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Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections

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The purpose of the present study was to use terminal restriction fragment length polymorphism analysis and the 16S rRNA gene clone library to investigate the diversity of the microbiota associated with asymptomatic and symptomatic endodontic infections and to compare the bacterial community structure in these two clinical conditions. Samples were taken from asymptomatic endodontic infections associated with chronic periradicular lesions and from symptomatic infections clinically diagnosed as acute abscesses. 16S rRNA genes from DNA isolated from clinical samples were used to construct clone libraries or were subjected to terminal restriction fragment length polymorphism analysis. Sequence analysis of 186 clones revealed 42 taxa; 23 (55%) were uncultivated phylotypes, of which seven were unique to endodontic infections. Clone sequencing and terminal restriction fragment length polymorphism analysis revealed that the most commonly detected taxa were *Fusobacterium nucleatum* (including terminal restriction fragment types 1 and 2), *Peptostreptococcus micros*/*Peptostreptococcus* sp. oral clone AJ062/BS044/FG014, *Prevotella* species, *Dialister* species, *Mogibacterium* species, *Lachnospiraceae* oral clone 55A-34, *Filifactor alocis*, *Megasphaera* sp. oral clone CS025/BS073, and *Veillonella* sp. oral clone BP1-85/*Veillonella dispar*/*V. parvula*. *Bacteroides*-like sp. oral clone X083/*Bacteroidales* oral clone MCE7_20 and *Dialister* sp. oral clone BS016/MCE7_134 were detected only in asymptomatic teeth. On the other hand, *F. nucleatum* terminal restriction fragment type 2, *Prevotella intermedia*, *Dialister pneumosintes*, and some phylotypes were exclusively detected in symptomatic samples. Bacterial profiles of symptomatic endodontic infections generated by terminal restriction fragment length polymorphism analysis were clearly different from those of asymptomatic infections. Overall, the average number of terminal restriction fragments in symptomatic samples was significantly larger than in asymptomatic samples. Molecular analysis of the microbiota associated with symptomatic or asymptomatic endodontic infections indicates that the endodontic bacterial diversity is greater than previously described by culture methods and that the structure of the microbiota differ significantly between asymptomatic and symptomatic infections.

Key words: 16S rRNA gene clone library; endodontic microbiology; symptoms; terminal restriction fragment length polymorphism

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In the light of contemporary evidence, periradicular inflammatory diseases can be regarded as infectious disorders caused by endodontic infections (37). After pulpal necrosis, usually as a sequel to caries, the root canal environment provides a selective habitat for the establishment of a mixed microbiota conspicuously dominated by anaerobic bacteria (38). Microorganisms emanating from the root canal system elicit an inflammatory response at the periradicular tissues, which is mounted in an attempt to prevent spreading of the infectious process into bone tissue and beyond. Periradicular diseases can give rise to a multitude of clinical and radiographic presentations (30).

Traditionally, endodontic infections have been studied by culture-dependent methods (31). Published investigations of this issue are plagued by several problems,

including the probable contribution of viable but uncultivated bacteria to disease and insufficient bacterial characterization. Molecular genetic methods can sidestep many of these limitations associated with culture approaches (20). For instance, application of the 16S rRNA gene clone library revealed that approximately one-half of the bacterial species inhabiting the oral cavity have not been previously characterized (21, 22).

While the microbial causation of periradicular diseases is well established, it remains unclear whether the same species responsible for asymptomatic periradicular lesions also contribute to the development of symptomatic ones or whether a unique assemblage of microorganisms is responsible for the latter. Some species have been suggested to be involved with symptomatic lesions (37, 39). However, it has been revealed that the same species may also be present in similar frequencies in asymptomatic cases (2, 8, 35). Therefore, it appears that factors other than the mere presence of a given putative pathogenic species can play a role in the etiology of symptomatic endodontic infections. Indeed, a study using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) revealed that differences in the structure of the mixed bacterial consortium, represented by different types and load of the community members, may explain the development of symptoms (36). Thus, there appear to be significant differences in the predominant bacterial composition between asymptomatic and symptomatic lesions, with the latter harboring a higher number of species.

Terminal restriction fragment length polymorphism (T-RFLP) is a molecular approach that allows the assessment of the diversity of complex bacterial communities and rapid comparison of the community structure and diversity of different ecosystems (3, 16). After a PCR reaction in which the 5′ end of one of the primers is labeled with a fluorescent dye, PCR products are digested with judiciously selected restriction endonucleases, and the labeled terminal fragments can be specifically detected and their sizes determined by using automated DNA sequencer technology (17). The lengths of the terminal restriction fragment (T-RF) can be predicted from known 16S rRNA gene sequences. Studies have made use of this technique to characterize the human fecal microbiota (12) and the microbiota in saliva of healthy subjects and patients with periodontitis (26, 27). T-RFLP analysis has also been recently used to investigate the effect of the quality of coronal restorations on the composition of the root canal microbiota of untreated teeth and root-filled teeth associated with periradicular lesions and has revealed the presence of bacteria rarely described in infected root canals (14).

The present study intended to use T-RFLP analysis and the 16S rRNA gene clone library to study the diversity of the endodontic microbiota associated with asymptomatic and symptomatic periradicular lesions and to compare the bacterial community structure in these two clinical conditions. To the best of our knowledge, this is the first study using a combination of these two molecular genetic approaches to the analysis of the microbiota of asymptomatic and symptomatic endodontic infections.

Material and methods

Subjects and sampling procedures

This study was carried out in accordance with the guidelines of, and after approval by, the Ethical Committee at Estácio de Sa University, Rio de Janeiro, Brazil. Samples were taken from patients who had been referred for root canal treatment or emergency treatment to the Department of Endodontics, Estácio de Sá University. Only teeth from adult patients (aged 18–44 years), all of them having carious lesions, necrotic pulps, and radiographic evidence of periradicular diseases, were included in this study. Overall, 16 samples were obtained and grouped according to the clinical diagnoses: eight asymptomatic teeth with chronic periradicular lesions, and eight symptomatic teeth, clinically diagnosed as acute periradicular abscesses and showing spontaneous pain, exacerbated by mastication, and localized or diffuse swellings along with fever, lymphadenopathy, or malaise. No apparent communication from the abscess to the oral cavity or the skin surface was observed. Selected teeth showed no significant gingival recession and were free of periodontal pockets deeper than 4 mm.

Samples from teeth with asymptomatic periradicular lesions were taken from the root canals. Sampling procedures were carried out under strict asepsis as previously described (32, 33). A file with the handle cut off and two paper points were used to take samples from each canal which were then placed into cryotubes containing 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). Samples were immediately frozen at − 20°C.

Samples from teeth with symptomatic periradicular lesions were taken by aspiration of the purulent exudate from the swollen mucosa over each abscess. The overlying mucosa was disinfected with 2% chlorhexidine and a sterile disposable syringe was used to aspirate pus, which was immediately injected into cryotubes containing TE buffer. Pus samples were then immediately frozen.

DNA extraction

Samples were thawed to 37°C for 10 min and vortexed for 30 s. Afterwards, the microbial suspension was pelleted by centrifugation at $5000 \times g$ for 10 min. The pellet was then resuspended in 180 µl of buffer ATL supplied by QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and 20 µl of proteinase K (20 mg/ml) was added. Samples were incubated for 3 h at 56°C. Subsequently, total bacterial genomic DNA was isolated according to the protocol of QIAamp DNA Mini Kit. The total bacterial DNA was eluted with 200 µl of AE buffer (Qiagen) and stored at − 20°C.

T-RFLP analysis

T-RFLP analysis was performed as described previously (27). The primers used for the PCR amplification of 16S rRNA gene sequences were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (15). 27F was labeled at the 5′ end with 6′-carboxyfluorescein (6-FAM), which was synthesized by Applied Biosystems Japan (Tokyo, Japan). Amplification reactions were performed in a total volume of 50 µl containing 1–5 µl of dissolved DNA, 1.25 U of TaKaRa Ex Taq (TaKaRa Shuzo, Shiga, Japan), 5 µl of 10 × Ex Taq buffer, 4 µl of dNTP mixture (2.5 mM each), and 10 pmol of each primer. 16S rRNA genes were amplified in a Biometra Thermocycler TGradient (Biometra, Göttingen, Germany) using the following program: 95°C for 3 min, followed by 30 cycles consisting of 95°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, with a final extension period at 72°C for 10 min. Amplified DNA was verified by electrophoresis of aliquots of PCR mixtures (2 µl) in 1.5% agarose in 1 × TAE buffer. DNA was stained with ethidium bromide and visualized under short-wavelength UV light. PCR products were purified by the polyethylene glycol precipitation method (13) with some modifications. Briefly, a 50-µl aliquot of the 16S rRNA gene

solution was mixed with 30 μl of polyethylene glycol solution (40% polyethylene glycol 6000 and 10 mM $MgCl_2$) and 12 μl of 3 M sodium acetate, gently shaken for 10 min at room temperature, and centrifuged at 17,800 × *g* for 15 min. The supernatant was removed carefully by pipetting and the precipitated DNA was washed twice with 70% ethanol and redissolved in 20 μl of sterile distilled water. Purified 16S rRNA genes were stored at −20°C until analysis.

Purified PCR product (2 μl) was digested with 20 U of *Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I (TaKaRa Shuzo or ToYoBo, Osaka, Japan) in a total volume of 10 μl at 37°C for 3 h. The restriction digest product (1 μl) was mixed with 8 μl of Hi-Di Formamide (Applied Biosystems, Foster City, CA) and 1 μl of DNA fragment length standard. The standard size marker was a 1 : 1 mixture of the size standards GS 500 ROX and GS 1000 ROX (Applied Biosystems). Each sample was denatured at 95°C for 2 min and then immediately placed on ice. The length of T-RF was determined on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in GeneScan mode (15 kV, 100 μA, and 60°C for 40 min for each sample). Fragment sizes were estimated by using the Local Southern Method in GeneScan 3.7 software (Applied Biosystems). T-RFs with a peak area of less than 2% of total area were excluded from the analysis. Fragments were resolved to one base pair by manual alignment of the size standard peaks from different electropherograms. Predicted T-RFLP patterns of the 16S rRNA genes of known bacterial species were obtained using the GENETYX-MAC program (Software Developing Co., Tokyo, Japan).

Dendrogram analysis was performed using five T-RFLP patterns with five different restriction enzymes (*Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I) by BioNumerics version 3.5 software (Applied Maths, Kortrijk, Belgium). Dendrogram analysis was based on the similarity coefficient, for the objective interpretation of the difference of T-RFLP patterns. The distances between samples were represented graphically by constructing a dendrogram based on the Pearson's correlation coefficients of T-RFLP profiles. Unweighted pair-group method using arithmetic averages (UPGMA) was used for establishing the dendrogram type.

16S rRNA gene clone library analysis

16S rRNA gene clone library analysis was performed as described previously (28). The primers used for the PCR amplification of 16S rRNA gene sequences were 27F (without 6-FAM) and 1492R. 16S rRNA genes were amplified as described above. PCR products were purified using an UltraClean PCR Clean-up DNA purification kit (Mo Bio Laboratories, Inc., Carlsbad, CA). Purified amplicon was ligated into the plasmid vector pCR®2.1, and then transformed into One Shot® INVαF′ competent cells using the Original TA Cloning Kit (Invitrogen, San Diego, CA).

Plasmid DNAs were prepared using the TempliPhi DNA Amplification Kit (Amersham Biosciences, Piscataway, NJ) from randomly selected recombinants and used as templates for sequencing. Sequencing was conducted using the 27F and 519R primers (15), a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems), and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All sequences were checked for possible chimeric artifacts by the Chimera Check program of the Ribosomal Database Project-II (RDP-II) (4), and compared with similar sequences of the reference organisms by FASTA search (23). 16S rRNA gene sequence similarity of 98% was used as the cut-off for positive identification of taxa (19). A <98% identity in 16S rRNA gene sequence was the criterion used to identify a bacterium at the genus level.

Statistical analysis

Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Student's *t*-test. A *P*-value of less than 0.05 denoted a statistically significant difference.

Results

T-RFLP profile in asymptomatic and symptomatic endodontic infections

A total of 16 samples – eight asymptomatic cases and eight symptomatic cases – were subjected to PCR with 16S rRNA gene-specific primers. 16S rRNA genes were not amplified from two asymptomatic and one symptomatic sample. PCR products obtained from two other asymptomatic samples were not suitable for T-RFLP analysis because of weak amplification. Consequently, four asymptomatic samples and seven symptomatic samples were subjected to T-RFLP analysis. A dendrogram was constructed by combining five T-RFLP patterns with five different restriction enzymes (Fig. 1). Asymptomatic samples formed a cluster and were separate from the symptomatic samples, except for asymptomatic sample 10N. The numbers of T-RFs obtained for each sample using different enzymes are shown in Table 1. The number of *Alu*I-digested T-RFs was greater in symptomatic samples (12.3 ± 0.8) than in asymptomatic samples (8.8 ± 2.8) ($P = 0.043$). The number of *Rsa*I-digested T-RFs in symptomatic samples (14.3 ± 1.8) was significantly larger than in asymptomatic samples (10.3 ± 1.7) ($P = 0.0027$). Although the number of *Hha*I, *Msp*I, or *Hae*III-digested T-RFs in symptomatic samples (13.1 ± 4.4, 14.3 ± 3.1, 12.6 ± 2.5, respectively) was larger than in asymptomatic samples (11.8 ± 1.0,

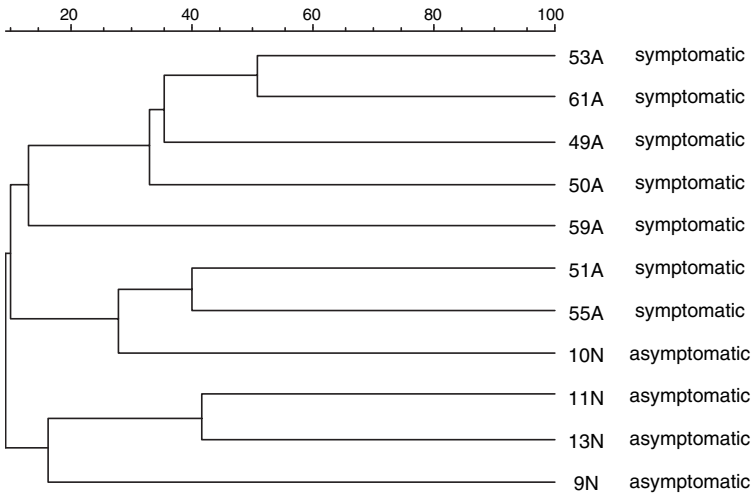

Fig. 1. Dendrogram showing the relationships among terminal restriction fragment length polymorphism (T-RFLP) patterns of asymptomatic and symptomatic samples. A dendrogram was constructed by combination of five T-RFLP patterns with five different restriction enzymes (*Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I).

Table 1. Number of T-RFs detected in four asymptomatic and seven symptomatic endodontic infections using five restriction enzymes

Sample	No. of T-RFs[a]					
	*Hha*I	*Msp*I	*Alu*I	*Hae*III	*Rsa*I	Average
Asymptomatic						
9N	12	13	12	10	11	11.6 ± 1.1
10N	13	11	10	15	12	12.2 ± 1.9
11N	11	10	6	8	10	9.0 ± 2.0
13N	11	12	7	11	8	9.8 ± 2.2
Symptomatic						
49A	12	15	13	12	15	13.4 ± 1.5
50A	18	13	11	15	14	14.2 ± 2.6
51A	12	18	12	15	13	14.0 ± 2.5
53A	12	18	13	12	16	14.2 ± 2.7
55A	20	15	13	15	16	15.8 ± 2.6
59A	11	10	12	9	15	11.4 ± 2.3
61A	7	11	12	10	11	10.2 ± 1.9

[a]T-RFs with a peak area of less than 2% of total area were excluded from the analysis.
T-RFs , terminal restriction fragments.

11.5 ± 1.3, 11.0 ± 2.9, respectively), the difference was not statistically significant ($P > 0.05$). Overall, the average number of five different restriction enzyme-digested T-RFs in symptomatic samples (13.3 ± 1.9) was significantly larger than in asymptomatic samples (10.7 ± 1.5) ($P = 0.020$).

Bacterial species and phylotypes identified using 16S rRNA gene clone library analysis

To validate the data obtained from T-RFLP analysis, we constructed 16S rRNA gene clone libraries of four samples (asymptomatic samples 9N and 13N, and symptomatic samples 51A and 55A). Forty-two different taxa were identified from 186 clones sequenced (Table 2). Twenty-three (55%) of the 42 taxa were identified as phylotypes, i.e. species that have not yet been cultivated and are known only by 16S rRNA gene sequences. Seven of these phylotypes were novel in that they have never been detected in other sites (Fig. 2). A mean of 12 taxa were detected in asymptomatic samples and a mean of 18 taxa were detected in symptomatic samples.

Computer-simulated T-RFLP analysis

On the basis of 16S rRNA gene clone library analysis data, clones were assigned to T-RFs in the T-RFLP patterns of two asymptomatic samples and two symptomatic samples. The typical T-RFLP pattern of each condition is shown in Fig. 3 and 4. Almost all the T-RFs were presumed to represent species or phylotypes detected by the 16S rRNA gene clone library analysis. In general, the most prevalent species or phylotypes (found in about one-half of the samples or more) included *Fusobacterium nucleatum* (including T-RF type 1 and 2), *Peptostreptococcus micros*/*Peptostreptococcus* sp. oral clone AJ062/BS044/FG014, *Mogibacterium pumilum, Lachnospiraceae* oral clone 55A-34, *Prevotella oralis*, *Prevotella multisaccharivorax*, a species proposed recently by Sakamoto et al. (29), *Dialister invisus*/*Dialister* sp. GBA27, *Filifactor alocis*, *Megasphaera* sp. oral clone CS025/BS073, and *Veillonella* sp. oral clone BP1-85/*Veillonella dispar*/*V. parvula*. *Bacteroides*-like sp. oral clone X083/*Bacteroidales* oral clone MCE7_20, and *Dialister* sp. oral clone BS016/MCE7_134 were detected only in asymptomatic teeth (four and three samples, respectively). On the other hand, some taxa were exclusively detected in symptomatic samples (three or more cases), including *F. nucleatum* T-RF type 2, *Prevotella intermedia*, *Dialister pneumosintes*, *Prevotella* sp. E9_42/*Prevotella* sp. oral clone PUS9.180 (both these phylotypes have > 99% identity in 16S rRNA gene sequence with the recently described *Prevotella baroniae* (7)), *Eubacterium* sp. oral clone BP1-89, and *Lachnospiraceae* oral clone MCE7_60. Detection frequencies of bacterial species or phylotypes associated with asymptomatic and symptomatic endodontic infections are shown in Table 3.

Discussion

Analysis of 16S rRNA gene sequences from 186 cloned inserts was carried out to determine species identity or closest relatives by comparison with sequences of known species or phylotypes. About one-half of the taxa detected have not been cultivated previously and seven phylotypes were unique to endodontic infections. These findings, showing a high proportion of as yet uncultivated and unrecognized bacteria, are in line with other studies using 16S rRNA gene clone library for the assessment of the diversity of the oral microbiota in health and disease (19, 21, 22, 25, 28). It is not unreasonable to assume that many of these as yet uncultivated phylotypes may participate in the pathogenesis of periradicular lesions.

All of the seven bacterial phyla previously recognized to have members among the endodontic microbiota were represented in this study (31, 34). Most of the inferred species belonged to the *Firmicutes* and *Bacteroidetes* phyla, which is in consonance with another molecular study of the endodontic microbiota (19). Clone analysis revealed that members of the *Firmicutes* phylum dominated (25 taxa), followed by members of the *Bacteroidetes* phylum (eight taxa) (Table 2). Relatively few taxa were associated with the *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Spirochaetes* phyla. Clone BA121 from the phylum *Synergistes* was detected in one case by T-RFLP analysis. All oral representatives of the *Deferribacteres* phylum were newly transferred to the *Synergistes* phylum (10) and a recent study using a nested PCR-DGGE assay revealed the occurrence of clone BA121 in several cases of endodontic infections (34).

In this study, we have not sequenced the entire 16S rRNA gene for every clone. Some species, clearly distinct by DNA–DNA homology comparisons, share identical 16S rRNA genes. Others are identical over the first ~ 500 bp of the gene, as used here, but can be distinguished over the full length of the gene. The range of possible identities of the source organisms is given in Table 2. In addition, we narrowed the possibilities down to a few species or phylotype (T-RF type) using computer-simulated T-RFLP analysis (Table 3).

Overall, the mean number of T-RFs after digestion with five enzymes was 13.3 in symptomatic samples and 10.7 in asymptomatic samples. By using 16S rRNA gene clone libraries of fewer samples we found a mean of 18 taxa in symptomatic samples and 12 taxa in asymptomatic samples. These figures are higher than found in other culture-dependent studies (11, 37) but are in agreement with results from previous studies using culture-independent approaches (19, 36). Munson et al. (19) used 16S rRNA gene sequence analysis and reported a mean of 20.2 (range 7–29) taxa in each asymptomatic sample. In a

Table 2. Bacterial species and phylotypes identified from two asymptomatic and two symptomatic endodontic infections by 16S rRNA gene sequencing analysis

	No. of clones (%)			
	Asymptomatic		Symptomatic	
Bacteria	9N	13N	51A	55A
Actinobacteria				
Atopobium rimae	1 (2.1)			1 (2.1)
Corynebacterium sp. oral clone 9N-22*	1 (2.1)			
Olsenella genomosp. C1	11 (23.4)			
Scardovia inopinata	1 (2.1)			
Bacteroidetes				
Bacteroidales oral clone MCE7_164/MCE3_262/MB4_G15			1 (2.2)	
Bacteroides-like sp. oral clone X083/*Bacteroidales* oral clone MCE7_20		13 (28.3)		
Porphyromonas endodontalis			10 (22.2)	
Prevotella denticola/*Prevotella* sp. oral clone AO036/AH005				2 (4.2)
Prevotella intermedia				3 (6.3)
Prevotella oralis		1 (2.2)	2 (4.4)	
Prevotella sp. E9_42/*Prevotella* sp. oral clone PUS9.180			1 (2.2)	1 (2.1)
Uncultured *Eubacterium* E1-K13				1 (2.1)
Firmicutes				
Dialister invisus/*Dialister* sp. GBA27/ *Dialister* sp. oral clone BS095/55A-29*/9N-1*	18 (38.3)		5 (11.1)	4 (8.4)
Dialister pneumosintes				3 (6.3)
Dialister sp. oral clone 9N-7*	1 (2.1)			
Dialister sp. oral clone BS016/MCE7_134		3 (6.5)		
Eubacterium infirmum/*Eubacterium sulci*	2 (4.3)		3 (6.7)	1 (2.1)
Eubacterium sp. oral clone BP1-89/*Eubacterium nodatum*			1 (2.2)	
Filifactor alocis	1 (2.1)		4 (8.9)	
Gemella morbillorum			2 (4.4)	
Lachnospiraceae oral clone 55A-34*				1 (2.1)
Lachnospiraceae oral clone MCE7_60			1 (2.2)	
Lactobacillus mucosae	1 (2.1)			
Megasphaera sp. oral clone CS025/BS073		4 (8.7)		
Mogibacterium neglectum/Mogibacterium pumilum/ Mogibacterium diversum/Mogibacterium vescum	1 (2.1)			1 (2.1)
Mogibacterium sp. oral clone BP1-36/*Mogibacterium timidum*	1 (2.1)			1 (2.1)
Peptostreptococcus micros/*Peptostreptococcus* sp. oral clone AJ062/BS044/FG014	3 (6.4)		2 (4.4)	1 (2.1)
Peptostreptococcus sp. oral clone CK035			2 (4.4)	9 (18.8)
Pseudoramibacter alactolyticus	3 (6.4)	21 (45.7)		
Selenomonas sputigena				1 (2.1)
Selenomonas sp. oral clone 55A-7*				1 (2.1)
Selenomonas sp. oral clone EQ054		1 (2.2)		
Solobacterium sp. oral clone K010/*Solobacterium moorei*			1 (2.2)	3 (6.3)
Streptococcus anginosus			2 (4.4)	
Streptococcus constellatus/*Streptococcus intermedius*				6 (12.5)
Veillonella sp. oral clone BP1-85/*Veillonella dispar*/*Veillonella parvula*		1 (2.2)		
Bacterium MDA2477/Bacterium MDA2477-like oral clone 51A-9*			5 (11.1)	
Fusobacteria				
Fusobacterium nucleatum	1 (2.1)	1 (2.2)	3 (6.7)	5 (10.5)
Proteobacteria				
Campylobacter gracilis		1 (2.2)		
Pseudomonas mephitica				2 (4.2)
Pseudomonas sp. LCY11				1 (2.1)
Spirochaetes				
Treponema denticola	1 (2.1)			
Total	47	46	45	48

*Identified in this study.

study using PCR-DGGE analysis, Siqueira et al. (36) reported a mean number of 12.1 (range 2–29) bands in symptomatic samples and 6.7 (range 2–11) bands in asymptomatic samples.

The structure of the endodontic bacterial community associated with asymptomatic and symptomatic infections as well as the number of participating species in the consortium were demonstrated to differ significantly. The bacterial profiles of symptomatic endodontic infections generated by T-RFLP analysis were clearly different from those of asymptomatic infections. Asymptomatic samples formed a cluster separated from the symptomatic samples, except for one sample. Overall, the average number of five different restriction enzyme-digested T-RFs in symptomatic samples was significantly larger than in asymptomatic samples. In a previous study, when PCR-DGGE was used to examine the structure of bacterial communities in samples taken from symptomatic and asymptomatic endodontic infections (36), the mean number of bands observed in the former was found to be higher than in the latter. Furthermore, clustering methods and principal component analysis of DGGE banding pattern placed the samples according to the presence or absence of symptoms. Although PCR-DGGE is a powerful methods

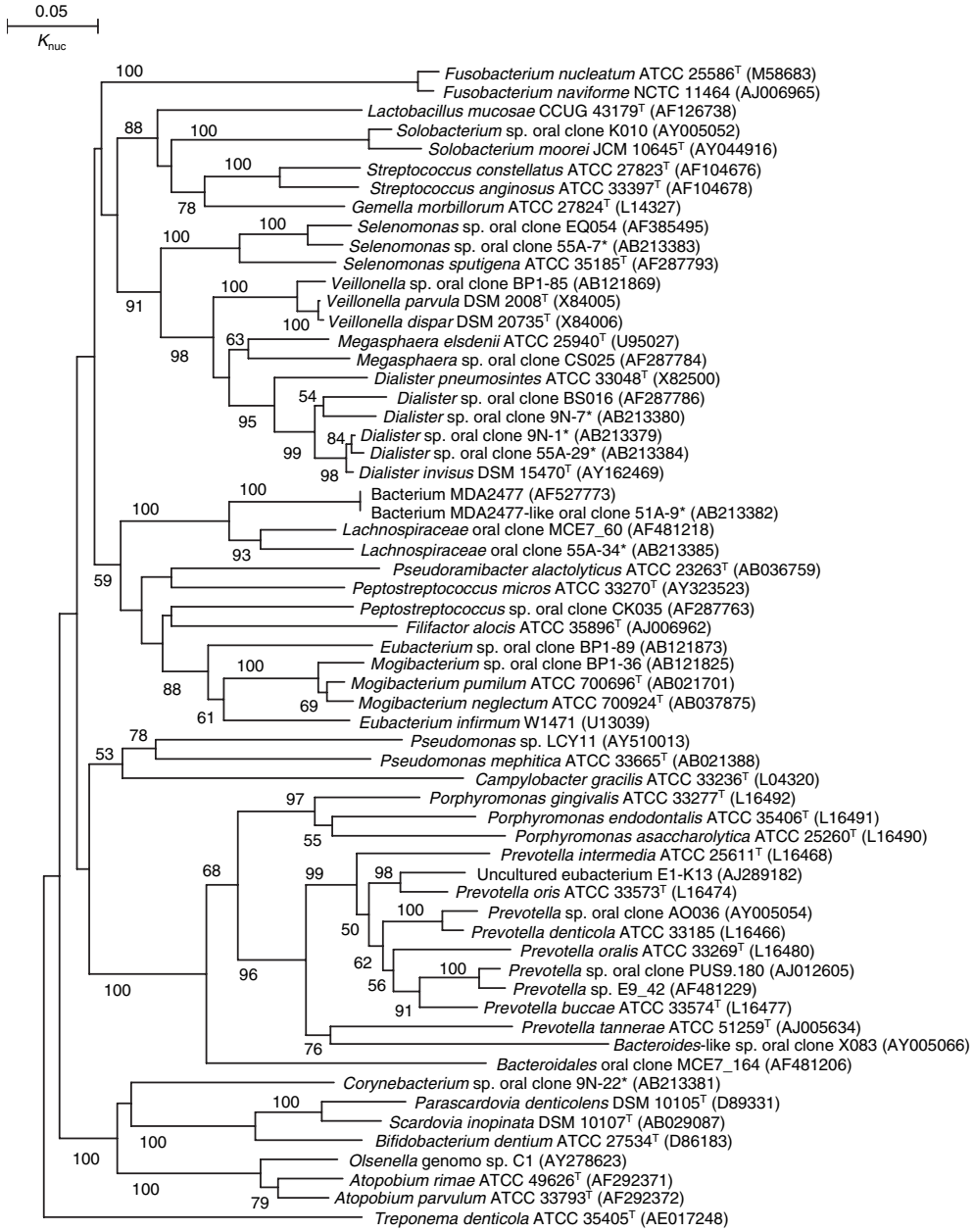

Fig. 2. Phylogenetic tree showing clones detected in the four libraries and related taxa. The tree was constructed by the neighbor-joining method based on 16S rRNA gene sequence comparisons (530 aligned bases). The scale bar represents 0.05 substitutions per nucleotide position. The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings. Accession numbers for 16S rRNA gene sequences are given for each strain. *****Identified in this study.

because individual bands can be cut out of the gel and sequenced to identify individual taxa (40), it is difficult to create a database from the band profiles obtained by DGGE analysis as compared with the T-RF profiles obtained by T-RFLP analysis. Even then, previous PCR-DGGE findings agree with the present T-RFLP analysis.

One might surmise that the differences observed in the present study were related to the fact that samples were taken from different sites, i.e. root canals in asymptomatic teeth and periradicular tissues in symptomatic teeth. In reality, samples from symptomatic teeth were taken by aspiration of purulent exudate from the swollen mucosa to increase the probability of detecting bacteria directly involved with the disease process. If one takes into account that bacteria detected in abscesses typically had their origin in the intraradicular infection, sampling the purulent exsudate will select out the species capable of invading the periradicular tissues. Had the samples been taken from the canals in abscessed cases, bacterial diversity could have been even greater due to the risks of also detecting possible bystanders. While bacteria colonizing the necrotic root canal face negligible host defenses, bacteria invading the periradicular tissues are under direct attack by host defense mechanisms. Therefore, it is noteworthy that symptomatic samples still showed greater bacterial diversity than did asymptomatic samples.

The T-RFLP technique is not only useful for bacterial community analysis;

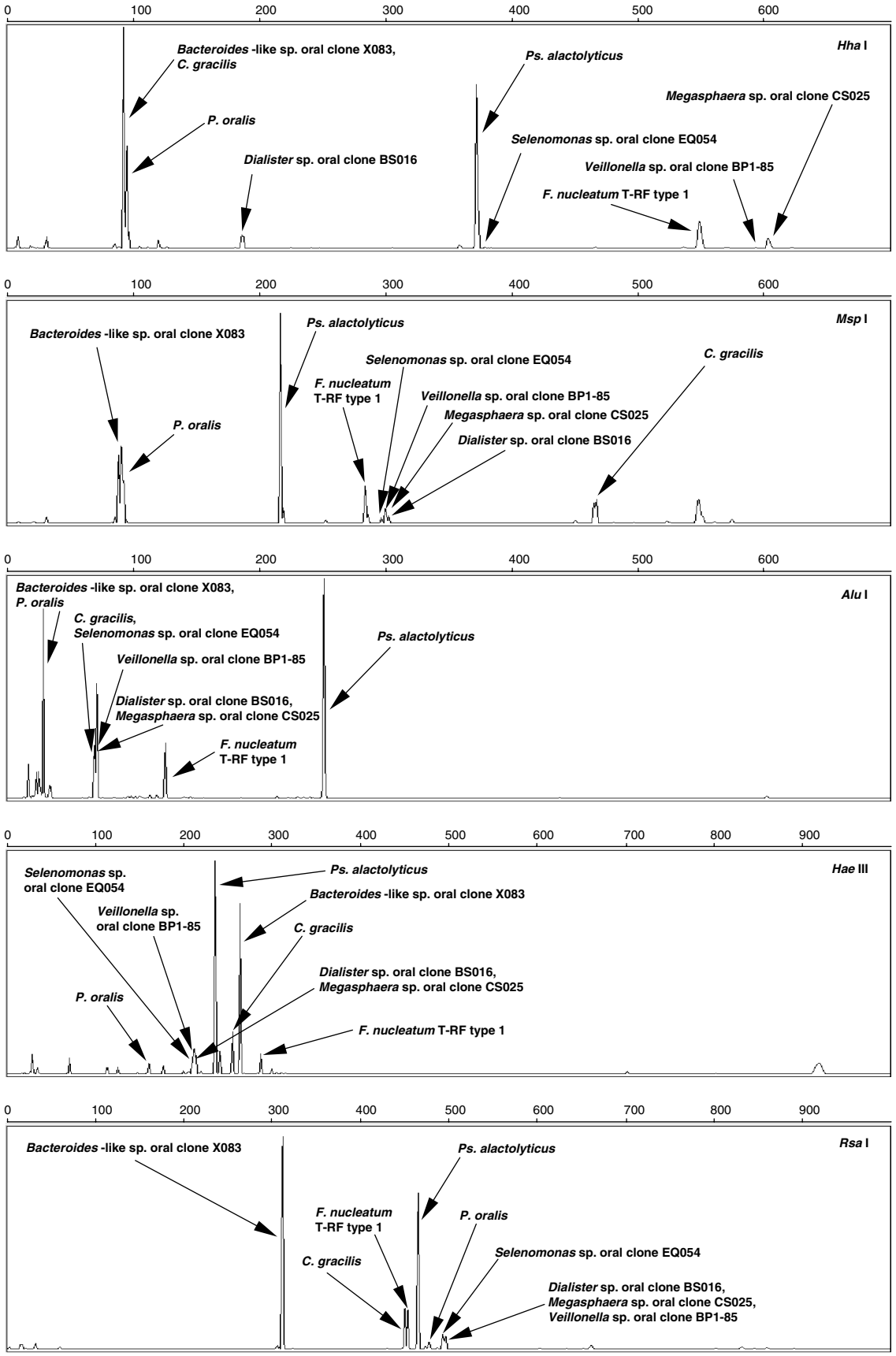

Fig. 3. T-RFLP patterns of 16S rRNA genes from asymptomatic sample 13N generated after digestion with five different restriction enzymes (*Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I). 16S rRNA genes were amplified with universal primers 27F and 1492R. Almost all the T-RFs were presumed to be species or phylotypes detected by the 16S rRNA gene clone library analysis. *C.*, *Campylobacter*; *F.*, *Fusobacterium*; *P.*, *Prevotella*; *Ps.*, *Pseudoramibacter.*

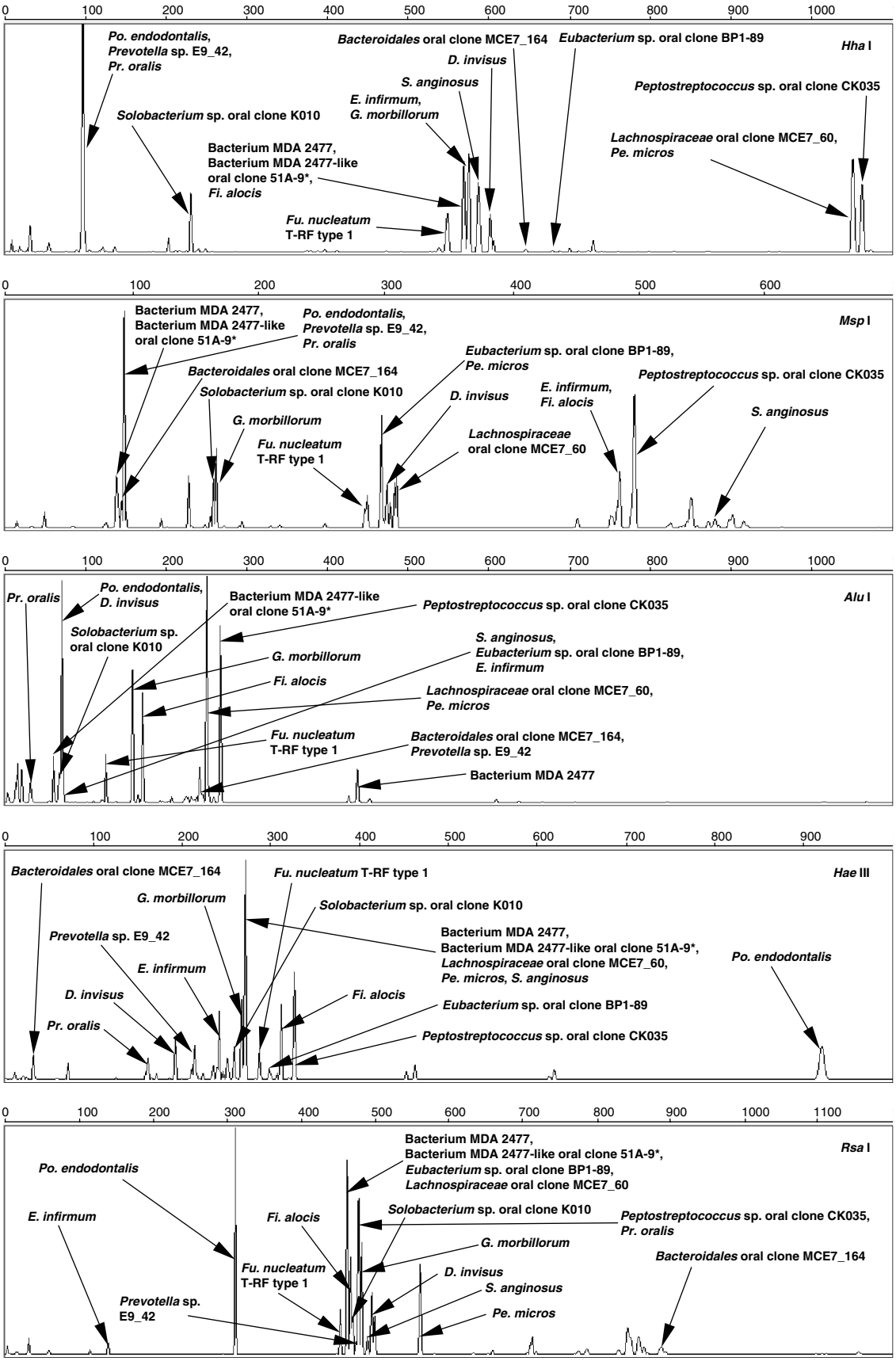

Fig. 4. Terminal restriction fragment length polymorphism patterns of 16S rRNA genes from symptomatic sample 51A generated after digestion with five different restriction enzymes (*Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I). 16S rRNA genes were amplified with universal primers 27F and 1492R. Almost all the terminal restriction fragments were presumed to be species or phylotypes detected by the 16S rRNA gene clone library analysis. *D.*, *Dialister*; *E.*, *Eubacterium*; *Fi.*, *Filifactor*; *Fu.*, *Fusobacterium*; *G.*, *Gemella*; *Pe.*, *Peptostreptococcus*; *Po.*, *Porphyromonas*; *Pr.*, *Prevotella*; *S.*, *Streptococcus*.

Table 3. Comparison of the microbiota associated with asymptomatic and symptomatic endodontic infections

Bacteria/T-RF type	Group	
	Asymptomatic ($n = 4$)	Symptomatic ($n = 7$)
Actinobacteria		
Atopobium rimae	2	1
Corynebacterium sp. oral clone 9N-22	1	0
Olsenella genomosp. C1	1	0
Scardovia inopinata	1	0
Bacteroidetes		
Bacteroidales oral clone MCE7_164/MCE3_262/MB4_G15	0	1
Bacteroides-like sp. oral clone X083/*Bacteroidales* oral clone MCE7_20	4	0
Porphyromonas endodontalis	1	1
Prevotella denticola/*Prevotella* sp. oral clone AO036	0	2
Prevotella intermedia	0	3
*Prevotella multisaccharivorax**	3	3
Prevotella oralis	3	3
Prevotella sp. E9_42/*Prevotella* sp. oral clone PUS9.180	0	3
Uncultured *Eubacterium* E1-K13	1	1
Firmicutes		
Dialister invisus/*Dialister* sp. GBA27	2	3
Dialister pneumosintes	0	3
Dialister sp. oral clone 55A-29	2	1
Dialister sp. oral clone 9N-1/9N-7	2	1
Dialister sp. oral clone BS016/MCE7_134	3	0
Eubacterium infirmum	2	2
Eubacterium sp. oral clone BP1-89	0	3
Filifactor alocis	2	3
Gemella morbillorum	0	1
Lachnospiraceae oral clone 55A-34	2	4
Lachnospiraceae oral clone MCE7_60	0	3
Lactobacillus mucosae	1	0
Megasphaera sp. oral clone CS025/BS073	3	2
Mogibacterium neglectum/*Mogibacterium diversum*/*Mogibacterium vescum*	0	1
Mogibacterium pumilum	3	3
Mogibacterium timidum	3	1
Mogibacterium sp. oral clone BP1-36	3	1
Peptostreptococcus micros/*Peptostreptococcus* sp. oral clone AJ062/BS044/FG014	2	4
Peptostreptococcus sp. oral clone CK035	2	2
Pseudoramibacter alactolyticus	3	1
Selenomonas sputigena	0	1
Selenomonas sp. oral clone 55A-7	0	2
Selenomonas sp. oral clone EQ054	1	0
Solobacterium sp. oral clone K010/*Solobacterium moorei*	1	3
Streptococcus anginosus	0	1
Streptococcus constellatus/*Streptococcus intermedius*	0	2
Veillonella sp. oral clone BP1-85/*Veillonella dispar*/*V. parvula*	3	2
Bacterium MDA2477	0	1
Bacterium MDA2477-like oral clone 51A-9	0	1
Fusobacteria		
Fusobacterium nucleatum T-RF type 1	2	6
Fusobacterium nucleatum T-RF type 2	0	5
Proteobacteria		
Campylobacter gracilis	1	1
Pseudomonas mephitica	0	1
Pseudomonas sp. LCY11	0	1
Spirochaetes		
Treponema denticola	1	1
Synergistes		
Synergistes oral clone BA121*	1	0

*Presumed on the basis of our database.
T-RF , terminal restriction fragment.

bacterial identity can also be inferred by T-RF lengths predicted from known 16S rRNA gene sequences. Although multiple species can show the same T-RF length after digestion with one enzyme, this shortcoming can be circumvented by analysis of digests with multiple restriction enzymes. Recently, we have used four restriction enzymes (*Hha*I, *Msp*I, *Alu*I, and *Hae*III) for T-RFLP analysis of the human colonic microbiota (18). Moreover, a phylogenetic assignment database for T-RFLP analysis of human colonic microbiota (PAD-HCM) was built to predict T-RFs at species level, including difficult-to-culture bacteria (18). In the present study, we used five restriction enzymes to improve the resolution of the method. The prediction of T-RFs was performed on the basis of five T-RFLP patterns with five different restriction enzymes using 16S rRNA gene clone library analysis data and a phylogenetic assignment database for T-RFLP analysis of the human oral microbiota

(M. Sakamoto, M. Umeda, I. Ishikawa, and Y. Benno, unpublished data).

Most of the named bacterial species detected herein have already been found in endodontic infections by culture-dependent and/or -independent approaches (1, 8, 9, 24, 32, 33), confirming their status as candidate endodontic pathogens. The most prevalent known cultivable species included *F. nucleatum, Dialister* species, *Prevotella* species, *Peptostreptococcus* species, *Mogibacterium* species, *F. alocis*, *Pseudoramibacter alactolyticus,* and *Eubacterium infirmum*. In addition, several uncultivated phylotypes were among the most prevalent taxa, with some of them being found in about one-half of the cases and others being exclusively detected in symptomatic infections. This raises the interesting possibility that they are previously unrecognized bacteria which may be involved with the pathogenesis of periradicular lesions.

In this study, two clones were closely related to *Fusobacterium naviforme* in the public databases. However, Conrads et al. (5) have presented convincing evidence showing that the 16S rRNA gene sequence of *F. naviforme* in the GenBank database is not derived from that species. Consequently, we treated these clones as *F. nucleatum*. The T-RF derived from *F. nucleatum* clone was treated as *F. nucleatum* T-RF type 1, and the T-RF derived from '*F. naviforme*-like clone' was treated as *F. nucleatum* T-RF type 2. It is difficult to differentiate the subspecies of *F. nucleatum* from each other on the basis of a partial sequence of the 16S rRNA gene (19). *F. nucleatum* T-RF type 1 was detected in eight of the 11 samples (73%) by T-RFLP analysis. Six of these samples were from abscesses. Other studies using culture-dependent or -independent approaches have also reported a high prevalence for this species in endodontic infections (8, 38). In addition, *F. nucleatum* T-RF type 2 was found in five symptomatic cases and in no asymptomatic case. These findings suggest that *Fusobacterium* species may be involved with the etiology of abscesses.

Dialister species/phylotypes were found in all samples obtained from asymptomatic and symptomatic endodontic infections. One of these, *D. invisus*/*Dialister* sp. GBA27, was found in three of the four samples by clone analysis and in five of the 11 samples by T-RFLP analysis. *D. invisus* is a newly named species (6), initially referred to as *Dialister* E1 and found by Munson et al. (19) in each of five samples from teeth with chronic periradicular lesions. Our findings indicate that *D. invisus* can be a common member of the endodontic microbiota not only in asymptomatic teeth but also in symptomatic ones and a pathogenetic role is suspected. Whereas *Dialister* sp. oral clone BS016/MCE7_134 was apparently associated with asymptomatic cases, another *Dialister* species, *D. pneumosintes*, was only found in symptomatic samples. A previous study has demonstrated a high prevalence of *D. pneumosintes* in abscessed teeth (24) and further studies should elaborate on the possible association of this species with symptoms.

In addition to *D. pneumosintes*, several other species/phylotypes were exclusively found in symptomatic infections (three cases or more). They included *F. nucleatum* T-RF type 2, *P. intermedia*, *Prevotella* sp. E9_42/*Prevotella* sp. oral clone PUS9.180, *Eubacterium* sp. oral clone BP1-89, and *Lachnospiraceae* oral clone MCE7_60. For many years, researchers have been trying to disclose associations of specific taxa with symptoms of endodontic origin (11, 37, 39). Data from most studies are rather conflicting and no single species has been definitely confirmed to be involved in the causation of symptoms (2, 8, 35). Even though our results suggest the association of some species or phylotypes with symptoms, further studies using a large sample size are warranted to confirm such an association and, more importantly, to infer causality.

Black-pigmented anaerobic bacteria have been suggested to be commonly associated with symptomatic endodontic infections (37, 39), but recent molecular data failed to show such an association (8, 35). *Prevotella* species were among the most prevalent bacteria identified in the present study, but only *P. intermedia* was suspected to be associated with symptoms. *Porphyromonas endodontalis* was detected in only two cases. In symptomatic sample 51A, 22.2% of the clones sequenced were found to be *P. endodontalis* (Table 2). A unique T-RF generated after digestion with *Hae*III (about 920 bp), which was presumed to represent *P. endodontalis*, was detected in the T-RFLP pattern of this sample (Fig. 4). The T-RF corresponding to *P. endodontalis* was not present in the other symptomatic samples (Table 3). In asymptomatic cases, the T-RF corresponding to *P. endodontalis* was only detected in sample 13N (Table 3 and Fig. 3), though *P. endodontalis* was not detected using 16S rRNA gene clone library analysis. Participation of these species in asymptomatic or symptomatic endodontic infections still remains to be clarified.

In conclusion, molecular analysis of the microbiota associated with symptomatic or asymptomatic endodontic infections demonstrated that the endodontic bacterial diversity is greater than previously described by culture methods and that about one-half of the microbiota is composed of uncultivated and as yet uncharacterized species. In addition, T-RFLP analysis was shown to be useful for assessment of diversity of the endodontic microbiota and rapid comparison of the community structure between different types of endodontic infections. T-RFLP data revealed that the structures of the microbiota in asymptomatic and symptomatic infections differ significantly. Although the subject population was small, the data reported herein bring important additional understanding to the etiology of asymptomatic and symptomatic endodontic infections. Based on knowledge of the bacterial structure and diversity in endodontic infections, future studies should focus on the bacterial profiles of a significant number of endodontic samples using molecular methods such as the reverse-capture checkerboard DNA–DNA hybridization or microarray technology.

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