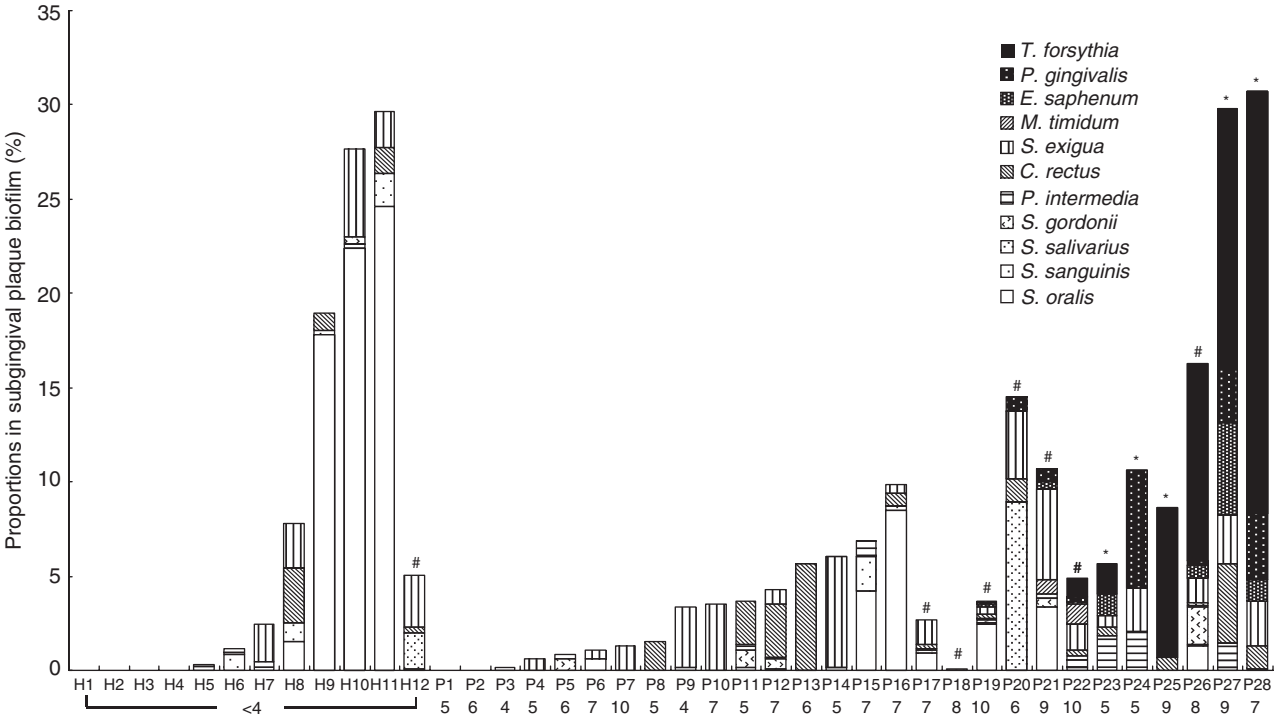

Fig. 4. Proportions of bacteria in the subgingival plaque biofilm of each periodontally healthy subject and each subject with periodontitis. *Any of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Eubacterium saphenum* was detected. #Streptococci and any of *P. gingivalis*, *T. forsythia* and *E. saphenum* were detected. Numerals (< 4 and 4–10) below each sample number indicate each probing depth (mm) of periodontally healthy subjects (H1–H12) and periodontitis subjects (P1–P28), respectively.

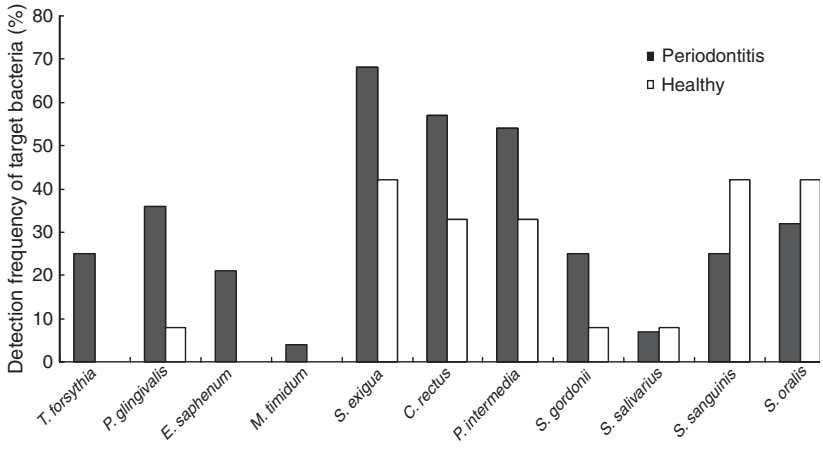

Fig. 5. Detection frequencies of target bacteria in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects.

anaerobes for each sample are shown in sequence in Fig. 4. *Streptococcus* species were specifically detected in the no-detection group and in the low-level group, but not in the high-level group, with the exception of sample P26 in the present study. These results suggest that environmental conditions, including oxygen concentration, nutritional supply and pH levels, are diverse among periodontal pockets and that the high-level group have an environment with low oxygen, nitrogenous-compound supply and stable neutral pH, which is suitable for the growth of periodontitis-associated bacteria (6,7).

The proportions of periodontitis-associated bacteria, such as *T. forsythia* and *P. gingivalis*, in subgingival plaque biofilm from subjects with periodontitis were relatively lower in the present study than in previous studies (18,23,24). This may be ascribed to the fact that the instruments used for sample collection in the present study (periodontal pocket probes), were different from those used in previous studies (Gracey curettes or paper points). Although it has been reported that the subgingival biofilm-forming bacteria detected were different when the sampling technique was different (25–27), the tendency to find higher proportions of periodontitis-associated bacteria in subgingival plaque biofilm from subjects with periodontitis was similar between the present study and previous studies. In addition, to our knowledge, this is the first study that specifically investigated, using real-time PCR, the proportions of early colonizers (such as *Streptococcus* species) in subgingival plaque biofilm isolated from the teeth of subjects with periodontitis.

Because we were unable to find periodontally healthy subjects in the elderly, the mean age of the perio-

dontally healthy subjects was 26 years in the present study. However, it has been reported that the subgingival microflora of periodontally healthy subjects does not change markedly with age (28,29), and thus the mean age of the periodontally healthy subjects may not influence greatly the results of the present study.

The present study demonstrated that bacterial species frequently detected in the subgingival plaque biofilm of subjects with periodontitis did not always represent a major proportion of the microflora (Figs 3 and 5). For example, the detection frequencies of *S. exigua*, *C. rectus* and *P. intermedia* were higher than those of *T. forsythia*, while the proportion of *T. forsythia* was higher than that of these three species. These results indicated the possibility that *T. forsythia* becomes predominant once it colonizes, although this bacterium cannot colonize efficiently, and suggest that the quantitative analysis is more reliable for evaluating bacterial status in the microflora.

In the present study, periodontal pocket depths were not found to correlate with either the total bacterial amounts or the proportions of individual bacteria in the microflora (Fig. 4). For instance, sample P7, obtained from a 10-mm periodontal pocket depth, did not contain *P. gingivalis*, *T. forsythia* or *E. saphenum*, while samples P23 and P24, obtained from a 5-mm periodontal pocket depth, contained large amounts of these bacteria. These results suggest that the depth of the periodontal pocket does not directly reflect the amount of bacteria found in the periodontal microflora.

In conclusion, facultative anaerobes, including *Streptococcus* species, were found to be the predominant bacterial species in subgingival plaque biofilm microflora from periodontally healthy subjects, whereas obligate anaerobes, including *T. forsythia*, *P. gingivalis* and *E. saphenum*, were the predominant bacterial species in subgingival plaque biofilm microflora from patients with periodontitis. The microbial diversity found in the present study suggests that environmental changes occur in the subgingival area, providing a more suitable environment for the survival of obligate anaerobes and thus the subsequent progression of periodontitis (6,7), and that the proportions of these obligate anaerobes in subgingival plaque from subjects with periodontitis are associated with the status of human periodontitis disease (21). The findings of the present study support the hypothesis that quantification of bacteria in subgingival plaque biofilm microflora is an appropriate tool for the diagnosis and prognosis of periodontitis (21). In addition to a large-scale study on the relationship between the periodontitis status and periodontitis-associated bacteria, a comparative and quantitative study on the changes observed in the oral microflora during the healing process, from periodontitis to healthy status, is required in order to verify the hypothesis.

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