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Quantification and characterization of *Synergistes* in endodontic infections

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Introduction: Bacterial species belonging to the poorly characterized division *Synergistes* have recently been reported in endodontic infections, and therefore may be part of the etiology of periradicular diseases. The objective of this study was to characterize and quantify the predominant *Synergistes* phylotypes in infected root canals.
Methods: We analyzed 32 necrotic teeth, each from a different patient, with radiographic evidence of apical periodontitis and with primary endodontic infections.
Results: Using real-time quantitative polymerase chain reaction based on *Synergistes*-specific primers, seven of the 32 cases were found to be positive. Comparative sequence analysis showed that each of the seven samples was infected by one numerically dominant phylotype. Diversity among phylotypes was such that they could be grouped into three major evolutionary branches within the *Synergistes* division. The size of the total *Synergistes* population ranged from 4.5×10^4 to 1.5×10^6 16S rRNA gene copies, and the median proportion accounted for 0.79% of the total bacterial community. For comparison, we also quantified such recognized endodontic pathogens as *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Treponema*. The first two species were found in five and nine cases, respectively, with a median proportion below 0.01%, while *Treponema* was found in 18 cases with a median proportion of 1.48%.
Conclusion: Thus, the prevalence and quantity of *Synergistes* was clearly within the range of the other analyzed pathogens, suggesting their clinical relevance in endodontic infections. Furthermore, the diversity of *Synergistes* found at the diseased sites designates infected root canals as an important human ecosystem providing several unique micro-niches for this novel group of bacteria.

Key words: endodontic infection; quantification of endodontopathogens; real-time quantitative polymerase chain reaction; *Synergistes*

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The bacterial division *Synergistes* constitutes a ubiquitous and diverse, yet largely uncharacterized, bacterial group represented by only a few pure culture isolates (5). Primarily known from environmental habitats, the first *Synergistes* isolates from human peritoneal fluid and soft tissue infection have recently been reported and described by our group (7). Furthermore, polymerase chain reaction (PCR) -based detection of 16S ribosomal RNA (rRNA) gene types in periodontal pockets and caries lesions has contributed to an emerging view of *Synergistes* as putative human pathogens and suggests that the oral cavity is a major reservoir for medically important phylotypes (7, 8, 12). However, oral *Synergistes* have previously been found either by chance in molecular inventories generated with universal 16S rRNA gene-targeting primers (12, 14), or through combination with nested PCR aimed at detecting a narrow range of only a few selected phylotypes (15, 17). For a more systematic exploration of human-associated *Synergistes*, we have recently designed a primer pair that covers a broad range of phylogenetically diverse *Synergistes* 16S rRNA gene types (7). Detection of the target group in the oral cavity was possible without the need of a nested PCR approach, making this primer system potentially suitable for application in real-time quantitative PCR (RTQ-PCR). Since *Synergistes* have recently been discovered in endodontic infections (17, 18), the aim of our study was the quantification and characterization of the predominant phylotypes in infected root canals containing

necrotic pulp tissue. This polymicrobial disease is of particular interest for two reasons: because it starts through infection of a tooth's root canal, a site normally devoid of microbes in a healthy state, and because only a limited number of bacterial species (relative to the total oral microflora) have been implicated with this disease (18). For comparison, we also determined the amounts of *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Treponema* as representative endodontic pathogens, along with assessing the total bacterial load using a set of different primer systems with proven target specificity, as published earlier (2, 3, 9, 13).

Materials and methods

Microbial strains

Bacterial strains used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA): *Actinomyces odontolyticus* ATCC 17929^T, *Enterococcus faecalis* ATCC 29212^T, *Fusobacterium nucleatum* ATCC 25586^T, *P. intermedia* ATCC 25611^T, *Prevotella nigrescens* ATCC 33563^T, *P. gingivalis* ATCC 33277^T, and *Tannerella forsythia* ATCC 43037^T. The type strain *Treponema denticola* DSM 14222^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Sample collection

Thirty-two patients attending the Piracicaba Dental School for root canal treatment, who were otherwise healthy and who had not received antibiotic treatment during the previous 3 months, were selected for this study. The patients ranged in age from 19 to 63 years old. The selected teeth (one tooth per patient) were uniradicular, containing a single root canal, presented with necrotic pulp tissue, and showed radiographic evidence of apical periodontitis but an absence of periodontal disease. All teeth were asymptomatic (chronic primary endodontic infection). A detailed medical and dental history was obtained from each patient. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved the protocol for specimen collection used in this investigation and all the patients signed an informed consent to participate in the study. The teeth were isolated with a rubber dam. The crown and surrounding rubber dam were disinfected with 30% H_2O_2 (volume/volume) for 30 s followed by 2.5% NaOCl for an additional 30 s. Subsequently, 5% sodium thiosulfate was used to neutralize the disinfectants (22). An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile saline. Before the pulp chamber was entered, the access cavity was disinfected following the same protocol as that described above. The sterility was checked by taking a swab sample of the cavity surface and streaking it on blood agar plates, which were incubated aerobically and anaerobically. All subsequent procedures were performed aseptically. The pulp chamber was accessed with sterile burs refrigerated in saline. The samples were collected with four sterile paper points, which were consecutively placed into the canal to the total length calculated from the preoperative radiograph. Afterwards, the four paper points per root canal were pooled in a sterile tube containing 1 ml reduced transport fluid (20). The samples were transported on dry ice by an overnight delivery service to the Division of Oral Microbiology and Immunology (RWTH Aachen University Hospital, Germany) for subsequent molecular analysis.

Extraction of total genomic DNA

Before extraction of the DNA, the frozen endodontic samples were thawed and dispersed by spinning for 15 s. Microbial DNA from endodontic samples as well as DNA from pure cultures were extracted and purified with a Qiamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The DNA concentration [absorbance at 260 nm (A_{260})] and the purity (A_{260}/A_{280}) were calculated using a Gene Quant II photometer (Pharmacia Biotech, Cambridge, UK).

General conditions for RTQ-PCR

Amplification and detection of DNA by RTQ-PCR were performed on a Light Cycler 2.0 (Roche Applied Science, Penzberg, Germany) using LightCycler Fast-Start DNA Master^{plus} SYBR Green I in a total volume of 20 µl. Final reactions contained 100 nm of each primer, and 3 µl of template DNA (approximately 75 ng). The assay primers and the temperature profiles used for quantification of *Synergistes*, *P. intermedia*, *P. gingivalis*, *Treponema* and total bacteria are specified in Table 1. Data acquisition and subsequent analysis were performed using LIGHTCYCLER Software 3.5 (Roche Applied Science). All samples were run in duplicate. Melting curve analysis was performed to determine the melting point of the amplification products and to assess reaction specificity. To avoid any possible primer dimer interference, the temperature at which the fluorescence was read during each cycle was adjusted to a degree just below the melting point of the amplification product, as specified in Table 1.

The amount of initial target DNA was calculated by determining the crossing point, the cycle at which the fluorescence exceeded a threshold value significantly higher than the background fluorescence. Quantification was performed using the automated (default) algorithm, a strategy that calculates the crossing point as the first maximum of the second derivative of the amplification curve. The conversion of crossing points to initial gene target molecule numbers was based on dilution series of target DNA with defined target molecule amounts as described below. The quantification data determined in this study for all bacterial groups will be referred to as 16S rRNA gene target molecule numbers.

Standard preparation

For quantification of *Synergistes*, endodontic sample E12 was selected for the preparation of standard reactions. Amplification products generated using the primer pair Syn360F/Syn961R were cloned into a plasmid using the TOPO TA Cloning Kit (Invitrogen Corp., San Diego, CA), following the manufacturer's protocol. After reamplification with vector-specific primers (M13F: 5′-GTAAAACGACGGCCAG-3′, and M13R: 5′-CAGGAAACAGCTATGAC-3′, Invitrogen Corp.), the PCR products were purified using the Qiagen Purification Kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were subsequently quantified with the PicoGreen dsDNA quantification kit (Molecular Probes, Leiden, The Netherlands). Knowing the exact size of the amplicons (Table 1) and using the average molecular weight of a single DNA base pair, the measured DNA value could then be converted to target molecule numbers per microliter. Dilution series of these PCR products were then used as calibration standards to measure samples with an unknown content of *Synergistes*.

For all other bacterial groups, target DNA from pure cultures was amplified with the universal bacterial primers PF1 (5′-AGA GTTTGATCCTGGCTCAG-3′) and PR1 (5′-GGCTACCTTGTTACGACTT-3′) (4). The temperature profile was 94°C, 2 min; followed by 33 cycles of 94°C for 1 min,

Table 1. Primer description and thermal profiles for real-time quantitative (RTQ) PCR

Primer	Sequence (5′ → 3′)	Reference	Target DNA (size in bp)	Temperature profile
Syn360F	GGAATATTGGGCAATGGG	Horz et al. 2006 (7)	16S rDNA of *Synergistes* (595)	RTQ-PCR: 95°C, 10 min; 40 cycles: 95°C,10 s; touch down: 66–64°C (with a decrease of 0.2°C after each cycle), 7 s; 72°C, 25 s; Fluorescence read at: 85°C
Syn961R	GTTCTTCGGTTTGCATCG			
PF1	AGAGTTTGATCCTGGCTCAG	Edwards et al. 1989 (4)		RTQ-PCR (combined with PF1): 95°C, 10 min; 40 cycles: 95°C, 10 s; 56°C, 10 s; 72°C, 27 s; Fluorescence read at: 84°C
Pi	GTTGCGTGCACTCAAGTCCGCC	Conrads et al. 1999 (2)	16S rDNA of *Prevotella intermedia* (660)	
PF1	AGAGTTTGATCCTGGCTCAG	Edwards et al. 1989 (4)		RTQ-PCR (combined with PF1): 95°C, 10 min; 42 cycles: 95°C, 10 s; 59°C, 10 s; 72°C, 20 s; Fluorescence read at: 83°C
Bg-1	CAATACTCGTATGCCCGTTATTC	Conrads et al. 1996 (3)	16S rDNA of *Porphyromonas gingivalis* (478)	
SGTrep0093aS19	TCTCCTAGAGYGGCGGACT	Lepp et al. 2004 (9)	16S rDNA of *Treponema* (674)	RTQ-PCR: 95°C, 10 min; 40 cycles: 95°C, 10 s; 59°C, 7 s; 72°C, 28 s; Fluorescence read at: 78°C
SGTrep0767aA20	TCCTGTTTGCTCCCCGCACY			
EuF	TCCTACGGGAGGCAGCAGT	Nadkarni et al. 2002 (13)	16S rDNA of total bacteria (466)	RTQ-PCR: 95°C, 10 min; 40 cycles 95°C, 10 s; 60°C, 10 s; 72°C, 25 s; Fluorescence read at: 80°C
EuR	GGACTACCAGGGTATCTAATCCTGTT			

55°C for 1 min and 72°C for 1.5 min, with a final extension of 72°C for 10 min. The resulting amplicons were purified and quantified as described above, again enabling the conversion of the amount of DNA to target molecule numbers. For quantification of *Treponema*, DNA from *T. denticola* was used as the standard. For quantification of total bacteria a set of five representative endodontic pathogens (i.e. *A. odontolyticus*, *E. faecalis*, *F. nucleatum*, *P. nigrescens*, and *T. forsythia*) was selected.

The linear scope of detection ranged from 10^2 to 10^9 target molecules, with an amplification efficiency of 1.7 (error 0.06) for *Synergistes*, 1.83 (error 0.01) for *P. intermedia*, 1.97 (error 0.01) for *P. gingivalis*, and 1.78 (error 0.01) for *Treponema*. For total bacteria, we assumed a mean amplification efficiency of 1.95 (tested for the five representative species, coefficient of variation: 2%, error range from 0.03 to 0.07).

Sequencing and phylogenetic analysis of *Synergistes*

The preparation of plasmid DNA, PCR amplification of cloned inserts, and non-radioactive sequencing were carried out as described previously (21). Sequencing was performed bi-directionally using a Big Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and an automatic capillary DNA sequencer (API Prism 310; Applied Biosystems). This led to ambiguity-free sequences that were approximately 595 base pairs (bp) in length. The identities of the *Synergistes* 16S rRNA gene sequences were confirmed by searching the international sequence databases using the BLAST program (URL: http://www.ncbi.nlm.nih.gov/blast/). The 16S rRNA gene sequences were integrated within the ARB program package (developed by the Technical University, Munich, Germany) (10), and sequences were analyzed and edited using the program's alignment tools. Incorrectly aligned positions were aligned manually on the basis of conserved primary sequence and secondary structure. Separate treeing analysis of the 5′ and 3′ halves of the sequence types was performed to test for the possible presence of chimeric artifacts. This test did not provide evidence that any of the clones analyzed was chimeric. Final phylogenetic tree reconstruction was performed using the neighbor-joining approach (16) with the Felsenstein correction.

Nucleotide sequence accession numbers of *Synergistes*

16S rRNA gene sequences have been deposited in the European Molecular Biology Laboratory (EMBL), GenBank and the DNA Data Base of Japan (DDBJ) nucleotide sequence databases under accession numbers from DQ643958 to DQ643964, EF104597 and EF104598.

Statistical analysis

The Spearman correlation test was used to check the hypothesis of correlation among *Synergistes*, *P. intermedia*, *P. gingivalis*, *Treponema*, and total bacteria.

Results

Prevalence of *Synergistes* and known endodontic pathogens

To determine if members of the *Synergistes* division could be directly detected in infected root canals (i.e. without nested PCR) we analyzed DNA extracts retrieved from 32 endodontic samples. Using the newly designed 16S rRNA gene targeting primer set Syn360F and Syn961R with intended target specificity for *Synergistes*, we found seven cases (22%) to be positive (root canal samples E7, E12, E16, E22, E24, E25, and E30). One defined melting point $Tm = 89°C$) was observed using melting curve analysis and the presence of a single DNA band of correct fragment size (approximately 595 bp) was confirmed by agarose gel electrophoresis for all the samples that tested positive (data not shown). For comparison, the incidence of *P. intermedia*, *P. gingivalis*, and *Treponema* was also tested using specifically targeted 16S rRNA gene PCR systems. Five samples (16%) were positive for *P. intermedia*, nine samples (28%) were positive for *P. gingivalis*, and 18 samples (56%) were positive for *Treponema* (Table 2).

Table 2. Absolute and relative abundance of *Synergistes*, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Treponema* in comparison to the total bacterial load in infected root canals containing necrotic pulp tissue, obtained by real-time quantitative PCR based determination of 16S rRNA gene target molecules[1]

Root canal samples	*Synergistes*	*P. intermedia*	*P. gingivalis*	*Treponema*	Total bacteria
E1	–	–	3.0×10^3	–	2.5×10^6
E2	–	–	–	7.5×10^5	3.5×10^6
E3	–	–	–	–	6.0×10^6
E4	–	–	–	–	3.2×10^6
E5	–	1.8×10^2	4.5×10^2	1.4×10^7	4.5×10^7
E6	–	–	–	7.8×10^5	8.2×10^6
E7	4.5×10^4	–	–	1.6×10^6	7.8×10^7
E8	–	–	–	6.9×10^6	8.1×10^6
E9	–	–	–	–	7.1×10^6
E10	–	–	3.0×10^2	9.6×10^4	1.0×10^8
E11	–	7.8×10^2	–	–	2.8×10^7
E12	2.2×10^5	–	–	1.2×10^6	2.8×10^7
E13	–	–	–	–	2.7×10^8
E14	–	–	–	1.4×10^5	2.6×10^8
E15	–	–	–	–	2.6×10^8
E16	1.5×10^6	–	–	1.1×10^6	1.2×10^8
E17	–	–	–	3.0×10^5	1.2×10^9
E18	–	–	–	1.1×10^5	3.2×10^6
E19	–	–	–	1.0×10^5	1.1×10^7
E20	–	1.8×10^2	9.0×10^2	–	4.2×10^7
E21	–	–	–	–	4.5×10^7
E22	9.3×10^5	–	–	–	5.1×10^7
E23	–	1.7×10^2	–	–	3.0×10^8
E24	3.6×10^5	–	–	3.9×10^5	6.0×10^8
E25	5.7×10^4	2.0×10^2	1.8×10^3	9.0×10^4	2.1×10^8
E26	–	–	9.6×10^3	2.9×10^7	1.0×10^8
E27	–	–	–	–	8.7×10^6
E28	–	–	7.8×10^3	9.0×10^3	3.7×10^7
E29	–	–	4.5×10^4	7.2×10^5	1.5×10^7
E30	2.6×10^5	–	3.6×10^3	2.9×10^4	8.3×10^6
E31	–	–	–	–	1.4×10^8
E32	–	–	–	–	6.2×10^8
Median[2]	2.6×10^5	1.8×10^2	3.0×10^3	5.6×10^5	4.4×10^7
Median proportion compared to total bacteria (%)[2]	0.79	<0.01	<0.01	1.48	

[1]Data are means of duplicate runs. The coefficient of variation was less then 10% between replicates.
[2]Median and median proportion were calculated from the positive cases.

Quantity of *Synergistes* and other bacteria in infected root canals

Total numbers of *Synergistes*, *P. intermedia*, *P. gingivalis*, and *Treponema*, as well as the entire bacterial load of infected root canals, were determined by RTQ-PCR. Among the 32 endodontic samples, the total bacterial load differed considerably and ranged from 2.5×10^6 to 1.2×10^9 16S rRNA gene target molecules, with a median of 4.4×10^7 (Table 2). The total load of *Synergistes* ranged from 4.5×10^4 to 1.5×10^6 16S rRNA gene target molecules with a median of 2.6×10^5. In contrast, the total amounts of *P. intermedia* and *P. gingivalis* were lower, ranging from 1.7×10^2 to 7.8×10^2 and 3.0×10^2 to 4.5×10^4 gene target molecules, respectively, with 1.8×10^2 and 3.0×10^3 as median values. The total amount of *Treponema* was comparable to *Synergistes*, ranging from 9.0×10^3 to 2.9×10^7 target molecules with a median of 5.6×10^5. Significant negative correlations were found between *Synergistes* and *P. intermedia* ($rs = -0.557$, $n = 13$, $P = 0.048$), *Synergistes* and *P. gingivalis* ($rs = -0.623$, $n = 15$, $P = 0.013$), and *P. intermedia* and *P. gingivalis* ($rs = -0.580$, $n = 13$, $P = 0.038$). There was no correlation between the amount of each of the four bacterial groups and total bacterial numbers.

The median proportion of *Synergistes* within the total microbial community was 0.79%. In contrast, the median proportion of both *P. intermedia* and *P. gingivalis* was <0.01%, while that of *Treponema* was 1.48%.

Identification of *Synergistes* 16S rRNA gene types

In order to identify the numerically dominant *Synergistes* phylotypes in the seven positive root canal samples, direct sequencing of the RTQ-PCR products was performed to produce ambiguity-free sequences of approximately 450 bp. All sequences were affiliated with the division *Synergistes,* according to the GenBank database. For proper phylogenetic inference (based on the full-size amplicon of approximately 595 bp) and for further exploration of *Synergistes* diversity, clone libraries were generated and selected clones were used for comparative sequence analysis. In total, six clones per clone library (seven libraries, in total 42 clones) were analyzed. In five cases (i.e. in five root canal samples E7, E12, E16, E25, and E30) all six clones per library showed a sequence identity ≥99% matching the sequence type already determined by direct sequencing. Following the approach

of Lepp et al. 2004 (9) and Paster et al 2001 (14) sequences with an identity ≥99% were considered as a single phylotype. In two cases (E22 and E24) diversity was such that five clones each represented one single phylotype (identity ≥ 99% among clones) while one clone each represented a second phylotype. Fig. 1 depicts the phylogenetic position of the 16S rRNA gene sequence types in comparison with publicly available sequences of *Synergistes* strains and clones. Three sequence types recovered from samples E12, E24, and E30 could be assigned to *Synergistes* Cluster III (7) and were split into two sub-branches with sequence types from samples E24 and E30 sharing an identity of approximately 98% with each other and with the sequence type from sample E12 of approximately 95%. Five sequence types recovered from samples E7, E16, E22, E24, and E25 grouped tightly and were affiliated with *Synergistes* Cluster II showing a sequence identity of at least 98% to the oral clone BA121 (Fig. 1). In contrast, one sequence type recovered from sample E22 formed a separate lineage outside Cluster II and III, and was moderately related to *Synergistes jonesii* with a sequence identity of approximately 86%.

In summary, all seven root canal samples each contained one numerically dominant phylotype; among these phylotypes the broad genetic diversity grouped into three distinct lineages within the phylogenetic radiation of *Synergistes*.

Discussion

Synergistes in endodontic infections

The objective of the present study was to characterize the predominant *Synergistes* phylotypes in infected root canals in humans. It is noteworthy that the prevalence of *Synergistes* phylotypes in our positive samples (seven out of 32 tested cases) lies within the range that we determined for such classical endodontic pathogens as *P. intermedia* and *P. gingivalis,* and the genus *Treponema* (Table 2). While a microorganism's mere presence in infected root canals indicates the potential for invading this naturally closed system, the frequency of its occurrence could be a reflection of a species' relevance to the etiology of the disease. Another important aspect might be the quantity of individual bacterial groups. We found that the median load of *Synergistes* was two orders of magnitude higher than that of *P. gingivalis* and three orders higher than that of *P. intermedia*, in fact approaching the range of *Treponema* (Table 2). Even though pathogenicity or virulence factors of *Synergistes* have yet to be elucidated, their prevalence and abundance suggest that they might play a role in the etiology of apical periodontitis.

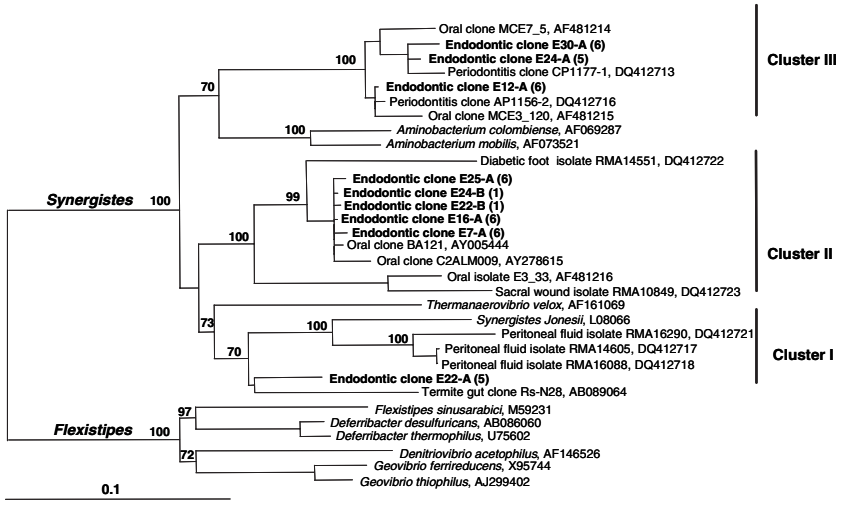

Fig. 1. Evolutionary distance tree showing the position of 16S rDNA clone sequences identified in infected root canals of human teeth in relation to representative sequences from the *Synergistes* and *Flexistipes* divisions. Clone sequences determined in this study are shown in bold type and the names start with the prefix E7, E12, E16, E22, E24, E25 and E30, respectively according to the names of the root canal samples from which they were recovered. The number behind each sequence indicates the number of clones that shared at least 99% identity to the displayed sequence type. A phylogenetic 'core' tree of *Synergistes* and *Flexistipes* 16S rDNA sequences was reconstructed by using the ARB software package (neighbor-joining, Felsenstein correction) of nearly full-length 16S rRNA gene sequences (>1350 bp; $n = 21$). *Synergistes* clone sequences determined in this study (amplicon size approximately 595 bp) as well as the clones CP1177-1 and AP1156-2 (7), were added using the ARB parsimony tool (10), which allows the addition of partial sequences to an existing 'core' tree without altering the global tree topology. The statistical significance levels of interior nodes shown as percentages were determined by performing bootstrap analyses (1000 replications, only values over 50% are shown). The scale bar corresponds to 0.1 substitutions per nucleotide.

Diversity of endodontic *Synergistes*

We found a remarkable diversity of *Synergistes* 16S rRNA gene types among the endodontic samples grouping them into three major evolutionary lineages within the *Synergistes* radiation. In contrast, diversity within samples appeared to be low, because in most cases all six clones analyzed per sample represented a single phylotype. However, it is possible that further *Synergistes* phylotypes were present in the clone libraries in low abundance but might have been overlooked because of the relatively low number of analyzed clones. Sequence types within Cluster III showed a higher degree of diversity than those from Cluster II and were related to clone sequence types previously identified in subgingival plaque (14). While the endodontic sequence types affiliated with Cluster II grouped tightly, the overall evolutionary scope of Cluster II is large because it consists of several clinically important and phylogenetically diverse *Synergistes* isolates from the oral cavity (12) and from soft tissue infections (7). For instance, the pure culture isolate, strain E3_33 (Cluster II), was recovered from an infected root canal (12) but shared an identity of only 87% with the Cluster II sequences determined in our study. This underlines the importance of different members of Cluster II for endodontic infections as well as their ability to be cultured from this human site. However, it remains unclear whether all members from Cluster II can be cultivated. In contrast, *Synergistes* Cluster III is so far without any representative pure culture isolate and is made up solely from clones obtained from the oral cavity.

The restriction of phylogenetic clades to specific ecosystems agrees with Godon et al. 2005 (5) who used 16S rDNA targeted PCR to explore 93 anaerobic environments, including the guts of 49 different animals and four specimens from human sources. The sequences from animal sources formed their own clustered groups, as did the sequences from anaerobic digestors, soil and from human subgingival plaque, suggesting that phylogenetically defined sub-groups of *Synergistes* occupy their own individual ecologic niches (5). Interestingly, one endodontic sample (E22) contained a remote grouping phylotype that was related to strains that were previously isolated from peritoneal fluid (7). This is striking because

such a phylotype has never been detected in the oral cavity and because it demonstrates that infected root canals are heterogeneous habitats that apparently provide unique ecologic niches for phylogenetically very diverse members of *Synergistes*.

Relative proportions and possible interactions among endodontic pathogens

The total bacterial load was in the range previously reported (21); however, the proportions of *Synergistes* and *Treponema* were around 1% and those of the other two pathogenic taxa investigated were even lower (Table 2). This indicates a high species complexity in infected root canals and/or the presence of other species that are numerically more dominant (e.g. those from the divisions *Firmicutes* or *Fusobacteria*). Relatively low proportions for *P. intermedia*, *P. gingivalis*, and *Treponema* are not too surprising because they have been linked with acute symptomatic endodontic infections (6, 19) and our study focused on chronic cases. Thus, a higher relative abundance of these bacterial groups could be expected in the acute infections. However, proportions of 1% or below of the total bacterial community do not necessarily imply minor importance in the etiology of the disease. At individual threshold levels certain bacterial groups might act as 'keystone species' (11) with their effect on the ecosystem being disproportionate to their relative numbers. Such a role is possible for *Synergistes* because it has been found in a large variety of anaerobic ecosystems, including in animals and humans, with an abundance generally below 1% according to the detection frequency in clone libraries (5). Interestingly, the proportion of *Synergistes* that we found in infected root canals (0.79%) exceeded significantly the proportions that we determined in a previous study in human oral plaque flora and fecal flora [0.04% and 0.01%, respectively (7)], giving additional support to the claim that they might represent true endodontic pathogens. Having adapted to individual ecologic niches during co-evolution along with their hosts, *Synergistes* seem to have evolved into specialists in anaerobic degradation of proteins and amino acids (1, 5). As such they may maintain a co-operative synergy with other microbes that utilize the end-products such as molecular hydrogen, which in turn could be vital for the polymicrobial infection process (9). We found significant negative correlations between *Synergistes* and *P. intermedia*, *Synergistes* and *P. gingivalis*, and between *P. intermedia* and *P. gingivalis*. Since the latter two species also have a proteolytic capacity, and because the small amount of available carbohydrates in an infected root canal is quickly depleted, substrate (i.e. proteins) competition among these groups might be the most plausible reason. For a future study, it would be interesting to determine the proportions of further pathogens and to assess how the structure of the bacterial community in endodontic infections varies with symptomatology, such as presence of pain, tenderness to percussion, or swelling.

Concluding remarks

To understand how the microbial community works in different phases or forms of endodontic disease, it is necessary to have some knowledge of species diversity and prevalence and also of the population size of individual bacterial species. Our study shows that members of the novel division *Synergistes* occur in proportions that are roughly equal to those of important endodontic pathogens and the broad diversity of the detected phylotypes suggests that infected root canals are highly heterogeneous and complex ecosystems.

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