Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries

Daniel Saito,¹ Renato de Toledo Leonardo,² Jorge Luiz Mazza Rodrigues,³ Siu Mui Tsai,³ José Francisco Höfling¹ and Reginaldo Bruno Gonçalves¹

Correspondence

Reginaldo Bruno Gonçalves

Reginald@fop.unicamp.br

¹Department of Oral Diagnosis, Dental School of Piracicaba, State University of Campinas (UNICAMP), Av. Limeira 90, Piracicaba, 13414-903 São Paulo, Brazil

²Department of Restorative Dentistry, Dental School of Araraquara, State University of São Paulo, Araraquara, São Paulo, Brazil

³Laboratory of Cell and Molecular Biology, Center of Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, São Paulo, Brazil

A significant proportion of oral bacteria are unable to undergo cultivation by existing techniques. In this regard, the microbiota from root canals still requires complementary characterization. The present study aimed at the identification of bacteria by sequence analysis of 16S rDNA clone libraries from seven endodontically infected teeth. Samples were collected from the root canals, subjected to the PCR with universal 16S rDNA primers, cloned and partially sequenced. Clones were clustered into groups of closely related sequences (phylotypes) and identification to the species level was performed by comparative analysis with the GenBank, EMBL and DDBJ databases, according to a 98 % minimum identity. All samples were positive for bacteria and the number of phylotypes detected per subject varied from two to 14. The majority of taxa (65.2 %) belonged to the phylum *Firmicutes* of the Gram-positive bacteria, followed by *Proteobacteria* (10.9 %), *Spirochaetes* (4.3 %), *Bacteroidetes* (6.5 %), *Actinobacteria* (2.2 %) and *Deferribacteres* (2.2 %). A total of 46 distinct taxonomic units was identified. Four clones with low similarity to sequences previously deposited in the databases were sequenced to nearly full extent and were classified taxonomically as novel representatives of the order *Clostridiales*, including a putative novel species of *Mogibacterium*. The identification of novel phylotypes associated with endodontic infections suggests that the endodontium may still harbour a relevant proportion of uncharacterized taxa.

Received 27 June 2005

Accepted 8 September 2005

INTRODUCTION

Although more than 150 species of bacteria have been identified in infected root canals, only a restricted number can be found simultaneously in the same tooth and a considerable variation of species is expected when analysing distinct clinical conditions, individuals or populations (Sundqvist, 1976; Molander *et al.*, 1998; Baumgartner *et al.*, 2004). Cultivation studies have shown a predominance of facultative and strict anaerobes in the endodontium, including representatives of *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, *Porphyromonas*, *Prevotella* and

Streptococcus (Sundqvist, 1992b; Le Goff *et al.*, 1997). Bacteria inside the canal are the major cause of periapical pathologies (Takehashi *et al.*, 1965) and, if not adequately treated, can give rise to dentoalveolar abscess, a condition that has ability to initiate morbidity, life-threatening illness (Walsh, 1997), and to predispose to transient bacteraemia during therapy (Savarrio *et al.*, 2005). Previous reports suggested that endodontic bacteria might be involved in extra-oral complications, such as chronic maxillary sinusitis (Melen *et al.*, 1986), orbital cellulitis (Ngeow, 1999), infective endocarditis (Bate *et al.*, 2000), rheumatoid arthritis (Breebaart *et al.*, 2002) and brain abscess (Henig *et al.*, 1978). In this regard, substantial understanding of the endodontic microbiota is an important requirement for both oral and medical microbiologists.

While it is common knowledge that the development of efficient treatment strategies relies on the characterization of the endodontic microbial communities in their entirety, cultivation-based techniques may cut down the range of detection, since a subset of oral inhabitants still cannot

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of uncultured *Clostridiales* bacterium clone AG_D03, uncultured *Clostridiaceae* bacterium clone AG_G04, uncultured *Streptococcaceae* bacterium clone AF_F05 and uncultured *Mogibacterium* sp. clone AF_H06 are AY821867, AY821868, AY821869 and AY821870, respectively.

PCR results with reference bacteria are available as supplementary material in JMM Online.

undergo cultivation (Paster *et al.*, 2001). In this new context, clone library analysis of rDNA, particularly the 16S rDNA, has become a trustworthy tool for determining bacterial diversity, often yielding more informative results when compared with cultivation alone (Kroes *et al.*, 1999; Rolph *et al.*, 2001). This broad-based cultivation-free approach has been employed in the investigation of polymicrobial human infections such as periodontal disease, childhood caries, dentoalveolar abscesses, maxillary sinusitis and noma lesions (Wade *et al.*, 1997; Paster *et al.*, 2001, 2002; Becker *et al.*, 2002; Hutter *et al.*, 2003; Paju *et al.*, 2003). Particularly in root canal infections, 16S rDNA sequence analysis has enabled detection of bacteria when culture had generated negative results and has permitted the identification of novel species in relatively small sets of samples (Rolph *et al.*, 2001; Munson *et al.*, 2002).

Here, we report the results of an investigation of the bacterial diversity of seven infected root canals by the analysis of 16S rDNA libraries, in an effort to contribute to the ongoing characterization of the root canal microbiota.

METHODS

Subjects. Seven patients, two males and five females, ranging from 15 to 42 years old (mean 27.7 ± 8.4 years) were included in the study. Subjects had been referred for endodontic treatment at the Dental School of Piracicaba and were selected for the presence of pulpal necrosis and chronic periapical lesions by clinical and radiographic evaluation. Subjects with periodontal pockets >3 mm, advanced bone loss, acute abscesses, tooth fractures or sinus tracts or those who had undergone antibiotic therapy within 2 months prior to collection were not included, as well as those who presented systemic diseases at the time of examination. Written informed consent was obtained from all individuals and ethical approval was granted by the Ethical Committee for Human Subjects of the Dental School of Piracicaba, State University of Campinas.

Sample collection. Each patient was submitted to local anaesthesia and the tooth was isolated with a rubber dam. In order to facilitate antiseptics of the operation field, only teeth without caries lesions and with unexposed pulp chambers were included. Cleaning of the tooth crown was performed to eliminate food debris and dental plaque. Antisepsis of the crown and operation field was conducted with 1.0% sodium hypochlorite for 1 min, followed by inactivation with 5% sodium thiosulfate. This protocol may have limitations in eliminating some resistant species of bacteria, but its use associated with careful selection of teeth should contribute to minimize contamination from saliva and other oral sources. Coronal access cavity was gained by high-speed bur irrigated with sterile saline solution. As the pulp chamber was reached, a sterile #15 K-file was introduced at 3 mm short of the root apex. After careful instrumentation, the active portion of the K-file was cut and placed in a test tube containing 1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Three sterile #15 paper points were introduced consecutively inside the canal for 20 s each and placed in the same test tube. Samples were transported immediately to the laboratory and stored at -20°C .

DNA extraction. Samples were thawed in a water bath at 37°C for 10 min and then vortexed for 30 s and the paper points and K-files were removed from the tubes. Bacterial cells were pelleted by centrifugation at 20 000 g for 10 min and the supernatant was discarded. A DNA extraction protocol based on chloroform/isoamyl alcohol

and CTAB was employed (Kuipers *et al.*, 1999; Smith *et al.*, 1989). DNA was resuspended in TE buffer with $10\text{ }\mu\text{g RNase ml}^{-1}$, incubated in a water bath at 37°C for 30 min and stored at -20°C until required.

16S rDNA amplification. PCR control tests for the eubacterial universal 16S rDNA primers fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991) were performed with 21 bacteria strains, yielding positive amplification for all DNA tested, as determined by visualization on agarose gel electrophoresis (available as Supplementary Fig. S1 in JMM Online). Polymicrobial 16S rDNA from the clinical samples was amplified by PCR in 25 μl mixtures as described previously (Rodrigues *et al.*, 2003), except that 2 mM MgCl_2 and 1.5 U *Taq* DNA polymerase were used. PCR products were examined by 1.0% low-melting-point agarose-gel electrophoresis with ethidium bromide staining. Amplification products (about 1500 bp) were purified with the GFX DNA Purification kit (Amersham Biosciences) according to the manufacturer's instructions.

Cloning of polymicrobial PCR products. An aliquot containing 65 ng of each 16S rDNA PCR product was ligated to pMOSBlue vector (Amersham Biosciences) and transformed in *Escherichia coli* DH10B cells, according to the manufacturer's instructions. Small-scale plasmid DNA preparations were conducted by an alkaline lysis protocol as described by Sambrook *et al.* (1989). Screening of recombinants was performed by 1.0% agarose-gel electrophoresis with ethidium bromide staining.

Partial 16S rDNA sequencing. Seventy recombinant clones per library were selected randomly for partial 16S rDNA sequencing reactions, performed in a DNA thermal cycler. The temperature profile for primers T7 (5'-TAATACGACTCACTATAGGG-3') and U19mer (5'-GTTTCCCAGTCACGACG-3') included an initial step at 96°C for 2 min, followed by 30 cycles of 96°C for 30 s, 50°C for 30 s and 60°C for 4 min. Reactions were performed in 10 μl mixtures containing 250 ng template DNA, 1 μl Big Dye Terminator Ready version 3.0 (Applied Biosystems), 0.5 μM primer and 3 μl sequencing buffer (200 mM Tris/HCl, pH 9.0, 5 mM MgCl_2).

16S rDNA sequence analysis. Sequences were analysed automatically in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems – Hitachi) and grouped into clusters (phylotypes) according to a 99% minimum similarity rule (Kroes *et al.*, 1999; Hutter *et al.*, 2003). One representative of each phylogroup was selected and submitted to the BLASTN algorithm (BLAST 2.0; <http://www.ncbi.nlm.nih.gov/blast>), allowing comparison with sequences present in the GenBank, DDBJ and EMBL databases. Only the highest-scored BLAST result was considered for phylogroup identification, with 98% minimum similarity (Stackebrandt & Goebel, 1994).

Phylogenetic analysis of novel phylotypes. Four clones with BLAST identities $\leq 97\%$ were considered as representatives of novel phylotypes and were sequenced with additional primers 341-357f (5'-CCTACGGGAGGCAGCAG-3'), 357-341r (5'-CTGCTGCCTCCGTAGG-3'), 685-704f (5'-GTAGSGGTGAAATSCGTAGA-3'), 704-685r (5'-TCTACGSATTTACCSCCTAC-3'), 1099-1114f (5'-GCAACGAGCGCAACCC-3') and 1114-1099r (5'-GGGTTGCGCTCGTTGC-3') (Lane, 1991). Contiguous sequences were assembled with the Phred/Phrap/Consed software package (<http://www.phrap.org>), followed by analysis with Chimera Check (RDP II; <http://rdp.cme.msu.edu>) to eliminate chimeric molecules. Novel phylotypes were assigned taxonomically with Naive Bayesian rRNA Classifier version 1.0 (RDP II) and submitted to phylogenetic analysis, along with closely related sequences from the order Clostridiales obtained by the Hierarchy Browser program (RDP II). All sequences were aligned with the CLUSTAL W software (Thompson *et al.*, 1994) and visualized with Bioedit 7.0.4 (<http://www.mbio.ncsu.edu/>

BioEdit). A phylogenetic tree was constructed with MEGA 2.1 (Kumar *et al.*, 2001), according to the calculation of a distance matrix (Jukes & Cantor, 1969) and tree reconstruction by the neighbour-joining method (Saitou & Nei, 1987). Bootstrap confidence values for branching nodes were inferred by the generation of 100 resampling trees.

RESULTS AND DISCUSSION

Nucleotide sequence analysis of 16S rDNA clone libraries was used to investigate the bacterial diversity of seven endodontically infected teeth. All teeth evaluated were positive for the presence of bacteria. Overall, 46 taxonomic units (phylotypes) were detected (Table 1). In spite of the relative homogeneity of our study group, composed of asymptomatic teeth associated with unexposed necrotic pulps, chronic periapical lesions and no periodontal disease, considerable variation in bacterial composition could be observed: 33 (71.7%) phylotypes were subject-exclusive, whereas only 13 (28.3%) could be detected in more than one patient. The number of phylotypes also ranged substantially among subjects, from two (patient AB) to 14 (patient AA) (mean 9.57 ± 3.91). This variation is in accordance with both cultivation- and molecular-based studies (Sundqvist, 1976; Jung *et al.*, 2000). Table 1 illustrates the relative distribution of phylotypes within each subject and is intended to provide a quantitative view of the results. This type of analysis makes an important contribution to our findings (Dewhirst *et al.*, 2000; Becker *et al.*, 2002; Munson *et al.*, 2002; Hutter *et al.*, 2003), but should be viewed with discretion, since multitemplate PCR can be subjected to bias in template-to-product ratios (Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998).

In general, the results of this investigation reflected results from previous cultivation- and molecular-based studies, with a predominance of anaerobic bacteria, especially from the phylum *Firmicutes* of Gram-positive bacteria (Sundqvist, 1992a; Munson *et al.*, 2002). Representatives of other phyla were found at much lower frequencies: *Proteobacteria* (10.9%), *Spirochaetes* (4.3%), *Bacteroidetes* (6.5%), *Actinobacteria* (2.2%) and *Deferribacteres* (2.2%); 9% of sequences could not be assigned at the phylum level. Common endodontic species, such as *Campylobacter gracilis*, *Eubacterium tardum*, *Peptostreptococcus anaerobius*, *Peptostreptococcus micros* and members of the *Lachnospiraceae*, were positively identified with high percentage identities ($\geq 99\%$). Recently reported phylotypes were also detected: *Bacteroidales* oral clone MCE7_164 E2b, *Lachnospiraceae* oral clone MCE7_60 E1, *Lachnospiraceae* oral clone MCE9_173 E4 and *Megasphaera* sp. oral clone MCE3_141 P1 (Munson *et al.*, 2002). *Dialister invisus*, a recently described oral Gram-negative coccobacillus (Downes *et al.*, 2003), was the most commonly found taxon (five out of seven subjects), followed by *Filifactor alocis* and *Eubacteriaceae* oral clone P2PB_46 P3 (four out of seven subjects). Accordingly, bacteria from the genus *Dialister* have been identified in oral infections with increasing frequencies (Contreras *et al.*, 2000; Munson

et al., 2002). *Dialister pneumosintes*, a species frequently associated with purulent infections, brain abscesses and bite wounds (Goldstein *et al.*, 1984; Rousée *et al.*, 2002), has also been considered as a putative pathogen in periodontal and endodontic infections (Ghayoumi *et al.*, 2002; Siqueira & Rôças, 2002) and could be detected in one subject.

Phylotypes corresponding to recently proposed pathogens of periodontal disease were identified, corroborating molecular data from Paster *et al.* (2001) and Hutter *et al.* (2003). Among those, *Treponema socranskii* has already been detected in the endodontium, being one of the most common root-canal treponemes (Baumgartner *et al.*, 2003), while uncultured *Eubacterium* clone PUS9.170 has also been found in dentoalveolar abscesses (Wade *et al.*, 1997). *Pseudoramibacter alactolyticus* and *Filifactor alocis* were positively detected with high percentage matches (99%) and have proven to be frequent inhabitants of root-filled, refractory cases (Siqueira & Rôças, 2004).

Forty-two (91.3%) clones were identified to the species level and four (8.7%) corresponded to sequences with no resemblance to any other previously deposited in the databases (Fig. 1), according to the established 98% nucleotide identity threshold. This parameter is in accordance with a previously proposed species definition, based on DNA-DNA reassociation assays (Stackebrandt & Goebel, 1994), and lies within the range of values employed by similar studies, which vary from 98% (Sakamoto *et al.*, 2000; Rolph *et al.*, 2001; Munson *et al.*, 2002) to 99% (Kroes *et al.*, 1999; Drancourt *et al.*, 2000; Hutter *et al.*, 2003). Applying a 97% threshold value did not alter our findings, whereas a value of 99% resulted in the detection of 15 potentially novel species, as opposed to only four. The application of such a stringent condition was nonetheless rejected, since it could generate redundancies in the results, due to intragenomic heterogeneities in 16S rRNA operons (Coenye & Vandamme, 2003; Acinas *et al.*, 2004).

Among the novel phylotypes detected (Fig. 1), uncultured *Mogibacterium* sp. clone AF_H06 (AY821870) was the only one to be assigned taxonomically to the genus level. *Mogibacterium* is a genus of anaerobic, Gram-positive bacilli, originally isolated from periodontal pockets and infected root canals (Nakazawa *et al.*, 2000). Representatives of this genus were shown to be frequent in endodontic infections, as observed by Rolph *et al.* (2001), who identified clones closely related to *Mogibacterium neglectum*, *M. vescum* and *M. diversum*, with 97% nucleotide identities. The epidemiological importance of this group of bacteria in endodontic infections is still to be investigated.

Interestingly, some important endodontic species, such as *Fusobacterium nucleatum* and the black-pigmented anaerobes *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia*, could not be detected. Lack of primer specificity was readily discounted, since we had successfully tested our primers with *F. nucleatum* subsp. *polymorphum* ATCC 10953^T and several species within the

Table 1. Highest-scored BLAST search results from 46 taxonomic units detected in unexposed root canal infections by sequence analysis of 16S rDNA clonal libraries and distribution of phylotypes per subject

Search result	Identity (%)	Phylotype distribution per subject (%)						
		AA	AB	AC	AD	AE	AF	AG
<i>Bacteroidales</i> oral clone MCE7_120 E3	98					4.2		
<i>Bacteroidales</i> oral clone MCE7_164 E2b	99				2.9			
<i>Bacteroides</i> -like sp. oral clone X083	98	1.4						
<i>Burkholderia fungorum</i> strain NW-2	100			7.4				
<i>Burkholderia phenazinium</i>	99				5.7			
<i>Burkholderia</i> sp. Cl6	98					4.2		
<i>Campylobacter gracilis</i>	99	1.4						
<i>Catabacter hongkongensis</i> HKU16	100				2.9			
<i>Clostridiales</i> oral clone MCE3_9 E1	93						9.1	15.0
<i>Clostridiales</i> oral clone P4PB_122 P3	92							5.0
<i>Desulfobulbus</i> sp. oral clone R004	98					20.8		
<i>Dialister invisus</i>	100	28.8			2.9	4.2	3.0	10.0
<i>Dialister pneumosintes</i>	99							5.0
<i>Eubacteriaceae</i> oral clone P2PB_46 P3	99				20.0	33.3	6.1	15.0
<i>Eubacterium</i> sp. oral clone BU061	99	2.7						
<i>Eubacterium</i> sp. oral clone CK047	99					4.2		
<i>Eubacterium</i> sp. oral clone DA014	94						6.1	
<i>Eubacterium</i> sp. oral clone FX028	98					4.2		
<i>Eubacterium tardum</i> SC87K	99	23.3			11.4		12.1	
<i>Eubacterium timidum</i>	99						27.3	10.0
<i>Filifactor alocis</i>	99	1.4		22.2		12.5		20.0
<i>Filifactor</i> sp. oral clone BP1-58	99	1.4						
<i>Filifactor</i> sp. oral clone BP1-88	99			22.2				
<i>Flexistipes</i> sp. E3_33 oral isolate	99				2.9			
<i>Lachnospiraceae</i> oral clone BP1-14	99				5.7			
<i>Lachnospiraceae</i> oral clone MCE7_60 E1	99				2.9			
<i>Lachnospiraceae</i> oral clone MCE9_173 E4	99				34.3	16.7		
<i>Lactobacillus panis</i> DAF 1	98		12.5					
<i>Megasphaera</i> sp. oral clone BB166	98			7.4				5.0
<i>Megasphaera</i> sp. oral clone MCE3_141 P1	99	11.0		3.7				
<i>Mogibacterium neglectum</i>	93						3.0	
<i>Peptostreptococcus micros</i> ATCC 33270 ^T	100						3.0	
<i>Peptostreptococcus anaerobius</i> clone LK54	99	1.4		14.8				
<i>Peptostreptococcus</i> sp. oral clone CK035	99							15.0
<i>Pseudoramibacter alactolyticus</i> ATCC 23263 ^T	99	15.1			8.6			
<i>Selenomonas</i> sp. oral clone JS031	98			3.7				
<i>Selenomonas</i> -like sp. oral strain FNA3	98			7.4				
<i>Treponema socranskii</i> subsp. 04	99						3.0	
<i>Treponema</i> sp. 6:H:D15A-4	98	4.1						
Uncultured actinobacterium clone APe2_64	98						12.1	
Uncultured bacterium BH017	99						6.1	
Uncultured bacterium inhufec A-37	99						6.1	
Uncultured bacterium W090	99	2.7					3.0	
Uncultured <i>Eubacterium</i> clone PUS9.170	99	2.7		11.1				
Unidentified oral bacterium RP55-6	98	2.7						
<i>Veillonella</i> sp. ADV 360.00	99		87.5					
Total number of phylotypes		14	2	9	11	9	13	9

phylum *Bacteroidetes*, including *Porphyromonas gingivalis* ATCC 33277^T, *Porphyromonas endodontalis* ATCC 35406^T and *Prevotella intermedia* ATCC 25611^T (Supplementary

Fig. S1). Similar root-canal studies were also unable to find any *Porphyromonas gingivalis* phylotypes (Rolph *et al.*, 2001; Munson *et al.*, 2002). Fusobacteria have frequently been

Fig. 1. 16S rDNA phylogenetic interrelationships of four novel phylotypes from unexposed endodontic infections, indicated in bold type, and reference strains from the order *Clostridiales* (accession numbers in parentheses). The phylogenetic tree was constructed as described in Methods and rooted for *Escherichia coli* K-12 (not shown). Bootstrap confidence values were generated over 100 tree replications (values above 50 % are shown). Bar, 0.05 nucleotide substitutions per site.

encountered in root canals by cultivation (Sundqvist, 1992a) and PCR assays (Fouad *et al.*, 2002), but their prevalence seems to be relatively low in 16S rDNA-based studies. Accordingly, Munson *et al.* (2002) identified one *Fusobacterium* clone out of 624 sequenced, whereas Rolph *et al.* (2001) detected *F. nucleatum* solely in the refractory subset of cases. The absence of some bacterial species in the present study might be attributed to intrinsic technique limitations or to the small sample investigated.

In conclusion, the results of this study are in accordance with those from similar research, revealing a predominance of anaerobic species from the phylum *Firmicutes* of the Gram-positive bacteria in infected root canals, particularly from the class *Clostridia*. The identification of uncultured clones originally encountered in the endodontium, saliva and subgingival plaque demonstrates, again, that the endodontic and periodontal microbial communities might share a relevant proportion of bacteria, despite their established anatomical interrelationships (Kerekes & Olsen, 1990; Rupf

et al., 2000). The identification of novel phylotypes adds to the concept that the endodontium might still harbour a relevant proportion of uncharacterized taxa.

ACKNOWLEDGEMENTS

This work was supported by grants from the Brazilian funding agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 04/01674-6, 04/13548-5, 00/10168-6), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We are very grateful to Ms Fabiana de Souza Cannavan and Mr José Elias Gomes for technical support.

REFERENCES

- Acinas, S. G., Marcelino, L. A., Klepac-Ceraj, V. & Polz, M. F. (2004). Divergence and redundancy of 16S rRNA sequences in genomes with multiple *rrn* operons. *J Bacteriol* **186**, 2629–2635.

- Bate, A. L., Ma, J. K. & Pitt Ford, T. R. (2000). Detection of bacterial virulence genes associated with infective endocarditis in infected root canals. *Int Endod J* **33**, 194–203.
- Baumgartner, J. C., Khemaleelakul, S. U. & Xia, T. (2003). Identification of spirochetes (treponemes) in endodontic infections. *J Endod* **29**, 794–797.
- Baumgartner, J. C., Siqueira, J. F., Jr, Xia, T. & Rôças, I. N. (2004). Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction. *J Endod* **30**, 141–144.
- Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E. & Griffen, A. L. (2002). Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* **40**, 1001–1009.
- Breebaart, A. C., Bijlsma, J. W. & van Eden, W. (2002). 16-year remission of rheumatoid arthritis after unusually vigorous treatment of closed dental foci. *Clin Exp Rheumatol* **20**, 555–557.
- Coenye, T. & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol Lett* **228**, 45–49.
- Contreras, A., Doan, N., Chen, C., Rusitanonta, T., Flynn, M. J. & Slots, J. (2000). Importance of *Dialister pneumosintes* in human periodontitis. *Oral Microbiol Immunol* **15**, 269–272.
- Dewhirst, F. E., Tamer, M. A., Ericson, R. E., Lau, C. N., Levanos, V. A., Boches, S. K., Galvin, J. L. & Paster, B. J. (2000). The diversity of periodontal spirochetes by 16S rRNA analysis. *Oral Microbiol Immunol* **15**, 196–202.
- Downes, J., Munson, M. & Wade, W. G. (2003). *Dialister invisus* sp. nov., isolated from the human oral cavity. *Int J Syst Evol Microbiol* **53**, 1937–1940.
- Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J. & Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol* **38**, 3623–3630.
- Fouad, A. F., Barry, J., Caimano, M., Clawson, M., Zhu, Q., Carver, R., Hazlett, K. & Radolf, J. D. (2002). PCR-based identification of bacteria associated with endodontic infections. *J Clin Microbiol* **40**, 3223–3231.
- Ghayoumi, N., Chen, C. & Slots, J. (2002). *Dialister pneumosintes*, a new putative periodontal pathogen. *J Periodontol Res* **37**, 75–78.
- Goldstein, E. J., Citron, D. M. & Finegold, S. M. (1984). Role of anaerobic bacteria in bite-wound infections. *Rev Infect Dis* **6**, 177–183.
- Henig, E. F., Derschowitz, T., Shalit, M., Toledo, E., Tikva, P. & Aviv, T. (1978). Brain abscess following dental infection. *Oral Surg Oral Med Oral Pathol* **45**, 955–958.
- Hutter, G., Schlagenhauf, U., Valenza, G., Horn, M., Burgemeister, S., Claus, H. & Vogel, U. (2003). Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens. *Microbiology* **149**, 67–75.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Jung, I. Y., Choi, B. K., Kum, K. Y., Roh, B. D., Lee, S. J., Lee, C. Y. & Park, D. S. (2000). Molecular epidemiology and association of putative pathogens in root canal infection. *J Endod* **26**, 599–604.
- Kakehashi, S., Stanley, H. R. & Fitzgerald, R. J. (1965). The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* **20**, 340–349.
- Kerekes, K. & Olsen, I. (1990). Similarities in the microfloras of root canals and deep periodontal pockets. *Endod Dent Traumatol* **6**, 1–5.
- Kroes, I., Lepp, P. W. & Relman, D. A. (1999). Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci U S A* **96**, 14547–14552.
- Kuipers, J. G., Niefeld, L., Dreses-Werringloer, U., Koehler, L., Wollenhaupt, J., Zeidler, H. & Hammer, M. (1999). Optimised sample preparation of synovial fluid for detection of *Chlamydia trachomatis* DNA by polymerase chain reaction. *Ann Rheum Dis* **58**, 103–108.
- Kumar, S., Tamura, K., Jakobsen, I.-B. & Nei, M. (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.
- Lane, D. J. (1991). 16S/ 23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Le Goff, A., Bunetel, L., Mouton, C. & Bonnaure-Mallet, M. (1997). Evaluation of root canal bacteria and their antimicrobial susceptibility in teeth with necrotic pulp. *Oral Microbiol Immunol* **12**, 318–322.
- Melen, I., Lindahl, L., Andreasson, L. & Rundcrantz, H. (1986). Chronic maxillary sinusitis. Definition, diagnosis and relation to dental infections and nasal polyposis. *Acta Otolaryngol* **101**, 320–327.
- Molander, A., Reit, C., Dahlén, G. & Kvist, T. (1998). Microbiological status of root-filled teeth with apical periodontitis. *Int Endod J* **31**, 1–7.
- Munson, M. A., Pitt-Ford, T., Chong, B., Weightman, A. & Wade, W. G. (2002). Molecular and cultural analysis of the microflora associated with endodontic infections. *J Dent Res* **81**, 761–766.
- Nakazawa, F., Sato, M., Poco, S. E., Hashimura, T., Ikeda, T., Kalfas, S., Sundqvist, G. & Hoshino, E. (2000). Description of *Mogibacterium pumilum* gen. nov., sp. nov. and *Mogibacterium vescum* gen. nov., sp. nov., and reclassification of *Eubacterium timidum* (Holdeman *et al.* 1980) as *Mogibacterium timidum* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 679–688.
- Ngeow, W. C. (1999). Orbital cellulitis as a sole symptom of odontogenic infection. *Singapore Med J* **40**, 101–103.
- Paju, S., Bernstein, J. M., Haase, E. M. & Scannapieco, F. A. (2003). Molecular analysis of bacterial flora associated with chronically inflamed maxillary sinuses. *J Med Microbiol* **52**, 591–597.
- Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A. & Dewhirst, F. E. (2001). Bacterial diversity in human subgingival plaque. *J Bacteriol* **183**, 3770–3783.
- Paster, B. J., Falkler, W. A., Jr, Enwonwu, C. O. & 7 other authors (2002). Prevalent bacterial species and novel phylotypes in advanced noma lesions. *J Clin Microbiol* **40**, 2187–2191.
- Polz, M. F. & Cavanaugh, C. M. (1998). Bias in template-to-product ratios in multitemplate PCR. *Appl Environ Microbiol* **64**, 3724–3730.
- Rodrigues, J. L. M., Silva-Stenico, M. E., Gomes, J. E., Lopes, J. R. S. & Tsai, S. M. (2003). Detection and diversity assessment of *Xylella fastidiosa* in field-collected plant and insect samples by using 16S rRNA and *gyrB* sequences. *Appl Environ Microbiol* **69**, 4249–4255.
- Rolph, H. J., Lennon, A., Riggio, M. P., Saunders, W. P., MacKenzie, D., Coldero, L. & Bagg, J. (2001). Molecular identification of microorganisms from endodontic infections. *J Clin Microbiol* **39**, 3282–3289.
- Rousée, J. M., Bermond, D., Piemont, Y., Tournoud, C., Heller, R., Kehrl, P., Harlay, M. L., Monteil, H. & Jaulhac, B. (2002). *Dialister pneumosintes* associated with human brain abscesses. *J Clin Microbiol* **40**, 3871–3873.
- Rupf, S., Kannengiesser, S., Merte, K., Pfister, W., Sigusch, B. & Eschrich, K. (2000). Comparison of profiles of key periodontal pathogens in periodontium and endodontium. *Endod Dent Traumatol* **16**, 269–275.

- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sakamoto, M., Umeda, M., Ishikawa, I. & Benno, Y. (2000).** Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. *Microbiol Immunol* **44**, 643–652.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Savarrio, L., Mackenzie, D., Riggio, M., Saunders, W. P. & Bagg, J. (2005).** Detection of bacteraemias during non-surgical root canal treatment. *J Dent* **33**, 293–303.
- Siqueira, J. F., Jr & Rôças, I. N. (2002).** *Dialister pneumosintes* can be a suspected endodontic pathogen. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **94**, 494–498.
- Siqueira, J. F., Jr & Rôças, I. N. (2004).** Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **97**, 85–94.
- Smith, G. L., Socransky, S. S. & Smith, C. M. (1989).** Rapid method for the purification of DNA from subgingival microorganisms. *Oral Microbiol Immunol* **4**, 47–51.
- Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Sundqvist, G. (1976).** *Bacteriological studies of necrotic dental pulps*. PhD dissertation, University of Umeå, Sweden.
- Sundqvist, G. (1992a).** Associations between microbial species in dental root canal infections. *Oral Microbiol Immunol* **7**, 257–262.
- Sundqvist, G. (1992b).** Ecology of the root canal flora. *J Endod* **18**, 427–430.
- Suzuki, M. T. & Giovannoni, S. J. (1996).** Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62**, 625–630.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wade, W. G., Spratt, D. A., Dymock, D. & Weightman, A. J. (1997).** Molecular detection of novel anaerobic species in dentoalveolar abscesses. *Clin Infect Dis* **25** (Suppl. 2), S235–S236.
- Walsh, L. J. (1997).** Serious complications of endodontic infections: some cautionary tales. *Aust Dent J* **42**, 156–159.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.