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Mini review

Molecular analysis of human oral microbiota

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Objectives: The application of molecular, mainly 16S ribosomal RNA (rRNA)-based approaches enables researchers to bypass the cultivation step and has proven its usefulness in studying the microbial composition in a variety of ecosystems, including the human oral cavity. In this mini-review, we describe the impact of these culture-independent approaches on our knowledge of the ecology of the human oral cavity and provide directions for future studies that should emphasize the role of specific strains, species and groups of microbes in periodontal disease.

Materials and methods: Recent findings are summarized to elucidate the relationship between periodontal disease and human oral microbiota, including as-yet-to-be-cultured organisms.

Results: The real-time polymerase chain reaction (PCR) method was developed to detect and quantify periodontopathic bacteria, such as *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*) and *Treponema denticola*. The checkerboard DNA–DNA hybridization technique allowed enumeration of large numbers of species in very large numbers of samples. 16S rRNA gene clone library analysis revealed the diversity of human oral microbiota and the existence of as-yet-to-be-cultured organisms that are presumed periodontal pathogens. In addition, terminal restriction fragment length polymorphism (T-RFLP) analysis was applied for assessment of diversity of human oral microbiota.

Conclusion: Culture-independent approaches are useful for studying the microbial ecology in the human oral cavity and should be useful in the future to elucidate the etiology of periodontal disease.

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Periodontal disease, a polymicrobial mixed infection, is a major oral disease. It is caused by several microbial species, such as *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*) (1) and *Treponema denticola*. 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 microorganisms of oral diseases including periodontal disease are, in general, restricted to cultivable species such as the aforementioned pathogens. It is therefore probable that a large number of as-yet-to-be-cultured organisms present in the human oral cavity may play a role in periodontal disease. The best model available at present for determining microbial diversity, without cultivation, is based on isolation of DNA from the target environment, polymerase chain reaction (PCR) amplification of the ribosomal RNA (rRNA) gene, cloning the amplicons into *Escherichia coli*, and sequence analysis of the cloned 16S rRNA gene inserts (2). These culture-independent approaches have been used to determine the diversity of spirochetes in the subgingival pocket of subjects with a range of periodontal

conditions, including two healthy, one adult periodontitis, three acute necrotizing ulcerative gingivitis, eight refractory periodontitis, and one human immunodeficiency virus (HIV) periodontitis, and the prevalence of cultivable and uncultivable treponemes in oral diseases (3). In this mini-review, we discuss the relationships between periodontal disease and human oral microbiota including as-yet-to-be-cultured organisms.

Human oral spirochetes

The bacterial flora associated with gingivitis (4, 5) and periodontitis (6) have been investigated. Spirochetes are the predominant microorganisms known to proliferate in periodontal disease sites among the bacterial flora. Although the relationship between periodontitis and oral treponemes has been emphasized clinically, cultivation studies of oral treponemes are limited because of the oxygen sensitivity and unique nutritional requirements of these microorganisms and the long cultivation period (7, 8). The following species of cultivable oral treponemes have been validated: *Treponema amylovorum* (9), *T. denticola* (10), *T. lecithinolyticum* (11), *T. maltophilum* (12), *T. medium* (13, 14), *T. parvum* (15), *T. pectinovorum* (16), *T. putidum* (17), *T. socranskii* (18), and '*T. vincentii*'. (The last treponeme has not been validated in a peer-reviewed publication. It has been published however, in *Bergey's Manual of Systematic Bacteriology* (19) and is commonly used.) These species are classified into two groups according to the fermentation of carbohydrates. The saccharolytic oral treponemes contain six species (*T. amylovorum*, *T. lecithinolyticum*, *T. maltophilum*, *T. parvum*, *T. pectinovorum*, and *T. socranskii*), and the asaccharolytic oral treponemes contain four species (*T. denticola*, *T. medium*, *T. putidum*, and '*T. vincentii*'). Paster *et al.* (20) reported the phylogeny of cultivable oral treponemes isolated by Robert Smibert (Virginia Polytechnic Institute, Blacksburg, VA, USA). They proposed three novel species (*Treponema* Smibert-2, *Treponema* Smibert-3, and *Treponema* Smibert-5) based on 16S rRNA gene sequence comparisons. *Treponema* Smibert-2 was later considered a novel species, *Treponema parvum* (15). The taxonomy of oral spirochetes has been discussed in a mini-review article (21).

Among the cultivable oral treponemes, *T. denticola* is frequently isolated from sites of severe infection in patients with periodontitis (22), and many studies have attempted to elucidate the role of *T. denticola* in periodontitis (23–25). Although *T. socranskii* is frequently isolated from the subgingival plaque samples of periodontitis patients, in addition to *T. denticola*, it is difficult to cultivate and identify (26, 27). The PCR technique can be used to detect and identify *T. socranskii* (28). This technique is a rapid and reliable method for differentiating *T. socranskii* from other cultivable oral treponemes. Takeuchi *et al.* (29) used this PCR technique to identify *T. socranskii* in addition to *T. denticola* and *P. gingivalis* and to clarify the relationship between the presence of these microorganisms and the severity of clinical periodontal parameters. Their findings suggest that *T. socranskii*, *T. denticola*, and *P. gingivalis* are associated with the severity of periodontal tissue destruction. In addition, restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes amplified by PCR was used to differentiate three subspecies of *T. socranskii* (28). Recently, the relationship between *T. socranskii* ssp. *buccale* and periodontal disease was emphasized (30, 31). 16S rRNA gene PCR-RFLP analysis was also used to differentiate cultivable oral treponemes, including *T. denticola*, *T. medium*, *T. pectinovorum*, *T. socranskii*, and '*T. vincentii*' (32). Furthermore, species-specific nested PCR was used to detect *T. amylovorum*, *T. denticola*, *T. maltophilum*, *T. medium*, *T. pectinovorum*, *T. socranskii*, and '*T. vincentii*' in dental plaques (33).

Detection and quantification of periodontopathic bacteria

The relationship between periodontal disease and detection frequency of putative periodontal pathogens was exhaustively evaluated using PCR of the 16S rRNA genes (34–38). These findings suggest that several species are strongly associated with periodontitis.

Accurate quantification of periodontal pathogens in clinical samples (saliva and subgingival plaque) is needed for understanding the etiologic role of these bacteria. The conventional PCR (endpoint PCR) method detects the plateau phase of the reaction, but is not suitable for quantification of the pathogens. In contrast, the real-time PCR method allows monitoring of the exponential phase. This method allows rapid detection and quantification of the bacteria in clinical samples. Real-time PCR using the TaqMan system was first used to quantitate *T. forsythensis* in subgingival plaque (39). Subsequently, this system was used to determine both the density of *P. gingivalis* and the total number of bacterial cells in plaque samples (40). In addition, real-time PCR using SYBR Green dye and LightCycler system (Roche Diagnostics, Mannheim, Germany) was first used to detect and quantify periodontopathic bacteria, such as *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythensis*, *T. denticola*, and *T. socranskii*, in saliva and subgingival plaque samples (41). Using the LightCycler system, it is possible to determine the amount of periodontopathic bacteria within 1 hour (*A. actinomycetemcomitans*: 40 min, *P. gingivalis*: 34 min, *T. forsythensis*: 40 min, *T. denticola*: 32 min, *T. socranskii*: 46 min). Maeda *et al.* (42) suggested that there was no significant difference between the TaqMan and SYBR Green chemistry in their specificity, quantitativity, and sensitivity. In addition, they suggested that, since the TaqMan assay required additional manipulation and cost for the probe, the SYBR Green assay might be suitable for routine clinical examinations. Currently, detection and quantification of periodontopathic bacteria by real-time PCR method are generalized in this field and many studies have reported the usefulness of real-time PCR (43–52). We anticipate that the real-time PCR method will become in the future an indispensable method for the diagnosis of periodontal disease,

evaluation of treatment, and prognostic judgment.

Enumeration of bacterial species in complex microbial ecosystems using checkerboard DNA–DNA hybridization

It has been difficult to conduct large-scale studies of microbiologically complex ecosystems using conventional microbiological techniques. The real-time PCR technique mentioned above is not particularly suitable for the examination of large numbers of samples for large numbers of different species. In contrast, molecular identification techniques in new probe-target formats, such as checkerboard DNA–DNA hybridization, permit enumeration of large numbers of species in very large numbers of samples (53). The checkerboard DNA–DNA hybridization technique was first described in 1994 by Socransky *et al*. (54). Using 40 species-specific DNA–DNA hybridization probes to detect oral bacteria, it was revealed that subgingival plaque contains bacterial species in different complexes (55). Socransky *et al*. (55) observed five major complexes using cluster analysis (Table 1). The red complex, consisting of *P. gingivalis*, *T. forsythensis*, and *T. denticola*, showed the strongest relationship with clinical measures of peridontal disease, particularly pocket depth and bleeding on probing. The checkerboard DNA–DNA hybridization technique has been used to comprehensively examine the microbial composition of supra and subgingival plaque in subjects in health and periodontitis (56, 57), the salivary microbiota levels in relation to periodontal status (58), the relationship of cigarette smoking to the composition of the subgingival microbiota (59, 60), the differences between the subgingival microbiota in subjects from different geographic locations (61), the relationship of ethnic/racial group, occupational and periodontal disease status (62), and effects of different periodontal therapies (63, 64). Recently, it was reported that the checkerboard DNA–DNA hybridization technique is useful for the enumeration of bacterial species in microbiologically complex systems (53). This technique is rapid, sensitive, and relatively inexpensive. It overcomes many of the limitations of cultivation-based approaches. Paster *et al*. (65) developed a PCR-based, reverse capture, checkerboard hybridization protocol to differentiate between species of oral streptococci, which are very closely related phylogenetically. Based on these techniques, DNA microarray will be developed in the near future.

Table 1. Microbial complexes in subgingival plaque

Complex	Species
Red complex	*Porphyromonas gingivalis*
	Tannerella forsythensis
	Treponema denticola
Orange complex	*Campylobacter gracilis*
	Campylobacter rectus
	Campylobacter showae
	Eubacterium nodatum
	Fusobacterium nucleatum ssp. *nucleatum*
	Fusobacterium nucleatum ssp. *polymorphum*
	Fusobacterium nucleatum ssp. *vincentii*
	Fusobacterium periodonticum
	Peptostreptococcus micros
	Prevotella intermedia
	Prevotella nigrescens
	Streptococcus constellatus
Green complex	*Actinobacillus actinomycetemcomitans* serotype a
	Campylobacter concisus
	Capnocytophaga gingivalis
	Capnocytophaga ochracea
	Capnocytophaga sputigena
	Eikenella corrodens
Yellow complex	*Streptococcus gordonii*
	Streptococcus intermedius
	Streptococcus mitis
	Streptococcus oralis
	Streptococcus sanguis
Purple complex	*Actinomyces odontolyticus*
	Veillonella parvula
Other species	*Actinobacillus actinomycetemcomitans* serotype b
	Actinomyces naeslundii genospecies 2 (*A. viscosus*)
	Selenomonas noxia

Bacterial diversity in the human oral cavity

16S rRNA gene clone library analysis was first used in 1994 to determine the genetic diversity of cultivable and uncultivable spirochetes in the gingival crevice of a patient with severe periodontitis by Choi *et al*. (66). These investigators found that the clones fell into 23 clusters differing by about 1–2%. Their findings indicate an unexpected diversity of oral treponemes from a single patient. Thereafter, this method was applied to analyze the diversity of asaccharolytic *Eubacterium* species (67). In addition, Kroes *et al*. (68) used this method to characterize the breadth of bacterial diversity within the human subgingival crevice. Although the subject population was small, Sakamoto *et al*. (69) also used this method to compare the oral microbiota in the saliva from two patients with periodontitis and from a periodontally healthy subject. There was no clonal sequence affiliated with periodontopathic bacteria in the saliva from the healthy subject, whereas a number of periodontal pathogens such as *Campylobacter rectus*, *P. intermedia*, *P. gingivalis*, and *T. socranskii* were detected in the saliva from the patients with periodontitis. In addition, a number of previously uncharacterized and uncultured microorganisms were recognized. Subsequently, Paster *et al*.

(31) 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. A similar technique was also used to compare the bacteria found in children with severe caries to those found in caries-free children (70) and to determine the prevalent species and phylotypes in advanced lesions of children with noma (71). According to the most recent report (72), it is presumed that over 700 bacterial species (phylotypes are included) inhabit the oral cavity, and more than half of these cannot be cultivated.

Detection of novel oral phylotypes associated with periodontitis

Leys *et al*. (73) investigated the relationship between the presence of *T. forsythensis* and a novel phylotype, oral clone BU063 identified by Paster *et al*. (31), and periodontal health status. Harper-Owen *et al*. (74) designed and validated phylotype-specific PCR primers for phylotypes PUS3.42, PUS9.170, and PUS9.180 identified by Dymock *et al*. (75) and determined their incidences in subgingival plaque samples from subjects with periodontitis and from healthy controls. In a previous study (69), a number of novel oral phylotypes, representing as yet uncultured organisms, were identified. Among these phylotypes, Sakamoto *et al*. (76) designed specific PCR primers for five phylotypes AP12, AP21, AP24, AP50, and RP58, which are deeply branched particularly in the phylogenetic tree, and determined the prevalence of these phylotypes in 45 patients with periodontitis and 18 healthy subjects. Among the phylotypes tested, phylotype AP24, which is closely related to oral clone DA014 (99% sequence similarity) reported previously (31), was significantly associated with saliva and subgingival plaque samples from patients with periodontitis ($p < 0.01$), but the difference was not statistically significant in the presence of other phylotypes. These data suggest that phylotype AP24 may play an important role in periodontal disease. It is important to examine not only known periodontopathic bacteria but also as-yet-to-be-cultured organisms in the study of periodontal disease. Although attempts have been made to isolate phylotype AP24 from subgingival plaque and saliva samples, such attempts have not yet been successful. However, novel *Prevotella* species were isolated from the human oral cavity in the process of the research (77, 78).

Recently, it was reported that members of the uncultivated bacterial division TM7 (79), which have been detected in the human oral cavity (31), might play a role in the multifactorial process leading to periodontitis (80). In contrast, several phylotypes were associated with periodontal health (73, 81). Kumar *et al*. (81) reported that clone W090 from the *Deferribacteres* phylum and clone BU063 from the *Bacteroidetes* phylum were associated with periodontal health. In the future, as a new index of periodontal disease, it is expected that the relationship between periodontal disease and other novel phylotypes is investigated in more detail.

Application of terminal restriction fragment length polymorphism analysis in periodontics

A phylogenetic approach based on 16S rRNA has been applied to investigate the diversity of cultivable and uncultivable species in the human oral cavity, without requiring cultivation (30, 31, 68, 69). 16S rRNA gene clone library analysis can provide direct sequence information. However, analysis of individual 16S rRNA clones is an expensive and extremely inefficient approach for comparison of a multitude of bacterial communities.

Terminal restriction fragment length polymorphism (T-RFLP) is an alternative 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 (82). This technique has been used for assessing the diversity and structure of complex bacterial communities in various environments (83–90) and has been evaluated in separate review articles (91, 92). In addition, the T-RFLP analysis program (TAP) has been developed and published on the worldwide web (http://rdp.cme.msu.edu/html/analyses.html) (93).

Sakamoto *et al*. (94) used T-RFLP analysis to characterize and compare oral microbiota present in saliva samples of 18 healthy subjects and 18 patients with periodontitis. They presented the first report on characterization of oral microbiota based on T-RFLP patterns. Their study indicated that T-RFLP analysis is useful for the assessment of diversity of oral microbiota and rapid comparison of the community structure between subjects with and without periodontitis. In contrast, two groups (95, 96) 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 compared 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. Multiple species can be predicted for the same T-RF length, but it is possible to identify bacterial species by analysis of digests with multiple restriction enzymes. Changes in the subgingival microbiota in adult Down's syndrome patients with periodontitis (63), and adult periodontitis patients after scaling and root planing (97) or antibiotic (amoxicillin or metronidazole) therapy combined with scaling and root planing (62) have been investigated using checkerboard DNA–DNA hybridization or PCR techniques. However, these studies report changes in only a limited part, which represents the cultivable known species, of the subgingival microbiota. Recently, Zijnge *et al*. (96) used DGGE analysis, which takes into account the presence of unidentified and hard-to-cultivate species present in the subgingival plaque (like T-RFLP analysis), to study shifts in the subgingival microbiota before, 1 day after and 3 months after

treatment. Although the subject population was small, Sakamoto *et al.* (98) used T-RFLP analysis to study the change of oral microbiota in saliva and subgingival plaque samples of patients with periodontitis before and 3 months after periodontal treatment. Significant changes in the T-RFLP patterns of subgingival plaque samples of the patients were noted after 3 months of improved oral hygiene, and full mouth supra- and subgingival scaling and root planing (Fig. 1). 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 from the patients before treatment, the proportions of

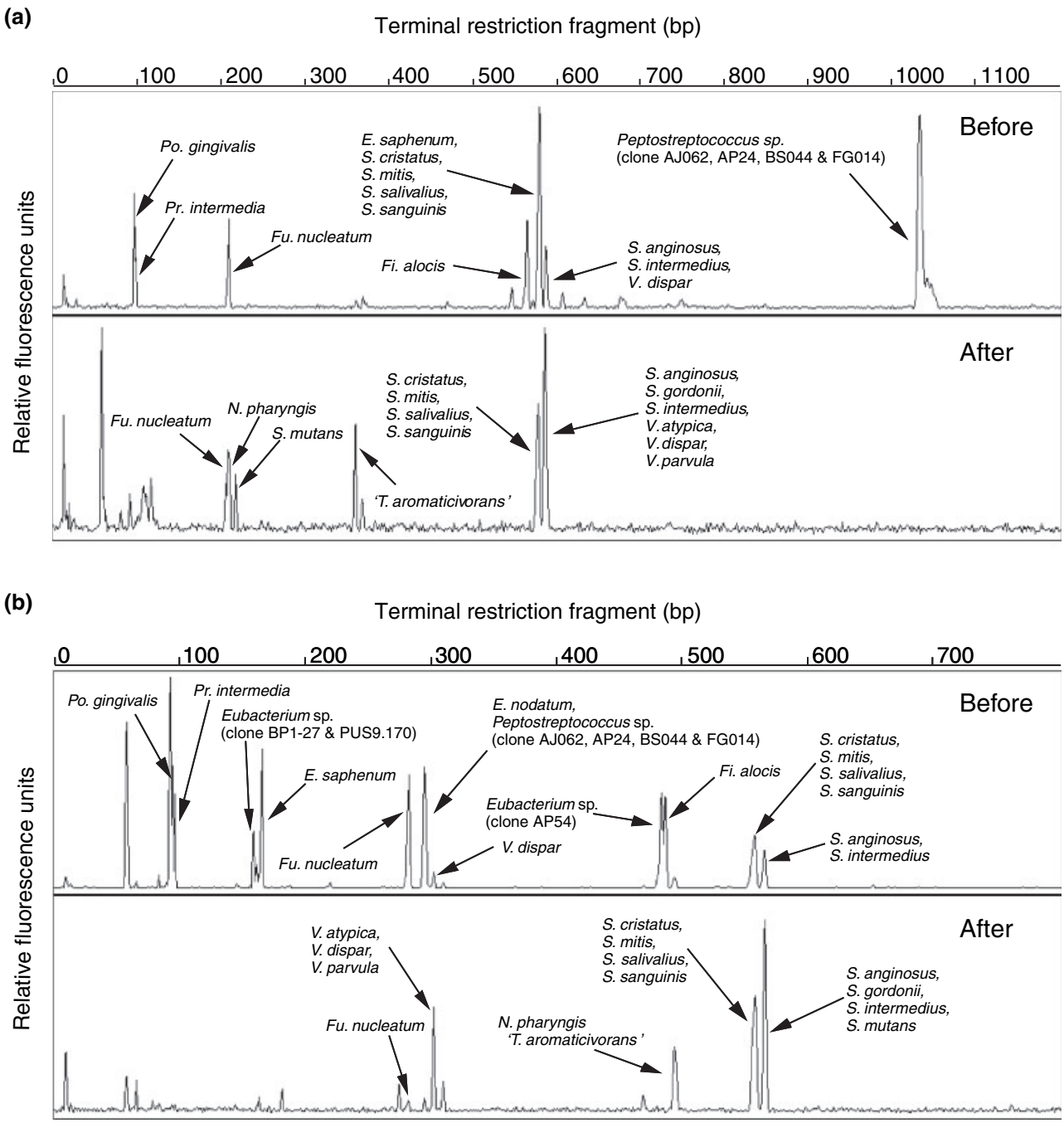

Fig. 1. Terminal restriction fragment length polymorphism patterns of 16S rRNA genes from subgingival plaque samples of a patient with periodontitis taken before treatment and after treatment generated after digestion with *Hha*I (a) and *Msp*I (b). 16S rRNA genes were amplified with universal primers 27F and 1492R. Almost all the terminal restriction fragments were presumed to be 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*. Reproduced with permission from Sakamoto *et al.* (98).

these T-RFs were significantly reduced or not detected after treatment. T-RFs larger than 1000 bp comprised several phylotypes of *Peptostreptococcus* species, including phylotype AP24 (76). *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 (99). Consequently, monitoring of the proportion of T-RFs larger than 1000 bp in the T-RFLP pattern may be useful for the evaluation of the prognosis of periodontal disease. T-RFLP analysis data were in agreement with real-time PCR and 16S rRNA gene clone library analysis data. After 3 months, the *P. gingivalis* population was markedly reduced (3.1×10^{-3}%), although the proportion of *P. gingivalis* before treatment was 7.6%. The proportion of the T-RF presumed to be *P. gingivalis* was 5.9%, and became undetected after 3 months. In addition, T-RF presumed to be *P. intermedia*, which is an important periodontal pathogen, could be differentiated from the T-RF presumed to be *P. gingivalis* by being 2-bp larger. Before treatment, the proportion of the T-RF presumed to be *P. intermedia* was 2.8%. After 3 months, this T-RF was not detected. These findings indicate that T-RFLP analysis is useful for evaluation of the effects of medical treatment of periodontitis. However, further analyzes of digests with multiple restriction enzymes (four or five) are necessary because multiple species, which belong to different genera, were detected from the same T-RF.

Conclusion

With the advancement of molecular biology in recent years, the initiation and progression mechanisms of periodontitis are becoming clearer gradually. As the culture-independent approaches have revealed the diversity of human oral microbiota and the existence of a large number of as-yet-to-be-cultured organisms which are presumed as periodontal pathogens, the researches on periodontal disease and human oral microbiota are coming to a new turn. Using the sequence information of 16S rRNA gene obtained, it is possible to detect not only known oral species but also the newly identified uncultivated species (phylotypes) directly in clinical samples. At present, a Human Oral Microbe Identification Microarray (HOMIM) slide system for the identification of essentially all of the more than 600 species encountered in the oral cavity is under development (100). This microarray should be extremely useful in clinical studies to simultaneously examine the roles of all bacterial species present at sites of oral diseases. The culture-independent approaches described in this mini-review will become indispensable in the future to elucidate the etiology of periodontal disease.

References

1. Sakamoto M, Suzuki M, Umeda M, Ishikawa I, Benno Y. Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. *Int J Syst Evol Microbiol* 2002;**52:**841–849.
2. Olsen GJ, Lane DJ, Giovannoni SJ, Pace NR. Microbial ecology and evolution: a ribosomal RNA approach. *Annu Rev Microbiol* 1986;**40:**337–365.
3. Dewhirst FE, Tamer MA, Ericson RE *et al.* The diversity of periodontal spirochetes by 16S rRNA analysis. *Oral Microbiol Immunol* 2000;**15:**196–202.
4. Moore LVH, Moore WEC, Cato EP *et al.* Bacteriology of human gingivitis. *J Dent Res* 1987;**66:**989–995.
5. Moore WEC, Holdeman LV, Smibert RM *et al.* Bacteriology of experimental gingivitis in children. *Infect Immun* 1984;**46:**1–6.
6. Moore WEC, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR. Bacteriology of severe periodontitis in young adult humans. *Infect Immun* 1982;**38:**1137–1148.
7. Miller JN, Smibert RM, Norris SJ. The genus *Treponema*. In: Balows A, Trüper HG, Dworkin M, Harder W, Shleifer KH, eds. *The prokaryotes*, 2nd edn. New York: Springer-Verlag, 1992: 3537–3559.
8. Smibert RM. Anaerobic spirochetes. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, eds. *Manual of clinical microbiology*, 5th edition. Washington DC: American Society for Microbiology,1991: 572–578.
9. Wyss C, Choi BK, Schüpbach P, Guggenheim B, Göbel UB. *Treponema amylovorum* sp. nov., a saccharolytic spirochete of medium size isolated from an advanced human periodontal lesion. *Int J Syst Bacteriol* 1997;**47:**842–845.
10. Chan ECS, Siboo R, Keng T *et al. Treponema denticola* (ex Brumpt 1925) sp. nov., nom. rev. and identification of new spirochete isolates from periodontal pockets. *Int J Syst Bacteriol* 1993;**43:**196–203.
11. Wyss C, Choi BK, Schüpbach P, Moter A, Guggenheim B, Göbel UB. *Treponema lecithinolyticum* sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases. *Int J Syst Bacteriol* 1999;**49:**1329–1339.
12. Wyss C, Choi BK, Schüpbach P, Guggenheim B, Göbel UB. *Treponema maltophilum* sp. nov., a small oral spirochete isolated from human periodontal lesions. *Int J Syst Bacteriol* 1996;**46:**745–752.
13. Umemoto T, Nakazawa F, Hoshino E, Okada K, Fukunaga M, Namikawa I. *Treponema medium* sp. nov., isolated from human subgingival dental plaque. *Int J Syst Bacteriol* 1997;**47:**67–72.
14. Nakazawa F, Hoshino E, Fukunaga M *et al.* Amended biochemical characteristics and phylogenetic position of *Treponema medium*. *Oral Microbiol Immunol* 2003;**18:**127–130.
15. Wyss C, Dewhirst FE, Gmür R *et al. Treponema parvum* sp. nov., a small, glucuronic or galacturonic acid-dependent oral spirochaete from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. *Int J Syst Evol Microbiol* 2001;**51:**955–962.
16. Smibert RM, Burmeister JA. *Treponema pectinovorum* sp. nov. isolated from humans with periodontitis. *Int J Syst Bacteriol* 1983;**33:**852–856.
17. Wyss C, Moter A, Choi BK *et al. Treponema putidum* sp. nov., a medium-sized proteolytic spirochaete isolated from lesions of human periodontitis and acute necrotizing ulcerative gingivitis. *Int J Syst Evol Microbiol* 2004;**54:**1117–1122.
18. Smibert RM, Johnson JL, Ranney RR. *Treponema socranskii* sp. nov., *Treponema socranskii* subsp. *socranskii* subsp. nov., *Treponema socranskii* subsp. *buccale* subsp. nov. & *Treponema socranskii* subsp. *paredis* subsp. nov. isolated from the human periodontia. *Int J Syst Bacteriol* 1984;**34:**457–462.
19. Smibert RM. Genus III. *Treponema* Schaudinn 1905, 1728^{AL}. In: Krieg NR, Holt JG, eds. *Bergey's manual of systematic bacteriology*, **Vol. 1**. Baltimore**:** Williams & Wilkins 1984: 49–57.
20. Paster BJ, Dewhirst FE, Coleman BC, Lau CN, Ericson RL. Phylogenetic analysis of cultivable oral treponemes

from the Smibert collection. *Int J Syst Bacteriol* 1998;**48:**713–722.
21. Chan ECS, McLaughlin R. Taxonomy and virulence of oral spirochetes. *Oral Microbiol Immunol* 2000;**15:**1–9.
22. Riviere GR, Elliot KS, Adams DF *et al*. Relative proportions of pathogen-related oral spirochetes (PROS) and *Treponema denticola* in supragingival and subgingival plaque from patients with periodontitis. *J Periodontol* 1992;**63:**131–136.
23. Ding Y, Uitto VJ, Haapasalo M *et al*. Membrane components of *Treponema denticola* trigger proteinase release from human polymorphonuclear leukocytes. *J Dent Res* 1996;**75:**1986–1993.
24. Ishihara K, Okuda K. Molecular pathogenesis of the cell surface proteins and lipids from *Treponema denticola*. *FEMS Microbiol Lett* 1999;**181:**199–204.
25. Simonson LG, Goodman CH, Bial JJ, Morton HE. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. *Infect Immun* 1988; **56:**726–728.
26. Koseki T, Ishikawa I, Umeda M, Benno Y. Characterization of oral treponemes isolated from human periodontal pockets. *Oral Microbiol Immunol* 1995;**10:**271–277.
27. Sakamoto M, Koseki T, Umeda M, Ishikawa I, Benno Y, Nakase T. Phylogenetic analysis of saccharolytic oral treponemes isolated from human subgingival plaque. *Microbiol Immunol* 1999;**43:**711–716.
28. 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.
29. 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.
30. Hutter G, Schlagenhauf U, Valenza G *et al*. Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. *Microbiology* 2003;**149:**67–75.
31. Paster BJ, Boches SK, Galvin JL *et al*. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.
32. Sato T, Kuramitsu HK. Restriction fragment-length polymorphism analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction for rapid identification of cultivable oral treponemes. *Oral Microbiol Immunol* 1999;**14:**117–121.
33. 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.
34. 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.
35. Mayanagi G, Sato T, Shimauchi H, Takahashi N. Detection frequency of periodontitis-associated bacteria in subgingival and supragingival plaque of periodontitis and healthy subjects. *Oral Microbiol Immunol* 2004;**19:**379–385.
36. Takeuchi Y, Umeda M, Ishizuka M, Huang Y, Ishikawa I. Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Japanese population. *J Periodontol* 2003;**74:**1460–1469.
37. Umeda M, Chen C, Bakker I, Contreras A, Morrison JL, Slots J. Risk indicators for harboring periodontal pathogens. *J Periodontol* 1998;**69:**1111–1118.
38. Umeda M, Miwa Z, Takeuchi Y *et al*. The distribution of periodontopathic bacteria among Japanese children and their parents. *J Periodont Res* 2004;**39:**398–404.
39. Shelburne CE, Prabhu A, Gleason RM, Mullally BH, Coulter WA. Quantitation of *Bacteroides forsythus* in subgingival plaque. Comparison of immunoassay and quantitative polymerase chain reaction. *J Microbiol Methods* 2000;**39:**97–107.
40. Lyons SR, Griffen AL, Leys EJ. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J Clin Microbiol* 2000;**38:**2362–2365.
41. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. *Microbiol Immunol* 2001;**45:**39–44.
42. Maeda H, Fujimoto C, Haruki Y *et al*. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol Med Microbiol* 2003;**39:**81–86.
43. Asai Y, Jinno T, Igarashi H, Ohyama Y, Ogawa T. Detection and quantification of oral treponemes in subgingival plaque by real-time PCR. *J Clin Microbiol* 2002;**40:**3334–3340.
44. Boutaga K, van Winkelhoff AJ, Vandenbroucke-Grauls CMJE, Savelkoul PHM. Comparison of real-time PCR and culture for detection of *Porphyromonas gingivalis* in subgingival plaque samples. *J Clin Microbiol* 2003;**41:**4950–4954.
45. Kawada M, Yoshida A, Suzuki N *et al*. Prevalence of *Porphyromonas gingivalis* in relation to periodontal status assessed by real-time PCR. *Oral Microbiol Immunol* 2004;**19:**289–292.
46. Kuboniwa M, Amano A, Kimura RK *et al*. Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with TaqMan probes. *Oral Microbiol Immunol* 2004;**19:**168–176.
47. Morillo JM, Lau L, Sanz M, Herrera D, Silva A. Quantitative real-time PCR based on single copy gene sequence for detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J Periodont Res* 2003;**38:**518–524.
48. Nonnenmacher C, Dalpke A, Mutters R, Heeg K. Quantitative detection of periodontopathogens by real-time PCR. *J Microbiol Methods* 2004;**59:**117–125.
49. Suzuki N, Yoshida A, Saito T, Kawada M, Nakano Y. Quantitative microbiological study of subgingival plaque by real-time PCR shows correlation between levels of *Tannerella forsythensis* and *Fusobacterium* spp. *J Clin Microbiol* 2004;**42:**2255–2257.
50. Yoshida A, Suzuki N, Nakano Y, Oho T, Kawada M, Koga T. Development of a 5′ fluorogenic nuclease-based real-time PCR assay for quantitative detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *J Clin Microbiol* 2003;**41:**863–866.
51. Yoshida A, Kawada M, Suzuki N *et al*. TaqMan real-time polymerase chain reaction assay for the correlation of *Treponema denticola* numbers with the severity of periodontal disease. *Oral Microbiol Immunol* 2004;**19:**196–200.
52. van der Ploeg JR, Giertsen E, Lüdin B, Mörgeli C, Zinkernagel AS, Gmür R. Quantitative detection of *Porphyromonas gingivalis fimA* genotypes in dental plaque. *FEMS Microbiol Lett* 2004;**232:**31–37.
53. Socransky SS, Haffajee AD, Smith C *et al*. Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems. *Oral Microbiol Immunol* 2004;**19:**352–362.
54. Socransky SS, Smith C, Martin L, Paster BJ, Dewhirst FE, Levin AE. 'Checkerboard' DNA–DNA hybridization. *Biotechniques* 1994;**17:**788–792.
55. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
56. Ximénez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in subjects in health and periodontits. *J Clin Periodontol* 2000;**27:**648–657.
57. Ximénez-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.

58. Darout IA, Albandar JM, Skaug N, Ali RW. Salivary microbiota levels in relation to periodontal status, experience of caries and miswak use in Sudanese adults. *J Clin Periodontol* 2002;**29:**411–420.
59. Boström L, Bergström J, Dahlén G, Linder LE. Smoking and subgingival microflora in periodontal disease. *J Clin Periodontol* 2001;**28:**212–219.
60. Haffajee AD, Socransky SS. Relationship of cigarette smoking to the subgingival microbiota. *J Clin Periodontol* 2001;**28:** 377–388.
61. Haffajee AD, Bogren A, Hasturk H, Feres M, Lopez NJ, Socransky SS. Subgingival microbiota of chronic periodontitis subjects from different geographic locations. *J Clin Periodontol* 2004;**31:** 996–1002.
62. Craig RG, Boylan R, Yip J *et al*. Prevalence and risk indicators for destructive periodontal diseases in 3 urban American minority populations. *J Clin Periodontol* 2001;**28:**524–535.
63. Feres M, Haffajee AD, Allard K, Som S, Socransky SS. Change in subgingival microbial profiles in adult periodontitis subjects receiving either systemically-administered amoxicillin or metronidazole. *J Clin Periodontol* 2001;**28:** 597–609.
64. Sakellari D, Belibasakis G, Chadjipadelis T, Arapostathis K, Konstantinidis A. Supragingival and subgingival microbiota of adult patients with Down's syndrome. Changes after periodontal treatment. *Oral Microbiol Immunol* 2001;**16:**376–382.
65. Paster BJ, Bartoszyk IM, Dewhirst FE. Identification of oral streptococci using PCR-based, reverse-capture, checkerboard hybridization. *Methods Cell Sci* 1998;**20:**223–231.
66. Choi BK, Paster BJ, Dewhirst FE, Göbel UB. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. *Infect Immun* 1994;**62:**1889–1895.
67. Spratt DA, Weightman AJ, Wade WG. Diversity of oral asaccharolytic *Eubacterium* species in periodontitis – identification of novel phylotypes representing uncultivated taxa. *Oral Microbiol Immunol* 1999;**14:**56–59.
68. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci USA* 1999;**96:**14547–14552.
69. Sakamoto M, Umeda M, Ishikawa I, Benno Y. Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. *Microbiol Immunol* 2000;**44:**643–652.
70. Becker MR, Paster BJ, Leys EJ *et al*. Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* 2002;**40:**1001–1009.
71. Paster BJ, Falkler WA Jr, Enwonwu CO *et al*. Prevalent bacterial species and novel phylotypes in advanced noma lesions. *J Clin Microbiol* 2002;**40:**2187–2191.
72. Kazor CE, Mitchell PM, Lee AM *et al*. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J Clin Microbiol* 2003;**41:**558–563.
73. Leys EJ, Lyons SR, Moeschberger ML, Rumpf RW, Griffen AL. Association of *Bacteroides forsythus* and a novel *Bacteroides* phylotype with periodontitis. *J Clin Microbiol* 2002;**40:**821–825.
74. Harper-Owen R, Dymock D, Booth V, Weightman AJ, Wade WG. Detection of unculturable bacteria in periodontal health and disease by PCR. *J Clin Microbiol* 1999;**37:**1469–1473.
75. Dymock D, Weightman AJ, Scully C, Wade WG. Molecular analysis of microflora associated with dentoalveolar abscesses. *J Clin Microbiol* 1996;**34:**537–542.
76. Sakamoto M, Huang Y, Umeda M, Ishikawa I, Benno Y. Detection of novel oral phylotypes associated with periodontitis. *FEMS Microbiol Lett* 2002; **217:**65–69.
77. Sakamoto M, Suzuki M, Huang Y, Umeda M, Ishikawa I, Benno Y. *Prevotella shahii* sp. nov. and *Prevotella salivae* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* 2004; **54:**877–883.
78. Sakamoto M, Huang Y, Umeda M, Ishikawa I, Benno Y. *Prevotella multiformis* sp. nov., isolated from human subgingival plaque. *Int J Syst Evol Microbiol*;**55:**815–819.
79. Hugenholtz P, Goebel BM, Pace NR. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* 1998; **180:**4765–4774.
80. Brinig MM, Lepp PW, Ouverney CC, Armitage GC, Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl Environ Microbiol* 2003; **69:**1687–1694.
81. 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.
82. Liu WT, Marsh TL, Cheng H, Forney LJ. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol* 1997;**63:**4516–4522.
83. Clement BG, Kehl LE, DeBord KL, Kitts CL. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J Microbiol Methods* 1998;**31:**135–142.
84. Dunbar J, Ticknor LO, Kuske CR. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl Environ Microbiol* 2000;**66:**2943–2950.
85. González JM, Simó R, Massana R *et al*. Bacterial community structure associated with a dimethylsulfonipropionate-producing north atlantic algal bloom. *Appl Environ Microbiol* 2000;**66:**4237–4246.
86. Hiraishi A, Iwasaki M, Shinjo H. Terminal restriction pattern analysis of 16S rRNA genes for the characterization of bacterial communities of activated sludge. *J Biosci Bioeng* 2000;**90:**148–156.
87. Kaplan CW, Astaire JC, Sanders ME, Reddy BS, Kitts CL. 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* 2001;**67:**1935–1939.
88. Leser TD, Lindecrona RH, Jensen TK, Jensen BB, Møller K. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodysenteriae*. *Appl Environ Microbiol* 2000;**66:**3290–3296.
89. Lukow T, Dunfield PF, Liesack W. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiol Ecol* 2000;**32:**241–247.
90. Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 1999;**65:**3518–3525.
91. Kitts CL. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr Issues Intest Microbiol* 2001;**2:**17–25.
92. Marsh TL. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr Opin Microbiol* 1999;**2:**323–327.
93. Marsh TL, Saxman P, Cole J, Tiedje J. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl Environ Microbiol* 2000;**66:**3616–3620.

94. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. *J Med Microbiol* 2003;**52:**79–89.
95. Fujimoto C, Maeda H, Kokeguchi S *et al*. Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of microbial communities of subgingival plaque. *J Periodont Res* 2003;**38:**440–445.
96. Zijnge V, Harmsen HJM, Kleinfelder JW, van der Rest ME, Degener JE, Welling GW. Denaturing gradient gel electrophoresis analysis to study bacterial community structure in pockets of periodontitis patients. *Oral Microbiol Immunol* 2003;**18:**59–65.
97. Darby IB, Mooney J, Kinane DF. Changes in subgingival microflora and humoral immune response following periodontal therapy. *J Clin Periodontol* 2001;**28:**796–805.
98. Sakamoto M, Huang Y, Ohnishi M, Umeda M, Ishikawa I, Benno Y. Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes. *J Med Microbiol* 2004;**53:**563–571.
99. Rams TE, Feik D, Listgarten MA, Slots J. *Peptostreptococcus micros* in human periodontitis. *Oral Microbiol Immunol* 1992;**7:**1–6.
100. Boches SK, Lee AM, Paster BJ, Dewhirst FE. Development of a Human Oral Microbe Identification Microarray. *J Dent Res* 2004;**83**(Spec Iss A)**:** IADR Abstr. 2263. http://www.dentalresearch.org