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Bacteria are involved in the onset and progression of periodontitis. A promising molecular technique, denaturing gradient gel electrophoresis (DGGE), to study microbial population dynamics in the subgingival pocket is presented. Twenty-three samples were taken from the subgingival pockets of nine patients and six healthy family members. From four periodontitis patients, 12 samples were evaluated before, 1 day after and 3 months after treatment. Part of the 16S rRNA gene of all bacteria was amplified by PCR and separated by DGGE, creating banding patterns representative of the community structure. Shifts in composition and diversity of the microbial population could be determined semiquantitatively, and this showed that treatment resulted in a decrease in the diversity of the population. After 3 months a microbial population 33–47% different from the population before treatment had re-established. Intense bands representing *Exiguobacterium aurantiacum* were present in 13 out of 25 samples, indicating that this species may play a role in periodontal disease.

Key words: bacteria; denaturing gradient gel electrophoresis; periodontitis

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Periodontal diseases refer to inflammatory processes leading to destruction of the supporting tissues of the teeth and are the result of a mixed microbial infection (12). According to the specific plaque hypothesis, the inflammatory response in subgingival tissues is caused by a limited number of bacterial species present in subgingival plaque (19). Several species are putative periodontopathogens based on frequency of isolation in lesion sites. Among these are *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis, Prevotella intermedia*, *Bacteroides forsythus*, *Treponema denticola*, *Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Eikenella corrodens* and *Selenomonas sputigena* (36). Efforts have been made to determine the prevalence of these species in periodontal lesions (2, 4, 8–10, 28, 29). However, no single bacterial species could be identified that is uniquely involved in periodontitis. Using 40 species-specific DNA-DNA hybridization probes to detect oral bacteria, it was shown that subgingival plaque contains bacterial species in different complexes (36). Socransky et al. (36) observed five major complexes using cluster analysis. The complex consisting of *B. forsythus*, *P. gingivalis* and *T. denticola* showed the strongest relationship with clinical features. Nevertheless, the probes used comprised a limited part of the subgingival population and only partially took into account the diversity of the subgingival plaque, as over 500 species can be recovered from the oral cavity (16, 27). Moreover, molecular techniques, for example cloning and se-

quencing, revealed the presence of yet uncultivable or difficult to cultivate bacterial species such as *Eubacterium* species, *Coriobacteriaceae* and treponemes in subgingival plaque (1, 5, 6, 37). However, information about the contribution of these species to the initiation and progression of periodontitis is limited (6).

Here we present one of the first applications of denaturing gradient gel electrophoresis (DGGE) in periodontal research to study the mixed nature of periodontal disease. This technique takes into account the presence of unidentified and hard to cultivate species present in subgingival plaque. DGGE was introduced in microbial ecology a decade ago by Muyzer et al. (23) and has proved to be useful in detecting and identifying mixed microbial infections from the ocular environment (33). DGGE facilitates profiling of mixed microbial populations by the separation of amplified 16S rRNA gene fragments of the same length by differences in GC content (24). Separation of 16S rRNA gene fragments on a polyacrylamide gel containing a linearly increasing gradient of denaturant (urea and formamide) results in a pattern of bands that corresponds to a number of predominant members of the microbial community (25). Further population information can be obtained by determining the sequence of eluted DNA from bands excised from the gel (21).

In the present study, the DGGE technique was used for the analysis of the microbial population obtained from subgingival plaque samples from patients with advanced forms of inflammatory periodontal disease. A number of those study subjects were sampled at different times in order to identify the predominant species before and after treatment, and to study shifts in the microbial population.

Materials and methods

Study subjects

A total of 15 subjects were enrolled in the study. The minimum number of teeth the patients possessed was 20. Nine out of the 15 subjects were selected from adult patients with previously untreated advanced periodontitis referred by general practitioners to the Department of Dentistry, University of Groningen, the Netherlands for periodontal therapy. The remaining six subjects were periodontally healthy family members (siblings or children) or spouses of these nine patients. Individuals with any of the following conditions were excluded from the study: use of systemic antibiotics in the previous 6 months; requirement for antibiotic premedication; allergy to metronidazole; blood disorders; liver and kidney disorders; central nervous system disorders; intake of any medications; pregnancy or lactation. All periodontitis patients received oral hygiene instruction and full mouth supra- and subgingival scaling for 4 h. In patients not responding to scaling and root planing, systemic metronidazole was administered, 250 mg 3 times a day for 1 week. Where necessary, periodontal surgery was performed. Following active treatment, the patients were scheduled to receive maintenance therapy every 3 months.

Clinical and radiographic assessments

Probing pocket depth and probing attachment level were measured at six sites per tooth using a periodontal probe calibrated in millimeters. Plaque index (34), gingival index (18) and bleeding on probing were also assessed at six sites per tooth. The X-ray status of each patient was recorded. The diagnosis 'advanced periodontitis' was made if (a) the patients showed periodontal bone loss around at least two single-rooted teeth per quadrant reaching the middle third of the roots, and (b) these two teeth showed sites with pocket depths of $\geq$6 mm and loss of attachment of $\geq$7 mm.

Sample processing

Subgingival plaque samples were taken from all 15 subjects including healthy relatives. In the nine patients, the deepest site of each quadrant was identified (32). Four sites showed probing pocket depths of $\geq$6 mm and loss of probing attachment of $\geq$7 mm. Prior to sampling, supragingival plaque was removed using hand instruments and a sterile cotton roll was inserted to prevent saliva from disturbing sampling. Subgingival plaque samples were taken at the selected sites by inserting sterile endodontic absorbing points (xx fine; Roeko, Ulm, Germany) to the bottom of the periodontal pocket for 20 s. In the six family members, subgingival plaque samples were taken at the mesial site of each first molar. The four paper points of each subject were removed, stored on ice and transported to the laboratory. In order to obtain some insight into the potential usefulness of the molecular monitoring method applied, four out of the nine periodontitis patients were sampled at three different times: at baseline, 1 day after and 3 months after treatment. Subgingival plaque samples were again taken at the four preselected sites and pooled. Hence, 23 bacterial samples (17 from patients and six from family members) were available for further processing.

DNA extraction

DNA was extracted from the paper points using a modification of the phenol/chloroform extraction method described by Stephen et al. (38). Briefly, a mixture of glass beads, paper points and 500 µl sterile milli-Q water was vortexed twice for 30 s and incubated overnight at 4°C. The paper points were removed and 200 µl phenol was added, after which the samples were mechanically disrupted in a Mini-BeadBeater-8TM (BioSpec Products Inc., Bartlesville, OK) with zirconium beads (0.11 mm) twice for 30 s. After disruption, 200 µl chloroform/iso-amylalcohol (24 : 1 volume/volume) was added and samples were centrifuged for 5 min at 14 000 *g*. A second phenol/chloroform extraction was performed and, after collecting the supernatant, DNA was precipitated with 500 µl isopropanol after standing at −20°C for 2 h. After centrifugation for 15 min at 14 000 *g*, the supernatant was discarded and the pellet washed in 100 µl 70% alcohol. After further centrifugation for 15 min at 14 000 *g*, the supernatant was removed. The pellet was dissolved in 100 µl sterile milli-Q water and stored at −20°C.

PCR amplification

PCR was performed with a Tgradient thermocycler (Whatman Biometra, Göttingen, Germany). For amplification, the following general bacterial primers were used (26): F968-GC (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3′), containing a GC clamp which makes it suitable for DGGE, and R-1401 (5′ CGG TGT GTA CAA GAC CC 3′). The mixture contained 5.0 µl reaction buffer (100 mM Tris-HCl, pH

8.3, 500 mM KCl, and 15 mM $MgCl_2$), 200 µM of each dNTP, 35.5 µl of water, 200 nmol of each primer, 2.5 U Hotstart Taq polymerase (TaKaRa SHUZO Ltd, Otsu, Japan) and 1.0 µl of template DNA. The temperature profile included an additional denaturing step of 10 min at 96°C, followed by 34 cycles of a denaturing step at 96°C for 30 s, a primer annealing step at 56°C for 1 min, an extension step at 72°C for 1 min and a final extension step of 72°C for 5 min. PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 µg/ml ethidium bromide.

DGGE

DGGE of PCR products generated with the F968GC/R1401 primer set was performed as described by Muyzer et al. (23), with the use of a PhorU system (Ingeny, Goes, the Netherlands). PCR products were loaded on an 8% (w/v) polyacrylamide gel in 0.5 × TAE (1 × TAE is 0.04 M Tris base, 0.02 M acetic acid and 1.0 mM EDTA, pH 7.5). The denaturing gradient consisted of 30 to 70% denaturant (100% denaturant equals 7 M Urea and 40% formamide). Gels were poured using a gradient mixer. A 10-ml stacking gel without denaturant was added on top. Electrophoresis was performed for 16 h at 120 V and 60°C. Gels were stained with silver nitrate (3).

Analysis of the DGGE profiles

DGGE profiles were analyzed as described by Gillan et al. (11). The bands per lane are counted as a measure of the microbial diversity, and a coefficient of similarity (Cs) is calculated to compare the composition of two populations by the following formula:

$$Cs = 2j/(a + b) \times 100,$$

in which j is the number of common DGGE bands between two lanes, a is the number of bands in lane a, and b is the number of bands in lane b. A Cs value of 100% corresponds to two identical DGGE profiles.

Excision and sequence analysis of products

Bands of basic interest (based on intensity, dynamics in sequential samples, and similar migration to reference pathogens) were excised from the gel and put into cups containing 100 µl sterile milli-Q water and glass beads. After vortexing twice for 30 s, the samples were incubated overnight at 4°C. The cups were vortexed to enrich and purify the bands. A volume of 10 µl was used as a template in a universal DGGE-PCR reaction with the F986GC and R1401 primer set. Products were analyzed on DGGE gels, and bands at the same position on the gel were excised and processed again. After another round of PCR, products were checked on an agarose gel and purified using a multiscreen FB-96 plate (Millipore B.V., Etten-Leur, the Netherlands). Dideoxy-DNA sequence reactions were performed using a Dynamic ET-Terminator kit (Amersham Biosciences, Little Chalfort, UK). A MegaBACE 1000 (Amersham Biosciences) was used for sequence determination.

Phylogenetic analysis

All sequences were aligned against a representative selection of prokaryote 16S rRNA sequences extracted from the Ribosomal Database Project (RDP) database (21). Distance matrix analyses were performed using a Jukes-Cantor correction (30) and tree construction was carried out by neighbor-joining using the ARB software package (20). The phylogenetic associations of all representative sequences were determined from 100 bootstrapped replicates using a maximum-likelihood algorithm. Sequences with ≥99% identity were considered as a single phylotype.

Results

DGGE profiles

A total of 23 samples from nine patients and six relatives were analyzed using DGGE banding patterns. DGGE profiles from the 15 subjects contained intense and faint bands. Although profiles generally differed greatly amongst subjects, several intense bands were observed at the same position in almost every profile. Another interesting finding was the remarkable similarity in banding pattern and number of bands between one patient and their spouse. The similarity was less pronounced between another patient and his brother. Nevertheless, after the patient had been treated, the profile of his brother had also changed. Patients 1, 2, 3 (Fig. 1) and 4 (profile not shown) were analyzed in more detail. DGGE fingerprints of the microbial population at baseline (BL), 1 day after treatment (P00) and 3 months after treatment (P03) showed that profiles from the microbial population were highly diverse and varied in time and between individuals. Figure 1 shows two marker lanes containing PCR products of *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg) and *P. intermedia* (Pi) and bands at corresponding positions in the gel, for ex-

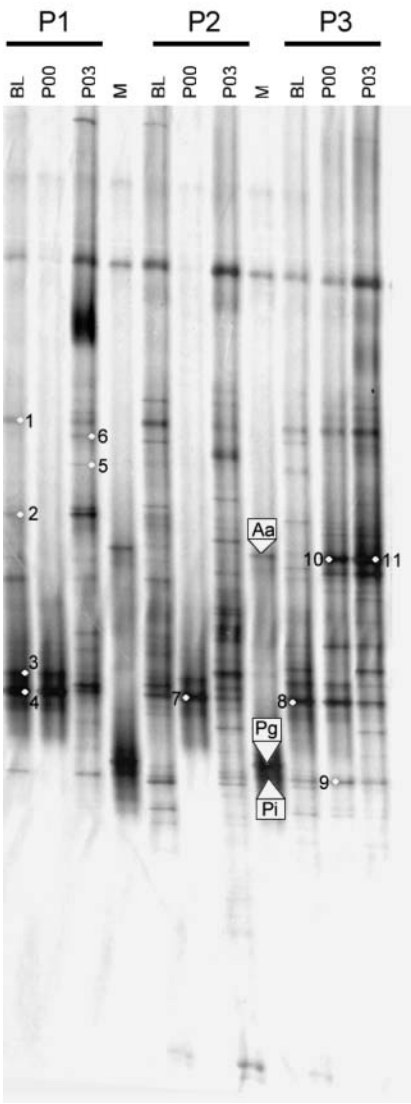

Fig. 1. PCR-DGGE profiles representing the bacterial diversity in patients 1, 2 and 3 (P1, P2 and P3) generated from samples taken before treatment (BL), 1 day after treatment (P00) and 3 months after treatment (P03). P1 did not receive antibiotic treatment. P2 and P3 were treated with metronidazole. Marker lanes (M) contain PCR products of *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg) and *P. intermedia* (Pi). The numbered bands are described in Table 3

Table 1. Total number of bands in DGGE analysis

Sample	Total number of bands			
	Patient			
	1	2	3	4
BL	23	39	35	28
P00	9	8	27	17
P03	37	47	39	24

BL, baseline; P00, 1 day after treatment; P03, 3 months after treatment.

Table 2. Coefficient of similarity (Cs) amongst lanes BL, P00 and P03 in DGGE analysis

	Coefficient of similarity (Cs) (%)			
	Patient			
	1	2	3	4
BL-P00	44	18	47	51
BL-P03	33	39	47	41
P00-P03	15	16	56	52

ample bands 9, 10 and 11. Bands migrating at the same position as bands 4, 7 and 8 were present in each lane except in lane P03 of patient 1 and lane BL of patient 2.

Treatment of the patients resulted in a decrease in the number of bands on P00 (see Table 1). The decrease was most pronounced for patients 1 and 2, from 23 to 9 bands and from 39 to 8 bands, respectively. After 3 months, the number of bands increased, for example to 47 for patient 2 and to 24 for patient 4. These changes in the number of bands suggest that the microbial population was suppressed by treatment and that after 3 months re-colonization of the site occurred.

DGGE profiles were also compared using the similarity coefficient (Cs), which takes into account the total number of bands per lane and the migration position of the bands, i.e. the number of bands two lanes have in common. Cs values therefore provide information about the composition of DGGE profiles and shifts in the microbial population. As shown in Table 2, DGGE profiles from all four patients changed at P00 compared with BL. The change in banding patterns was most striking for patients 1 and 2, as the Cs values were low: 44 and 18, respectively. This observation was consistent with the greatest reduction in number of bands for these two patients. After 3 months, DGGE profiles showed that, although there was an increase in the number of bands, a partially different microflora had colonized the subgingival pocket, as the similarities between BL and P03 were less than 100% in all four patients. Although the patterns were different,

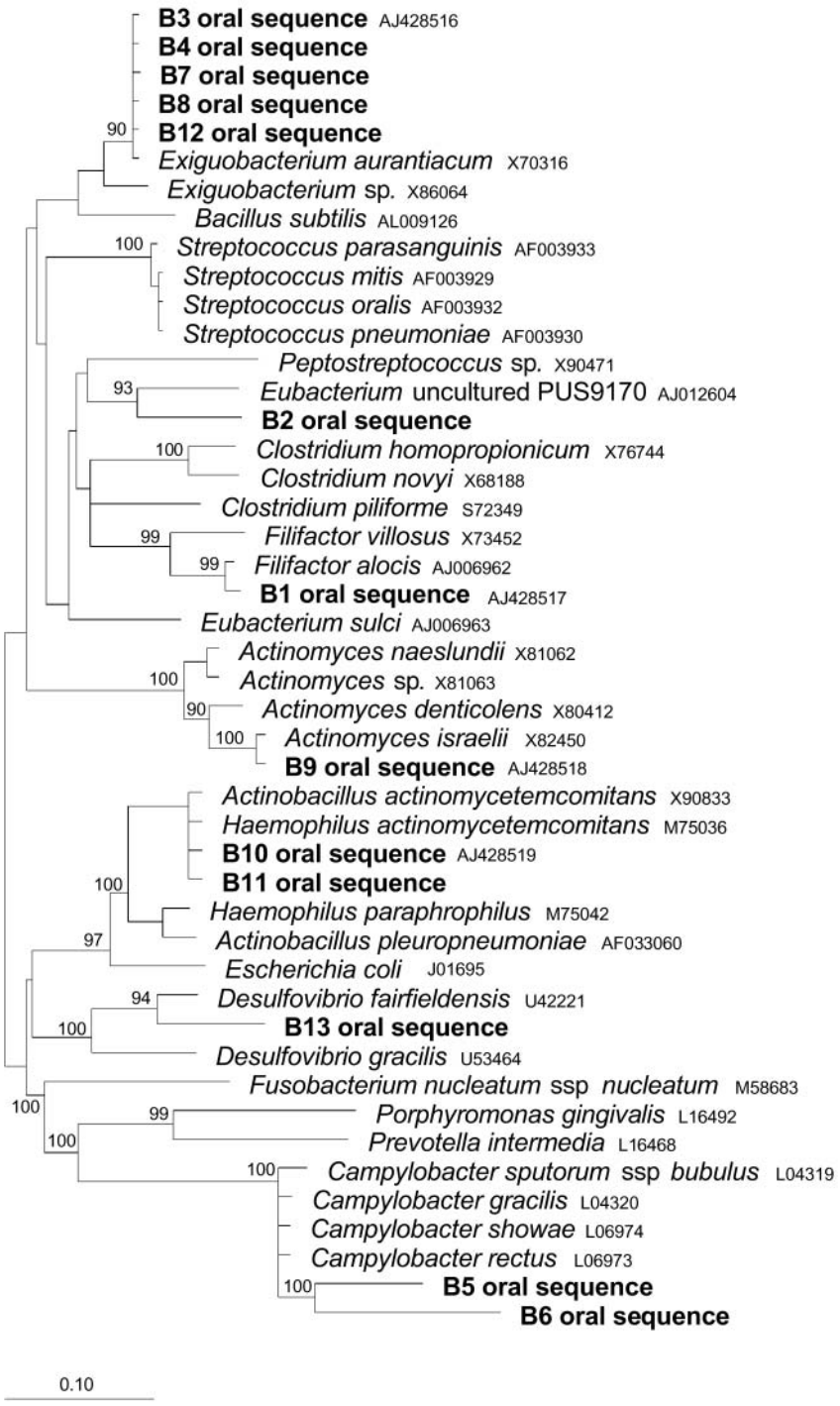

Fig. 2. Neighbor-joining tree based on partial 16S rDNA sequences from the clones and sequences derived from the RDP. This tree was constructed using 379 homologous sequence positions (1003–1381 *Escherichia coli* numbering). The numbers at the nodes of the tree indicate bootstrap values for each node out of 100 bootstrap resamplings (values below 90 are not shown). B1 to B13 oral sequences refer to bands 1–13 in Table 3 and Fig. 1. The scale bar corresponds to 0.10 substitutions per nucleotide position

some bands disappeared after treatment and reappeared after 3 months.

To identify species associated with the observed changes in the microbial population, the most intense bands from the profiles of patient 1 were excised and sequenced. A phylogenetic tree was constructed to identify the closest relative of each sequence (Fig. 2). The predominant bands of the population before treatment comprised bands 1–4. Analysis of the sequence of the excised DNA of band 1 showed that its closest relative (96% sequence similarity; Table 3) was *Filifactor alocis*, formerly known as *Fusobacterium alocis*, which has previously been isolated from gingival sulci of patients with gingivitis or periodontitis (13). Sequence 2 was related (86% sequence similarity) to an uncultured *Eubacterium* sp. isolate from dentoalveolar abscesses (39). Sequences of bands 3 and 4 were both related (98% sequence similarity) to *Exiguobacterium aurantiacum*, a motile, facultatively anaerobic coryneform bacterium with fermentative carbohydrate metabolism. This species has occasionally been isolated from clinical sources such as wounds and cerebrospinal fluid (6) but never from periodontal lesions. These relatively intense bands were also observed at corresponding positions after DGGE analysis of samples from all patients and three relatives. Therefore, bands 7, 8 (Fig. 1) and 12 (not shown) were excised and their sequences showed them to be related to *E. aurantiacum* (Fig. 2). The sequences of bands 3, 4, 7, 8 and 12 showed more than 99% sequence identity. Sequences 5 and 6 were contaminated with other sequences, but they were probably related to *C. rectus,* which is a suspected periodontopathogen (35). Despite the poor quality of sequences 5 and 6, identification of the bands as *C. rectus* was possible as the first 120 bases from the highly variable V6 region of the 16S rDNA gene were used for analysis. Because they appeared at the same position in the gel as *A. actinomycetemcomitans* or *P. intermedia* in the marker lane (M), bands 9, 10, 11 and 13 were excised and identified. The sequences of bands 10 and 11 positively confirmed this possibility by showing more than 99% sequence similarity with *A. actinomycetemcomitans.* However, the sequences of the DNA in bands 9 and 13 were identified as closely related to *Actinomyces israelii and Desulfovibrio fairfieldensis*, respectively.

Nucleotide sequence accession numbers

The sequences of the oral rDNA bands obtained from patients 1 and 3 (four sequences, 379–385 bp, indicated by an asterisk in Table 3) have been deposited in the GenBank database under accession numbers AJ428516, AJ428517, AJ428518 and AJ428519.

Discussion

Elucidation of whether periodontal disease is the result of plaque overgrowth or is a specific, albeit chronic infection is hindered by the lack of experiments in which the transition from gingivitis to periodontitis is documented. The trigger for this conversion is unknown. The formation, colonization and progression of the pocket represent an evolving process in which the host immune system, bacteria and their virulence factors play a delicate game of 'give and take'. As a result of changes in the environment, for example from aerobic to anaerobic conditions, the microbial population will shift from mainly *Actinomyces and Streptococcus* species towards an anaerobic gram-negative microflora (14, 15). Treatment is aimed at suppressing or eliminating the characteristic subgingival microflora. This study has shown that treatment does not result in the complete elimination of bacteria, although DGGE analysis and sequencing showed that the microbial populations changed upon treatment (Fig. 1). Predominant pathogenic species in the population before treatment were sequence 1 (*F. alocis*) and sequence 2 (uncultured *Eubacterium* sp. PUS 9.170). These sequences have been recovered from subgingival pockets before and could not be detected 1 day after treatment, as shown in Fig. 1, lane P00. Nevertheless, bands at corresponding positions in the gel reappeared after 3 months, indicating that these pathogenic species had re-established in the population.

Sequences 3 and 4 showed 99% sequence similarity and probably originated from one organism. Sequence heterogeneities in the 16S rDNA attributable to multiple gene copies may have resulted in different melting properties in DGGE. This has been described before for bifidobacterial species (31). Bands 3 and 4 were closely related to *E. aurantiacum* and were not eliminated from the population 1 day after treatment but were suppressed or eliminated after 3 months. This may have been a result of the presence of dead cells before treatment (P00) that were detected by PCR, or the microbial population after 3 months may have outcompeted this species. *E. aurantiacum* belongs to the *Eubacterium* group and the presence of very intense, dominant bands (bands 7, 8 and 12) in 13 of the 25 lanes is in agreement with previous reports emphasizing the possible role and importance of the *Eubacterium* group in periodontal disease (6, 37). Although the role of bacteria belonging to this group in periodontal disease is unclear, they are abundant in oral infections, but rarely found at healthy sites (22).

Table 3. Closest relative as determined by comparative sequence analysis, percentage similarity with this relative, length of the sequence, and accession number of each band identified in Fig. 1. Bands are indicated in Fig. 2 as B1-B13 oral sequence

Closest relative	Bands	% similarity	Length of sequence	Sequence accession number
Filifactor alocis	1*	96	385	AJ428517
Uncultured *Eubacterium* pus 9.170	2	86		–
Exiguobacterium aurantiacum	3*, 4, 7, 8, 12	98	379	AJ428516
Campylobacter rectus	5	46		–
Campylobacter rectus	6	55		–
Actinomyces israelii	9*	100	381	AJ428518
Actinobacillus actinomycetemcomitans	10*, 11	100	379	AJ428519
Desulfovibrio fairfieldensis	13	90		–

*These bands have been submitted to GenBank.

The predominant bands 5 and 6 were related to the putative periodontopathogen *Campylobacter* and appeared in the microbial population after 3 months. *Campylobacter* is a microaerophilic species, suggesting that an intermediate environment was created. Whether this situation will shift towards an anaerobic environment suitable for anaerobic gram-negative pathogens remains unclear.

The identification of bands 9 and 12 as related to *A. israelii* and *D. fairfieldensis* and not *P. intermedia* demonstrates the difficulty in identifying species based on their position in the gel. As shown in this study, the subgingival microbial community is prone to shifts in composition and diversity. Predominant bands of subgingival microflora were not related to the bacteria that are commonly associated with periodontitis (36). The detection of *E. aurantiacum* and *D. fairfieldensis* should focus our attention on previously undetected oral species such as *E. aurantiacum and* sulfate-reducing bacteria that have only recently been described as potential indicators for periodontal disease (17).

Attempts to determine the cause of periodontitis from the bacteria present in random samples of subgingival plaque does not take into account the dynamic strength and diversity of the microbial population. As shown in this study, DGGE offers a great opportunity to study shifts in the microbial composition at a population level, including identification of shifting species. Further research should be aimed at monitoring the microbial succession of the oral biofilm, in terms of both species diversity and activity. Because of the relationship that exists between rRNA and activity of cells, RT-PCR of rRNA will allow identification of the metabolic activity of the bacterial species present in the biofilm and thereby improve our knowledge of the species responsible for destructive activity.

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