Oral Microbiology Immunology 2004: 19: 379–385
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Detection frequency of periodontitis-associated bacteria by polymerase chain reaction in subgingival and supragingival plaque of periodontitis and healthy subjects

G. Mayanagi[1], T. Sato[2], H. Shimauchi[1], N. Takahashi[2]
[1]Division of Periodontology and Endodontology, [2]Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, Sendai, Japan

Mayanagi G, Sato T, Shimauchi H, Takahashi N. Detection frequency of periodontitis-associated bacteria by polymerase chain reaction in subgingival and supragingival plaque of periodontitis and healthy subjects.
Oral Microbiol Immunol 2004: 19: 379–385. © Blackwell Munksgaard, 2004.

The aim of this study was to compare the detection frequencies of 25 bacterial species in subgingival and supragingival plaque of 18 untreated periodontitis subjects and 12 periodontally healthy subjects. Genomic DNA was extracted from subgingival and supragingival plaque samples, and bacterial detection was performed by polymerase chain reaction of the 16S rRNA genes. Fourteen bacteria showed no relationship with periodontitis, and 11 of these 14 species were frequently detected (≥50%) in subgingival plaque in both periodontitis and healthy subjects. Nine bacteria such as *Eubacterium saphenum, Prevotella intermedia,* and *Treponema denticola* seemed to be related to periodontitis; their detection frequencies in subgingival plaque samples were higher in periodontitis than in healthy subjects, but these differences were not statistically significant by multiple comparisons ($0.002 \leq P < 0.05$). Two species (*Mogibacterium timidum* and *Porphyromonas gingivalis*) were detected significantly more frequently in subgingival plaque of periodontitis subjects than of healthy subjects ($P < 0.002$), with *P. gingivalis* being detected only in periodontitis subjects, suggesting that these two species are closely related to periodontitis. There were no significant differences in the detection frequencies of the 25 bacteria between subgingival and supragingival plaque, suggesting that the bacterial flora of supragingival plaque reflects that of subgingival plaque.

Key words: 16S rRNA genes; periodontitis; polymerase chain reaction; subgingival plaque; supragingival plaque

Nobuhiro Takahashi, DDS, PhD, Division of Oral Ecology and Biochemistry, Tohoku University Graduate School of Dentistry, Sendai 980–8575, Japan
Tel.: +81 22 717 8294;
fax: +81 22 717 8297;
e-mail: nobu-t@mail.tains.tohoku.ac.jp
Accepted for publication June 30, 2004

The composition of dental plaque microbiota in the human oral cavity is diverse and complex. It has been previously estimated by culturing and molecular biological methods that more than 600 species of bacteria inhabit the human oral cavity (13, 25, 27, 37, 49). The initiation and progression of periodontitis is thought to be caused by several species of these bacteria accumulating in subgingival periodontal pockets. *Porphyromonas gingivalis*, *Tannerella forsythia* (*Tannerella forsythensis*, formerly *Bacteroides forsythus*), and *Treponema denticola* are widely regarded as major periodontal pathogens (38), and numerous etiologic investigations of periodontal disease have therefore targeted these species. *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Micromonas micros* (formerly *Peptostreptococcus micros*), *Treponema amylovorum*,

Treponema maltophilum, *Treponema medium*, *Treponema socranskii*, *Treponema vincentii*, *Mogibacterium timidum* (formerly *Eubacterium timidum*), *Capnocytophaga ochracea*, and *Capnocytophaga sputigena* have also been recognized as pathogens of periodontitis based on their presence and relative numbers in healthy vs. diseased sites (18, 24, 48, 51). *Actinobacillus actinomycetemcomitans*, *Prevotella tannerae*, *Prevotella intermedia*, *Prevotella nigrescens*, *Centipeda periodontii*, and *Selenomonas sputigena* have been found in subgingival lesions of periodontitis patients (1, 23, 25, 36). *Dialister pneumosintes*, *Slackia exigua* (formerly *Eubacterium exiguum*), *Eubacterium saphenum, and Porphyromonas endodontalis* have also been considered to be associated with periodontitis (10, 18, 46).

Although all 25 bacterial species mentioned above are thought to be related to periodontitis, they have not been considered in the same study. Therefore, firstly, we undertook a comprehensive investigation of the 25 bacteria in subgingival and supragingival plaque using polymerase chain reaction (PCR) of the 16S rRNA genes. Secondly, we evaluated the relationship between periodontitis and the detection frequency of these bacteria. Thirdly, we compared the bacterial detection frequencies in subgingival plaque with the frequencies in supragingival plaque.

Material and methods

Subject population

Eighteen patients with periodontitis (mean age 63 ± 10.4 years; range 41–77 years) and 12 periodontally healthy subjects (mean age 27 ± 1.8 years; range 22–29 years) were randomly selected for this study. They had not received periodontal treatment or antimicrobial therapy for at least 6 months and were free of systemic diseases. Informed consent was obtained from each subject. Probing depths were measured in all teeth at six sites per tooth in each subject, and the teeth with the deepest probing depths were chosen as the target sites of sampling. The deepest probing depths were <4 mm (range 2.0–3.0 mm; mean 2.4 ± 0.5 mm) in periodontally healthy subjects (n = 12) and ≥4 mm (range 4.0–10.0 mm; mean 6.2 ± 2.1 mm) in subjects with periodontitis (n = 18).

Collection of samples

Supragingival plaque samples were taken with sterile explorers. For subgingival plaque samples, each of the target teeth was isolated with cotton rolls and air-dried after thorough removal of supragingival plaque with sterile cotton pellets. The samples were then collected using sterile periodontal pocket probes. In addition, alveolar mucosal samples (7 of 12 healthy subjects, 6 of 18 periodontitis subjects) were collected at the same sampling sites by swabbing 10 times with sterile swabs. All samples were immediately suspended in 1 ml of sterile distilled water and stored at −20°C before extraction of genomic DNA.

DNA extraction and nested PCR detection

After thawing, samples were centrifuged at 7740 × ***g*** for 5 min and the supernatants removed. Genomic DNA was then extracted from the pellets using an InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions.

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (19, 34, 35) and Taq DNA polymerase (HotstarTaq Master mix; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′- AGA GTT TGA TCC TGG CTC AG -3′; and 1492R, 5′- TAC GGG TAC CTT GTT ACG ACT T -3′. PCR mixtures were 5 μl of genomic DNA and 95 μl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 60°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted PCR product with the universal primers was 1505 bp in length.

The 25 bacteria were identified by amplification of the first PCR amplification products using species-specific primers (Table 1) based on 16S rRNA gene sequences. PCR mixtures were 1 μl of the first PCR amplification mixture and 24 μl of reaction mixture containing 1.5 mM $MgCl_2$. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1.

The PCR products were separated on 2% agarose gels (High Strength Analytical Grade Agarose, Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate; 1 mM EDTA, pH 8.4) stained with ethidium bromide and photographed under ultraviolet light. A 100-bp DNA Ladder (Invitrogen Corp., Carlsbad, CA) was used as a molecular size marker.

Data analysis

Fisher's exact probability tests were applied to compare the detection frequencies of the bacterial species between the healthy and periodontitis subjects, and between supragingival and subgingival plaques to evaluate significance. *P*-value was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons.

Results

The detection frequencies of target bacteria in subgingival and supragingival plaque are shown in Fig. 1. The bacterial species were listed in order of the differences (*P*-values) in their detection frequencies in subgingival plaque between healthy and periodontitis subjects. There were no significant differences ($P \geqslant 0.05$) in the detection frequencies of the first 14 bacteria between healthy and periodontitis subjects. Eleven of the 14 species (*F. nucleatum*, *C. rectus*, *E. corrodens*, *P. nigrescens*, *C. ochracea*, *T. maltophilum*, *S. exigua*, *M. micros*, *T. forsythia* (*T. forsythensis*), *C. sputigena*, and *C. periodontii*) were frequently detected in subgingival plaque samples (≥50%) of both periodontitis and healthy subjects. *A. actinomycetemcomitans*, *T. amylovorum*, and *T. vincentii* were infrequently detected in both periodontitis and healthy subjects (Fig. 1).

Of the last 11 species in Fig. 1, the detection frequencies of nine bacteria (*E. saphenum*, *P. intermedia*, *T. denticola*, *P. tannerae*, *T. medium*, *D. pneumosintes*, *T. socranskii*, *P. endodontalis*, and *S. sputigena*) seemed to be higher in subgingival plaque samples from periodontitis subjects than in healthy subjects, although these differences were not statistically significant by multiple comparisons ($0.002 \leq P < 0.05$, Fig. 1). The detection frequencies of *M. timidum* and *P. gingivalis* of periodontitis subjects were significantly higher ($P<0.002$) than those of healthy subjects. In addition, *P. gingivalis* was not detected in the subgingival plaque of healthy subjects (Fig. 1).

Table 1. Target bacteria and their species-specific primers

Species	Sequence (5'-3')	Size	References
Actinobacillus actinomycetemcomitans	CTC AGA GAT GGG TTT GTG CC AGA TTC ACT CCC CAT CGC TG	273 bp	(47)
Campylobacter rectus	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598 bp	(1)
Capnocytophaga ochracea	AGA GTT TGA TCC TGG CTC AG GAT GCC GTC CCT ATA TAC TAT GGG G	185 bp	(4)
Capnocytophaga sputigena	AGA GTT TGA TCC TGG CTC AG GAT GCC GCT CCT ATA TAC CAT TAG G	185 bp	(4)
Centipeda periodontii	AGA GTT TGA TCC TGG CTC AG TTA CAA AGG ATT ATT CGC CC	450 bp	(36)
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC GAT TTC GCT TCT CTT TGT TG	1105 bp	(6)
Eikenella corrodens	CGA TTA GCT GTT GGG CAA CTT ACC CTC TGT ACC GAC CAT TGT AT	410 bp	(9)
Eubacterium saphenum	TCT ACT AAG CGC GGG GTG A A CCC GAT TAA GGG TAC	430 bp	(12)
Fusobacterium nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705 bp	(31)
Micromonas micros	TCG AAC GTG ATT TTT GTG GA TCC AGA GTT CCC ACC TCT	1074 bp	(29)
Mogibacterium timidum	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524 bp	(12)
Porphyromonas endodontalis	GCT GCA GCT CAA CTG TAG TC CCG CTT CAT GTC ACC ATG TC	672 bp	(2)
Porphyromonas gingivalis	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518 bp	(47)
Prevotella intermedia	CGT GGA CCA AAG ATT CAT CGG TGG A CCG CTT TAC TCC CCA ACA AA	259 bp	(26)
Prevotella nigrescens	GTG TTT CAT TGA CGG CAT CCG ATA TGA AAC CA CGT CTC TGT GGG CTG CGA	828 bp	(26)
Prevotella tannerae	CTT AGC TTG CTA AGT ATG CCG AG CTG ACT TAT ACT CCC G	550 bp	(50)
Selenomonas sputigena	AGA GTT TGA TCC TGG CTC AG TC AAT ATT CTC AAG CTC GGT T	478 bp	(36)
Slackia exigua	GCC AAG CGG CCT CGT CGA AG C GGC TTT AAG GGA TTC GCT CG	697 bp	(12)
Tannerella forsythensis	AAA ACA GGG GTT CCG CAT GG C ACC GCG GAC TTA ACA GC	426 bp	(22)
Treponema amylovorum	AGA GTT TGA TCC TGG CTC AG C ACG CCT TTA TTC CGT GAG	193 bp	(48)
Treponema denticola	TAA TAC CGA ATG TGC TCA TTT ACA T TCA AAG AAG CAT TCC CTC TTC TTC TTA	316 bp	(1)
Treponema maltophilum	AGA GTT TGA TCC TGG CTC AG CT ATT GTG CTT ATT CAT CAG GC	438 bp	(48)
Treponema medium	CAC TCA GTG CTT CAT AAG GG CG GCC TTA TCT CTA AGA CC	856 bp	(33)
Treponema socranskii	AGG TAG ACA GCG GGA AAG GA AA CCC AAC ACC TCA CGG CA	902 bp	(32)
Treponema vincentii	GTC TCA ATG GTT CAT AAG AA CAA GCC TTA TCT CTA AGA CT	856 bp	(33)

There were no significant differences in the detection frequencies of the 25 bacteria between subgingival and supragingival plaque (Fig. 1). The detection frequencies in alveolar mucosal plaque samples were generally lower than those in the respective subgingival and supragingival plaque samples (Fig. 2).

The percent agreement of detection frequencies of the 25 bacteria was also evaluated. In subjects with periodontitis, the percent agreement of subgingival vs. supragingival plaque was higher (mean 81%, range 50–100%) than that of alveolar mucosal plaque vs. subgingival plaque (mean 63%, range 17–100%) and alveolar mucosal plaque vs. supragingival plaque (mean 60%, range 33–83%). This trend was also observed in healthy subjects (data not shown).

Discussion

In this study, the detection frequencies of 25 bacteria in subgingival, supragingival, and alveolar mucosal plaque of untreated periodontitis subjects and periodontally healthy subjects were obtained by nested PCR based on 16S rRNA genes. Since the subject's age is one of the most important risk factors of periodontitis, the periodontitis subjects were first divided into two groups according to age (41–64 years and 65–77 years). However, as the detection frequencies of the 25 bacterial species were quite similar in subgingival plaque samples from the two groups (data not shown), the groups were combined into one in the present study.

The first 14 bacteria in Fig. 1 showed no significant differences in detection frequencies in subgingival plaque between healthy and periodontitis subjects ($P \geqslant 0.05$); 11 of these bacteria were detected at high frequencies (≥50%) in both periodontitis and healthy subjects (Fig. 1). The detection rates of *C. rectus* and *E. corrodens* have been reported to be unrelated to the progression of attachment loss (28). Moreover, *C. rectus, E. corrodens, C. ochracea*, and *C. sputigena* have been frequently

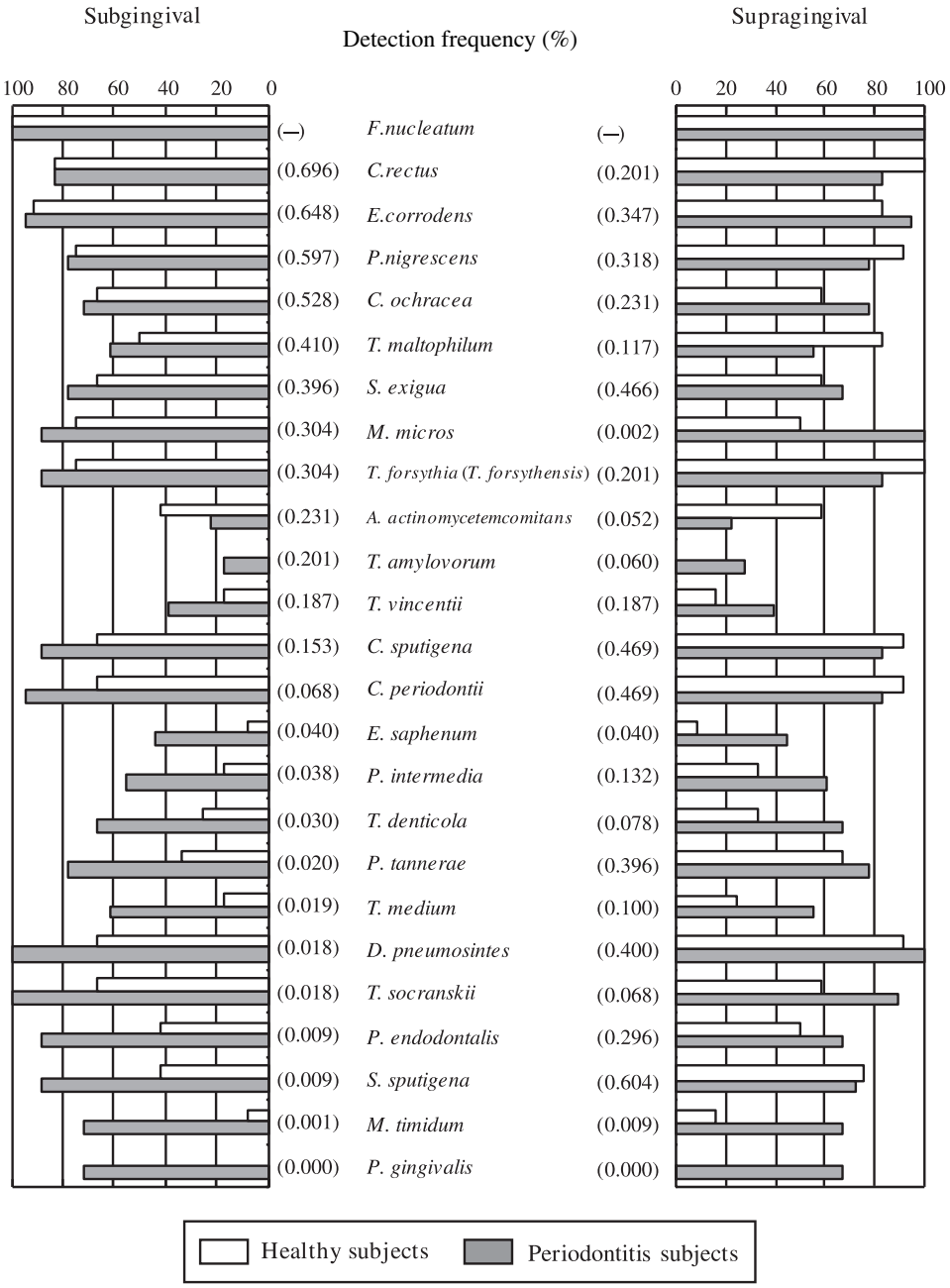

Fig. 1. Detection frequencies of target bacteria in subgingival and supragingival plaque. *P*-values of differences between healthy and periodontitis subjects are given in parentheses. Bacterial species are listed in order of *P*-values.

detected in plaque samples from healthy children (14). The results obtained in this study are therefore in accordance with those of previous studies, suggesting that these species are among the commensal bacteria of the oral cavity. *F. nucleatum* and *T. forsythia* (*T. forsythensis*) were also frequently detected in both healthy and periodontitis subjects in this study, suggesting that their relationship with periodontitis is uncertain. The *F. nucleatum* species consists of several subspecies (subspecies *nucleatum*, *polymorphum*, *fusiforme*, and *vincentii*) (7), and the *T. forsythia* (*T. forsythensis*) species has been reported to have *prtH* genetic subtypes (42). Thus, the pathogenicity of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) probably varies depending on the strain (8, 30, 42). Further study will be required to determine the subspecies and subtypes of *F. nucleatum* and *T. forsythia* (*T. forsythensis*) specific to periodontitis. *A. actinomycetemcomitans* was detected at the adult periodontitis sites in less than 25%, which was lower than at healthy sites in this study (Fig. 1). This is consistent with a recent study (11), although this species has been considered to be associated with juvenile and adult periodontitis (17, 20, 52, 53). This discrepancy could be due to differing pathogenicity of the various serotypes and genotypes of *A. actinomycetemcomitans* (3, 43).

Of the remaining 11 species, the detection frequencies of nine bacteria in subgingival plaque were higher in periodontitis subjects than in healthy subjects, but this difference was not

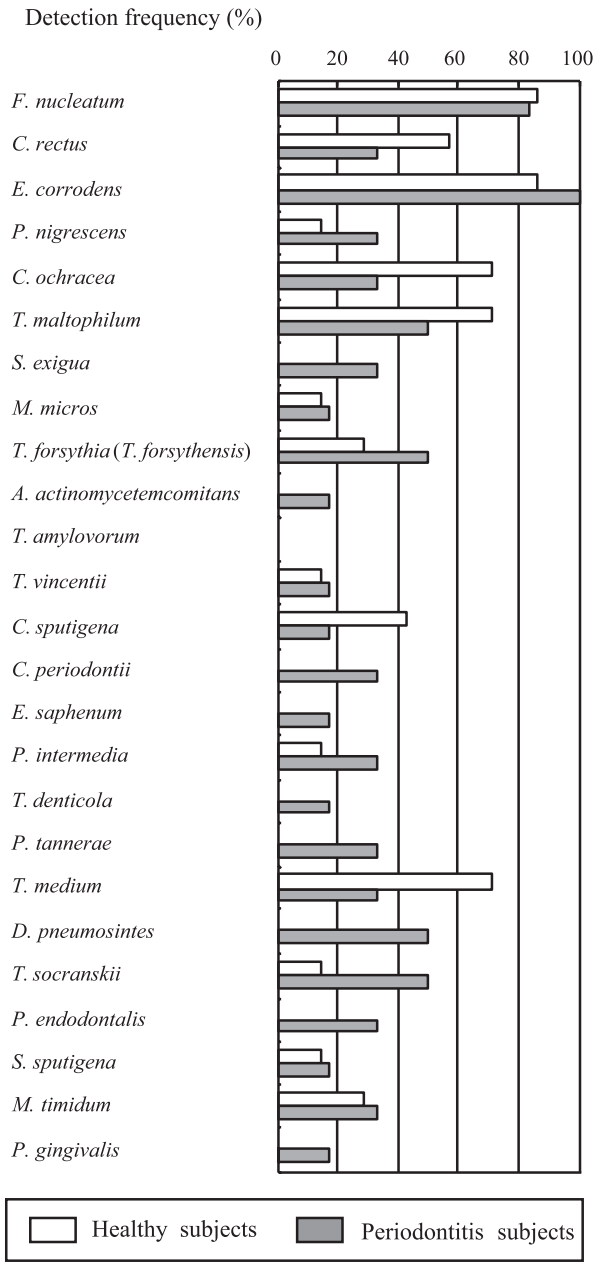

Fig. 2. Detection frequencies of target bacteria in alveolar mucosal plaque. Bacterial species are listed in the same order as in Fig. 1.

statistically significant ($0.002 \leq P < 0.05$) (Fig. 1). Two species (*M. timidum* and *P. gingivalis*) were detected significantly more frequently ($P < 0.002$) in subjects with periodontitis than in healthy subjects *P. gingivalis* was detected only in the periodontitis subjects. This is further evidence of an association of these two species with periodontitis, as reported previously for *P. gingivalis* (3, 18, 41, 51). *M. timidum* has also been isolated from deep periodontal pockets, supporting such an association (24, 45).

The detection frequencies of the bacteria in subgingival and supragingival plaque were similar in this study. A few studies have also suggested that the microbiota of subgingival and supragingival plaque were similar (16, 24, 51). Supragingival plaque may play an important role in bacterial invasion to subgingival sites, providing a reservoir of bacteria (51).

The recently proposed concept of 'biofilm' implies that bacteria establish a kind of supportive community (5). Based on this concept, bacterial species in dental plaque cooperate to make their environment anaerobic through oxygen consumption in their sugar and amino acid metabolic pathways (15, 21, 44) and maintain neutral pH via acid-neutralization by amino acid metabolism (39, 40), thus providing an environment where obligate anaerobic and acid-sensitive periodontopathic bacteria, such as *P. gingivalis*, are able to survive and grow. This may explain why *P. gingivalis* was detected at supragingival sites, which often become acidic and aerobic, in this study. From this point of view, the bacteria, frequently detected both in supragingival and subgingival plaque and at both healthy and periodontitis sites, are not individually pathogenic in the oral cavity, but rather may influence the initiation and progression of periodontitis when present together with periodontopathic bacteria.

The detection frequencies of bacteria in alveolar mucosa (Fig. 2) were generally lower than those in subgingival and supragingival plaque (Fig. 1), and the agreement rate of these bacteria at alveolar mucosa vs. subgingival (63%) or supragingival (60%) sites was also lower than those at subgingival vs. supragingival sites (81%). This suggests that alveolar mucosa has a microbiota composition that apparently differs from that of subgingival and supragingival plaque. This may be due to unique environmental factors of alveolar mucosa such as oxygen exposure and mobility, which result in less suitable environments for the target bacteria used in this study.

In conclusion, of 25 bacteria, 14 species showed no relationship with periodontitis, and 11 out of these 14 species were frequently detected (≥50%) in subgingival plaque in both periodontitis and healthy subjects. Nine bacteria seemed to be related to periodontitis, although their higher detection frequencies in subgingival plaque were not statistically significant by multiple comparisons. Only two species (*M. timidum* and *P. gingivalis*) in subgingival plaque were detected significantly more frequently in subjects with periodontitis than in healthy subjects, suggesting that the two species are closely related to periodontitis. In addition, the similarity of detection frequencies of the bacteria between subgingival and supragingival plaque suggests that analysis of supragingival plaque microbiota is useful in estimating the microbial composition of subgingival plaque.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (B)

(Nos. 13557187 and 16390611 to H.S.; Nos. 14370687 and 16390601 to N.T.); for Exploratory Research (No. 15659500 to N.T.); and for Young Scientists (B) (No. 14771000 to T.S.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. Oral Microbiol Immunol 1996: **11**: 266–273.
2. Bogen G, Slots J. Black-pigmented anaerobic rods in closed periapical lesions. Int Endod J 1999: **32**: 204–210.
3. Colombo AP, Teles RP, Torres MC, Souto R, Rosalem WJ, Mendes MC, et al. Subgingival microbiota of Brazilian subjects with untreated chronic periodontitis. J Periodontol 2002: **73**: 360–369.
4. Conrads G, Mutters R, Fischer J, Brauner A, Lutticken R, Lampert F. PCR reaction and dot-blot hybridization to monitor the distribution of oral pathogens within plaque samples of periodontally healthy individuals. J Periodontol 1996: **67**: 994–1003.
5. Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, et al. Bacterial biofilms in nature and disease. Ann Rev Microbiol 1987: **41**: 435–464.
6. Doan N, Contreras A, Flynn J, Slots J, Chen C. Molecular identification of *Dialister pneumosintes* in subgingival plaque of humans. J Clin Microbiol 2000: **38**: 3043–3047.
7. Dzink JL, Sheenan MT, Socransky SS. Proposal of three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. *nucleatum* subsp. nov., comb. nov., *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov. & *Fusobacterium nucleatum* subsp. *vincentii* subsp. nov., nom. rev., comb. nov. Int J Syst Bacteriol 1990: **40**: 74–78.
8. Dzink JL, Socransky SS, Haffajee AD. The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. J Clin Periodontol 1988: **15**: 316–323.
9. Furcht C, Eschrich K, Merte K. Detection of *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* by use of the polymerase chain reaction (PCR) *in vitro* and in subgingival plaque. J Clin Periodontol 1996: **23**: 891–897.
10. Ghayoumi N, Chen C, Slots J. *Dialister pneumosintes*, a new putative periodontal pathogen. J Periodontal Res 2002: **37**: 75–78.
11. Hamlet SM, Cullinan MP, Westerman B, Lindeman M, Bird PS, Palmer J, et al. Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. J Clin Periodontol 2001: **28**: 1163–1171.
12. Hashimura T, Sato M, Hoshino E. Detection of *Slackia exigua, Mogibacterium timidum* and *Eubacterium saphenum* from pulpal and periradicular samples using the polymerase chain reaction (PCR) method. Int Endod J 2001: **34**: 463–470.
13. Kazor CE, Mitchell PM, Lee AM, Stokes LN, Loesche WJ, Dewhirst FE, et al. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J Clin Microbiol 2003: **41**: 558–563.
14. Kimura S, Ooshima T, Takiguchi M, Sasaki Y, Amano A, Morisaki I, et al. Periodontopathic bacterial infection in childhood. J Periodontol 2002: **73**: 20–26.
15. Korayem M, Westbay G, Kleinberg I. Constituents of salivary supernatant responsible for stimulation of oxygen uptake by the bacteria in human salivary sediment. Arch Oral Biol 1990: **35**: 145–152.
16. Kornman KS. The role of supragingival plaque in the prevention and treatment of periodontal diseases. A review of current concepts. J Periodontal Res 1986: **21** (Suppl. 16): 5–22.
17. Kornman KS, Robertson PB. Clinical and microbiological evaluation of therapy for juvenile periodontitis. J Periodontol 1985: **56**: 443–446.
18. Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ. New bacterial species associated with chronic periodontitis. J Dent Res 2003: **82**: 338–344.
19. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, eds. Nucleic acid techniques in bacterial systematics. Chichester: John Wiley & Sons, 1991: 115–175.
20. Mandell RL, Socransky S. A selective medium for *Actinobacillus actinomycetemcomitans* and the incidence of the organism in juvenile periodontitis. J Periodontol 1981: **52**: 593–598.
21. Marquis RE. Oxygen-metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. J Ind Microbiol 1995: **15**: 198–207.
22. Meurman JH, Wahlfors J, Korhonen A, Alakuijala P, Vaisanen P, Torkko H, et al. Identification of *Bacteroides forsythus* in subgingival dental plaque with the aid of a rapid PCR method. J Dent Res 1997: **76**: 1376–1380.
23. Moore LV, Johnson JL, Moore WE. Description of *Prevotella tannerae* sp. nov. and *Prevotella enoeca* sp. nov. from the human gingival crevice and emendation of the description of *Prevotella zoogleoformans*. Int J Syst Bacteriol 1994: **44**: 599–602.
24. Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Ranney RR. Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infect Immun 1983: **42**: 510–515.
25. Moore WE, Moore LV. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–77.
26. Okamoto M, Maeda N, Kondo K, Leung KP. Hemolytic and hemagglutinating activities of *Prevotella intermedia* and *Prevotella nigrescens*. FEMS Microbiol Lett 1999: **178**: 299–304.
27. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
28. Renvert S, Dahlen G, Wikstrom M. Treatment of periodontal disease based on microbiological diagnosis. Relation between microbiological and clinical parameters during 5 years. J Periodontol 1996: **67**: 562–571.
29. Riggio MP, Lennon A, Smith A. Detection of *Peptostreptococcus micros* DNA in clinical samples by PCR. J Med Microbiol 2001: **50**: 249–254.
30. Roques CG, El Kaddouri S, Barthet P, Duffort JF, Arellano M. *Fusobacterium nucleatum* involvement in adult periodontitis and possible modification of strain classification. J Periodontol 2000: **71**: 1144–1150.
31. Rupf S, Merte K, Eschrich K. Quantification of bacteria in oral samples by competitive polymerase chain reaction. J Dent Res 1999: **78**: 850–856.
32. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y, Nakase T. Detection of *Treponema socranskii* associated with human periodontitis by PCR. Microbiol Immunol 1999: **43**: 485–490.
33. Sato T, Kuramitsu HK. Polymerase chain reaction for the detection of *fla*A-1 genes of oral spirochaetes in advanced periodontal pockets. Arch Oral Biol 2000: **45**: 921–925.
34. Sato T, Matsuyama J, Kumagai T, Mayanagi G, Yamaura M, Washio J, et al. Nested PCR for detection of mutans streptococci in dental plaque. Lett Appl Microbiol 2003: **37**: 66–69.
35. Sato T, Sato M, Matsuyama J, Hoshino E. PCR-restriction fragment length polymorphism analysis of genes coding for 16S rRNA in *Veillonella* spp. Int J Syst Bacteriol 1997: **47**: 1268–1270.
36. Sawada S, Kokeguchi S, Takashiba S, Murayama Y. Development of 16S rDNA-based PCR assay for detecting *Centipeda periodontii* and *Selenomonas sputigena*. Lett Appl Microbiol 2000: **30**: 423–426.
37. Socransky SS, Haffajee AD. Evidence of bacterial etiology: a historical perspective. Periodontol 2000 1994: **5**: 7–25.
38. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
39. Takahashi N. Acid-neutralizing activity during amino acid fermentation by *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. Oral Microbiol Immunol 2003: **18**: 109–113.
40. Takahashi N, Saito K, Schachtele CF, Yamada T. Acid tolerance and acid-neutralizing activity of *Porphyromonas gingivalis, Prevotella intermedia* and *Fusobacterium nucleatum*. Oral Microbiol Immunol 1997: **12**: 323–328.
41. Takeuchi Y, Umeda M, Sakamoto M, Benno Y, Huang Y, Ishikawa I. *Treponema socranskii, Treponema denticola*, and *Porphyromonas gingivalis* are associated with severity of periodontal tissue destruction. J Periodontol 2001: **72**: 1354–1363.
42. Tan KS, Song KP, Ong G. *Bacteroides forsythus prtH* genotype in periodontitis patients: occurrence and association with

periodontal disease. J Periodontal Res 2001: **36**: 398–403.
43. Tan KS, Song KP, Ong G. Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. J Periodontal Res 2002: **37**: 268–272.
44. Traudt M, Kleinberg I. Stoichiometry of oxygen consumption and sugar, organic acid and amino acid utilization in salivary sediment and pure cultures of oral bacteria. Arch Oral Biol 1996: **41**: 965–978.
45. Uematsu H, Hoshino E. Predominant obligate anaerobes in human periodontal pockets. J Periodontal Res 1992: **27**: 15–19.
46. Wade WG. The role of *Eubacterium* species in periodontal disease and other oral infections. Microb Ecol Health Dis 1996: **9**: 367–370.
47. Wahlfors J, Meurman JH, Vaisanen P, Alakuijala P, Korhonen A, Torkko H, et al. Simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by a rapid PCR method. J Dent Res 1995: **74**: 1796–1801.
48. Willis SG, Smith KS, Dunn VL, Gapter LA, Riviere KH, Riviere GR. Identification of seven *Treponema* species in health- and disease-associated dental plaque by nested PCR. J Clin Microbiol 1999: **37**: 867–869.
49. Wilson MJ, Weightman AJ, Wade WG. Applications of molecular ecology in the characterisation of uncultured microorganisms associated with human disease. Rev Med Microbiol 1997: **8**: 91–101.
50. Xia T, Baumgartner JC, David LL. Isolation and identification of *Prevotella tannerae* from endodontic infections. Oral Microbiol Immunol 2000: **15**: 273–275.
51. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. J Clin Periodontol 2000: **27**: 722–732.
52. Zambon JJ. *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol 1985: **12**: 1–20.
53. Zambon JJ, Christersson LA, Slots J. *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. J Periodontol 1983: **54**: 707–711.