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Molecular Analysis of Microflora Associated with Dentoalveolar Abscesses

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The microflora associated with three dentoalveolar abscesses was determined by cultural and molecular methods. 16S rRNA genes were randomly amplified by means of conserved eubacterial primers and cloned. Restriction fragment length polymorphism analysis of the clones and amplified genes encoding 16S rRNA from the cultured bacteria was used to detect putative unculturable bacteria. Clones representative of five predominant groups of uncultured organisms were sequenced. Two were identified as *Porphyromonas gingivalis* and *Prevotella oris*, and one was found to be closely related to *Peptostreptococcus micros*. The remaining two clones did not correspond to known, previously sequenced organisms. One was related to *Zoogloea ramigera*, a species of aerobic waterborne organisms, while the other was distantly related to the genus *Prevotella*. This study has demonstrated the possibility of the characterization of microflora associated with human infection by molecular methods without the inherent biases of culture.

Dentoalveolar abscesses result from infection around the apices of teeth. Bacteria gain access to this site either from the pulp secondary to dental caries or, rarely, via the periodontal route between the gingivae and the teeth. Dentoalveolar abscesses are acute conditions which may occasionally spread to other tissues, including the brain and nervous system, with serious consequences (5, 11).

The bacteria associated with dentoalveolar abscesses are principally viridans group streptococci, particularly the "*Streptococcus milleri*" group, *Fusobacterium* spp., *Prevotella* spp., *Porphyromonas* spp., and *Peptostreptococcus* spp. (4, 17). They are almost invariably mixed infections with, typically, three to four species recovered from individual abscesses by culture (13). However, it is estimated that approximately 50% of the oral flora is unculturable (1, 21). It is likely, therefore, that at least some members of the unculturable part of the flora are involved in disease and, indeed, may account for treatment failure since their susceptibility to antimicrobial agents would be unknown.

Molecular approaches to the study of unculturable bacteria have recently been developed. Direct amplification of 16S rRNA or DNA from biomass followed by cloning and sequencing has allowed the detection of unculturable bacteria from habitats such as photosynthetic microbial mats (24), seawater (8), and soil (14). Unculturable disease-causing bacteria from humans have also been identified in bacillary (epithelioid) angiomatosis (18) and Whipple's disease (26). The aim of this study was to employ this approach to study the microflora associated with dentoalveolar abscesses.

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MATERIALS AND METHODS

Sample collection, storage, and microbiological culture. Pus samples were obtained with informed consent from three patients with acute dentoalveolar abscesses attending the Primary Care Unit of the Dental Hospital, Bristol, United Kingdom. Sample 3 was taken from an abscess secondary to an infected root of an upper right premolar in a 34-year-old male, sample 8 was from a palatal abscess associated with the upper left lateral incisor in a 30-year-old male, and sample 10 was from a periapical abscess associated with an upper right premolar in a 32-year-old male. Prior to sample collection, the mucosa over each abscess was carefully cleaned with sterile cotton gauze to avoid salivary contamination of the sample. Pus was aspirated from the abscesses by means of a needle and syringe and taken immediately to the laboratory. A 100-μl sample of pus was placed in 2 ml of reduced transport medium (3), and 10-fold dilutions were prepared and used to inoculate duplicate sets of nonselective media. Fastidious Anaerobe Agar (LabM) with 5% sheep blood (FAA) plates were incubated for 7 days anaerobically, and Blood Agar Base no. 2 (LabM) with 5% sheep blood plates were incubated for 48 h in air plus 5% CO_2. Following incubation, plate counts were done and representative colonies were subcultured for identification by methods described previously (22). The remainder of the pus sample was centrifuged (10,000 × *g*, 5 min), the supernatant was removed, and 1 ml of saline-EDTA (0.15 M NaCl, 0.05 M EDTA) with proteinase K (0.5 mg/ml) was added to the pellet. The suspension was homogenized by vortex mixing and stored at −20°C.

DNA extraction. The influence of different methods for the isolation of genomic DNA from the pus samples and cultured bacteria on the results of the molecular analysis was assessed by comparing two protocols. Protocol A was a modification of a standard method (2). A 100-μl sample of pus was centrifuged (10,000 × *g*, 5 min), the pellet was resuspended in 310 μl of HTE buffer (50 mM Tris HCl, 20 mM EDTA; pH 8.0), the mixture was vortexed, and 350 μl of 2% sarcosyl was added. Cultivated bacteria were scraped from one FAA plate and treated similarly. A 5-μl volume of RNase A (10 mg/ml in TNE [10 mM Tris HCl, 10 mM NaCl, 0.1 mM EDTA; pH 8.0]) was added, and the tubes were incubated at 37°C for 15 min. A 35-μl volume of pronase E (10 mg/ml in TNE) was added, and the tubes were further incubated for 90 min at 50°C. A 175-μl volume of 5 M NaCl and 85 μl of 10% CTAB (hexadecyltrimethyl ammonium bromide) in 0.7 M NaCl were added, and the tubes were incubated at 65°C for 20 min. DNA was purified by phenol-chloroform extraction and precipitated with propan-2-ol. The DNA was pelleted by centrifugation (10,000 × *g*, 15 min), washed with cold 70% ethanol, dried in a vacuum desiccator, and then dissolved in sterile, molecular biology-grade water (BDH).

Protocol B was adapted from the method for extraction of DNA from gram-positive bacteria described by Grimont and Grimont (9). Pus samples and cultured bacteria were treated as in protocol A, and the pellets were suspended in 500 μl of TEST (10 mM Tris HCl, 5 mM EDTA, 1 mM NaCl, 0.5% Triton X-100; pH 8.0) with 10 mg of lysozyme per ml and vortex mixed. The tubes were incubated at 37°C for 30 min. A 50-μl volume of proteinase K (10 mg/ml) and 100 μl of 20% sarcosyl were added, and mixing was followed by an overnight incubation at 37°C. DNA was purified from the resulting lysates as described above for protocol A.

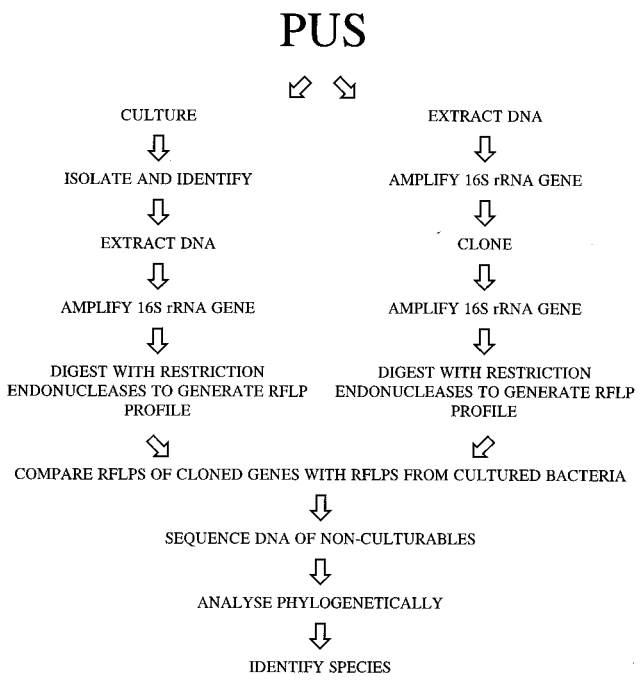

FIG. 1. Strategy for detection of unculturable bacteria in dentoalveolar abscesses.

PCR amplification of the 16S rRNA gene. PCR amplification of the 16S rRNA gene from dilutions of purified DNA was carried out by using a thermal reactor (Hybaid Ltd., Middlesex, England) and *Taq* polymerase (Boehringer Mannheim) according to the manufacturers' instructions except that 1.5 mM Triton X-100 was included in the reaction mix. There were 35 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, and an extension step at 72°C for 2 min. The primers used (19), modified from those described by Lane (12), were 27F (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′), 1392R (5′-CAC GGATCCACGGGCGGTGTGTRC-3′), and 1492R (5′-CACGGATCCTACG GGTACCTTGTTACGACTT-3′), where numbering refers to the position of the *Escherichia coli* 16S rRNA sequence to which the 3′ end of the primer anneals.

Cloning of amplified heterogeneous 16S rRNA gene products. Amplified 16S rRNA gene products were ligated into the vector pCRII, and recombinant molecules were transformed into the *E. coli* strain ONESHOT by means of the TA cloning kit (Invitrogen) according to the manufacturer's instructions.

Screening of 16S rRNA gene sequences in PCR products and clones. 16S rRNA gene PCR products obtained from amplification of DNA extracted from the cultured bacteria were digested with restriction endonucleases *Ban*I (New England Biolabs), *Cfo*I (Promega), *Hae*III (Promega), *Hph*I (New England Biolabs), and *Rsa*I (Promega) under the conditions recommended by the manufacturers. Restriction fragment length polymorphism (RFLP) profiles for cloned 16S rRNA gene products were obtained following PCR amplification by use of the 16S rRNA-specific primers used in the original amplification with boiled transformant cells in sterile water and digestion of the product with the restriction endonucleases described above.

Sequencing and phylogenetic analysis. Recombinant plasmids were purified from transformants by using Tip-20 columns (Qiagen, Dorking, United Kingdom), and the cloned 16S rRNA genes were sequenced by using the ALF (Pharmacia) automated sequencing system with the primers described by Lane (12). Preliminary analysis was carried out by using the services provided by the Ribosomal Database Project at the University of Illinois, Urbana-Champaign (16). The SIMILARITY_RANK program was used to determine sequences which were most similar to the submitted sequence, and CHECK_CHIMERA was used to test for sequences chimeric in structure.

Sequences were connected by using DNASIS (Hitachi) and aligned to each other and related species by means of Clustal V (10), and further analysis was performed with the PHYLIP suite of programs (7). More specifically, DNADIST was used to compare sequences by the Jukes Cantor algorithm and NEIGHBOR was used for neighbor-joining cluster analysis (20).

Nucleotide sequence accession numbers. GenBank accession numbers for the 16S rRNA genes sequenced in the study are as follows: PUS3.3, U43698; PUS3.23, U43697; PUS3.42, U43699; PUS8.29, U43700; and PUS10.40, U34035.

RESULTS

The strategy developed for the detection of unculturable bacteria is outlined in Fig. 1 and involved parallel microbiological and molecular analyses of the same three pus samples. A key step was screening by RFLP analysis to group the cloned 16S rRNA genes and matching the profiles of the clones with those of the bacteria cultured from the sample. Groups consisted of clones with RFLP profiles identical for all enzymes or with only minor differences (minor differences were defined as RFLP profiles which differed from those of the identical clones by a single restriction site for one or two enzymes). Major groups of clones which did not match with cultured bacteria were considered to represent putative uncultivable organisms. Figure 2 shows examples of the matching of RFLP profiles of cultivated organisms and clones. It can be seen that the profiles for *Peptostreptococcus micros* isolate 10/117 and *Fusobacterium nucleatum* 10/107 were identical to those of clones PUS10.60 and PUS10.48, respectively, for three enzymes. By contrast, clone PUS10.40 did not match with any of the profiles obtained from the cultured bacteria and was therefore considered to have been amplified from a putative unculturable organism.

The bacteria cultivated from sample 3 are described in Table 1. Twelve bacterial groups were identified by culture including gram-positive and gram-negative anaerobic cocci and bacilli and "*S. milleri*." The diverse flora found in this sample precluded an estimation of the relative proportions of the individual components. Table 2 shows the RFLP groups for sample 3 for two sets of PCR primers. Sixteen groups were seen with the primer pair 27F and 1492R, and 24 groups were seen with 27F and 1392R. When the RFLP profiles were compared with those obtained from the cultivated organisms, matches with *F. nucleatum*, a *Veillonella* sp., *Prevotella intermedia*, and *Dialister pneumosintes* isolates were seen. Groups 4 through 6 did not match any of the cultivated organisms, and 16S rRNA genes in representative clones were sequenced. Phylogenetic analysis of these sequences is shown in Fig. 3. Group 4 representative PUS3.3 clustered with *Prevotella oris*, and PUS3.23 (group 5) clustered with *Porphyromonas endodontalis*. The percent simi-

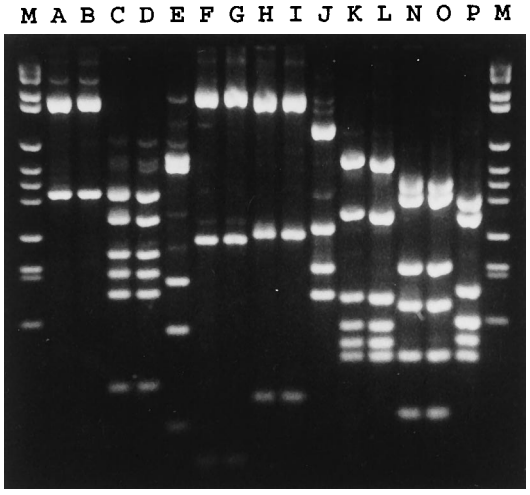

FIG. 2. Genes coding for 16S rRNA from cultured bacteria and clones amplified with primer pair 27F-1492R and digested with *Cfo*I (lanes A to E), *Hae*III (lanes F to J), and *Rsa*I (lanes K to P). Lanes A, F, and K, *P. micros* 10/117; lanes B, G, and L, clone PUS10.60; lanes C, H, and N, *F. nucleatum* 10/107; lanes D, I, and O, clone PUS10.48; lanes E, J, and P, clone PUS10.40; lanes M, molecular size markers (2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, 220, and 154 bp).

TABLE 1. Bacteria cultured from pus samples

Pus sample no.	Species	% of total cultivable population
3	*Campylobacter* sp.	ND[a]
	Dialister pneumosintes	ND
	Eubacterium sp.	ND
	Eubacterium timidum	ND
	Fusobacterium nucleatum	ND
	Fusobacterium sp.	ND
	Peptostreptococcus anaerobius	ND
	Prevotella buccalis or *P. veroralis*	ND
	Prevotella intermedia	ND
	Prevotella oris	ND
	"*Streptococcus milleri*"	ND
	Veillonella sp.	ND
8	*Actinomyces* sp.	33.6
	Fusobacterium nucleatum	0.2
	Peptostreptococcus micros	66.2
10	*Fusobacterium nucleatum*	5.0
	Peptostreptococcus micros	94.0
	Streptococcus mutans	1.0

[a] ND, not determined because of diversity of flora.

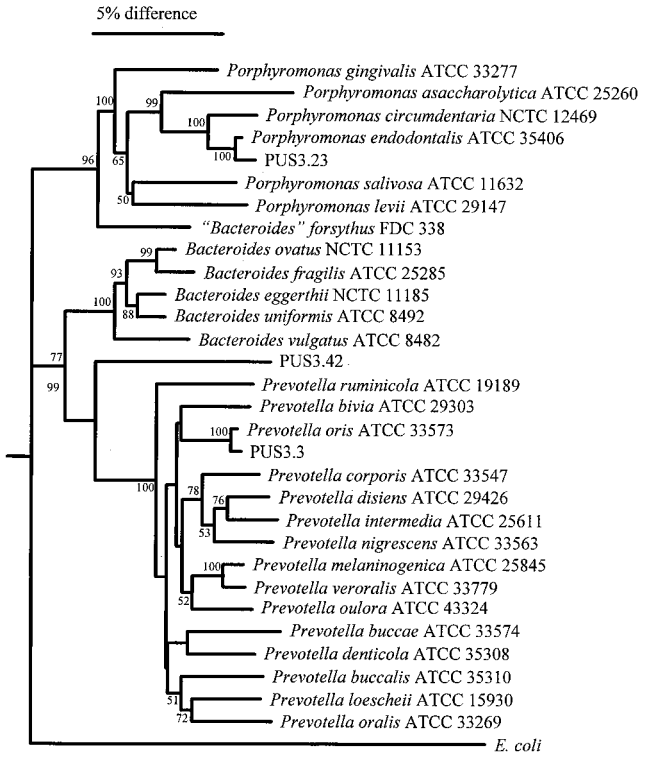

FIG. 3. Dendrogram constructed by the neighbor-joining method showing phylogenetic relationships of 16S rRNA gene sequences recovered from sample 3 and selected *Bacteroides*, *Prevotella*, and *Porphyromonas* spp. The analysis was a distance matrix method using 1,162 aligned bases. Bootstrap values shown at branch points are means of 100 replicates.

larities between these clones and the type strains of *P. oris* and *P. endodontalis* were 99.2 and 98.8%, respectively. Group 6 representative clone PUS3.42 did not cluster closely with any species found in the database but was phylogenetically most closely related to members of the genus *Prevotella*. The Ribosomal Database Project program SIMILARITY_RANK indicated that this sequence was most similar to that of *Prevotella oralis* but with a low similarity value of 82.7%.

The two primer pair combinations used produced broadly similar distributions of the RFLP groups. Thus, members of major RFLP groups 1 to 8 constituted over 90% of the sequences obtained with both primer pairs.

Sample 8 exhibited a more restricted flora than sample 3, which was dominated by *P. micros* and an *Actinomyces* species, with *F. nucleatum* as a minor component (Table 1). The molecular analysis (Table 3) showed that clones with RFLP profiles matching those obtained for the cultivated *F. nucleatum* were predominant, but no clones which matched with *P. micros*

TABLE 2. Groups of RFLP profiles of 16S rRNA genes amplified and cloned from sample 3 obtained with restriction endonucleases *Ban*I, *Cfo*I, *Hae*III, *Hph*I, and *Rsa*I[a]

Group no.	No. of clones with the indicated result with primer pair:				Cultivated organism with matching profile
	27F-1492R		27F-1392R		
	Identical profiles for 5 enzymes	Minor differences	Identical profiles for 5 enzymes	Minor differences	
1	6	4	4	6	*F. nucleatum*
2	5	4	8	1	*D. pneumosintes*
3	3	3	1	1	*Veillonella* sp.
4	11	5	13	6	NM[b]
5	9	0	20	7	NM
6	2	7	3	8	NM
7	1	0	3	3	NM
8	1	0	2	0	NM
9	2	0			NM
10	1	1			NM
11–16	1	0			NM
17			2	2	NM
18–20			1	1	NM
21			1	0	*P. intermedia*
22–32			1	0	NM

[a] DNA extraction protocol A was used.
[b] NM, no cultivated organism with matching profile.

TABLE 3. Groups of RFLP profiles of 16S rRNA genes amplified and cloned from sample 8 obtained with restriction endonucleases *Ban*I, *Cfo*I, *Hae*III, and *Rsa*I

Group no.	Protocol A				Protocol B with primer pair 27F-1492R		Cultivated organism with matching profile
	Primer pair 27F-1492R		Primer pair 27F-1392R				
	No. of clones with identical profiles for 4 enzymes	No. of clones with minor differences	No. of clones with identical profiles for 4 enzymes	No. of clones with minor differences	No. of clones with identical profiles for 4 enzymes	No. of clones with minor differences	
1	27	4	58	8	53	7	*F. nucleatum*
2	2	5	5	8	8	3	NM[a]
3	1	0					NM
4	1	0					NM
5	1	0					NM
6			1	1			NM
7–12			1	0			NM
13–15					1	0	NM

[a] NM, no cultivated organism with matching profile.

were seen. RFLP group 2 was the only other group represented by more than one clone. PUS8.29, an RFLP group 2 clone obtained with protocol B and the 27F-1492R primer pair, was sequenced. This was phylogenetically placed with some *Peptostreptococcus* species and was most similar to *P. micros* (97.1% similarity) (Fig. 4). There were no differences in the proportions of major RFLP groups 1 and 2 when different primer pairs or DNA extraction protocols were used (Table 3).

Sample 10 showed a restricted flora dominated by *P. micros*, with *F. nucleatum* and *Streptococcus mutans* also present as minor components (Table 1). The molecular analysis revealed groups of clones whose RFLP profiles matched those of the cultivated *P. micros* and *F. nucleatum* (Table 4). However, 18 other RFLP groups were identified, one of which, group 3, was a major component. Group 3 representative clone PUS10.40 was sequenced, and its phylogenetic assignment is shown in Fig. 5. The clone was placed in the β subdivision of the proteobacteria and was most similar to a strain of *Zoogloea ramigera*, a species of waterborne bacteria.

To confirm the validity of the sequence data obtained from the single clones discussed above, partial sequence data were obtained by using primer 27F for two additional clones from four RFLP groups: two from groups matching cultured bacteria (groups 1 and 2 from sample 10) and two from groups not matching cultured organisms (group 3, sample 10; group 4, sample 3). The similarities between the sequences are shown in Table 5. For six of the eight comparisons there was 100% similarity, and in the other two only one base differed between the sequences.

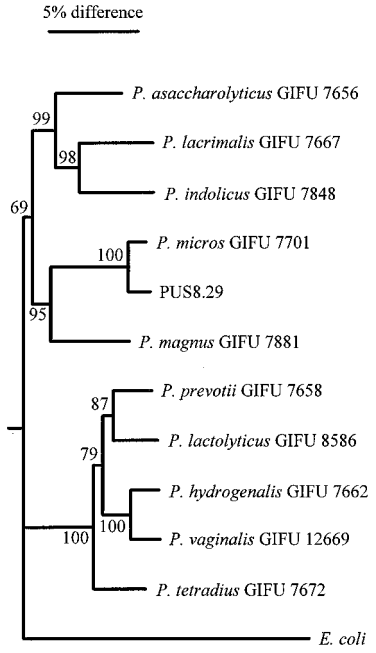

FIG. 4. Dendrogram constructed by the neighbor-joining method showing phylogenetic relationships of 16S rRNA gene sequences recovered from sample 8 and *Peptostreptococcus* spp. The analysis was a distance matrix method using 1,190 aligned bases. Bootstrap values shown at branch points are means of 100 replicates.

DISCUSSION

The cultivable flora from the dental abscesses reported in this paper was consistent with that described in the literature in that anaerobes were predominant, with streptococci additionally isolated in two of the three samples. The samples chosen, although few because of the complexity of the molecular analysis undertaken, were nevertheless considered to be representative of this type of acute infection. Nonselective media were used in the study in order to recover all of the organisms present. Inevitably, however, slowly growing organisms and/or those making up only a small proportion of the flora may not

TABLE 4. Groups of RFLP profiles of 16S rRNA genes amplified and cloned from sample 10 obtained with restriction endonucleases *Cfo*I, *Hae*III, and *Rsa*I[a]

Group no.	No. of clones with:		Cultivated organism with matching profile
	Identical profiles for 3 enzymes	Minor differences	
1	16	3	*P. micros*
2	7	1	*F. nucleatum*
3	22	5	NM[b]
4	2	2	NM
5	2	1	NM
6 and 7	2	0	NM
8	1	1	NM
9–21	1	0	NM

[a] DNA extraction protocol B was used with primers 27F and 1492R.
[b] NM, no cultivated organism with matching profile.

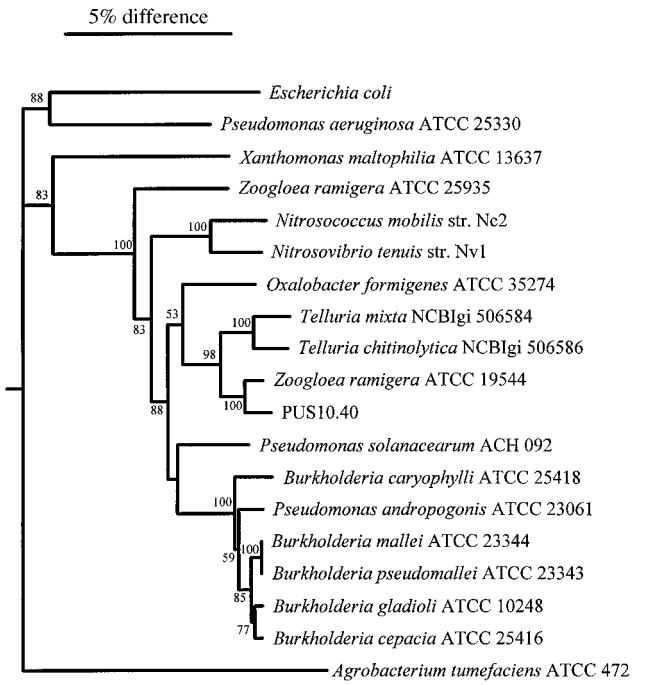

FIG. 5. Dendrogram constructed by the neighbor-joining method showing phylogenetic relationships of 16S rRNA gene sequences recovered from sample 10 and related proteobacteria. The analysis was a distance matrix method using 1,004 aligned bases. Bootstrap values shown at branch points are means of 100 replicates.

have been detected by cultivation. A panel of selective media may be useful in future studies to improve recovery of cultivable organisms.

The procedure used for the extraction of DNA from clinical specimens is obviously crucial, as this could be a source of substantial bias if, for example, gram-positive cells were not lysed. Two protocols were used in this study, one of which was optimized for gram-positive cell lysis, and there was no detectable difference between them in the populations of clones that were recovered.

Results summarized in Tables 2 to 4 clearly indicated that 16S rRNA gene amplification with different primer pair combinations did not influence the populations of sequences amplified. However, the choice of PCR primers for this type of study is of the utmost importance. An unnamed taxon of the genus *Eubacterium*, cluster 2, is particularly associated with dentoalveolar abscesses (23). The forward primer used in this study, 27F, does not anneal to the genes encoding 16S rRNA of this organism (unpublished observations). It was not surprising, therefore, that none of the clones in this study were identified as this organism. It may be that mixtures of PCR primers with broad specificity for different bacterial groups would be preferable to the use of single primer sets.

The strategy used to screen for unculturable bacteria was successful in that it was possible to match the RFLP profiles of major groups of clones with those of cultivable organisms. The validity of this approach was confirmed by the fact that the sequences of clones taken from the groups with identical profiles showed an extremely high degree of similarity. Interestingly, in almost every RFLP group containing more than one member, clones with minor differences in RFLP profile from those of the majority of strains were seen. These clones either may have arisen because of a lack of fidelity in the PCR when the technique was performed with mixed cultures or may represent chimeric products, as has been suggested previously (15). However, the fact that there was a numerically dominant group of identical profiles which proved to be identical in sequence suggests that the RFLP screening method used here is a reliable method for clone selection.

This study has revealed some phylotypes which are not closely related to any species currently represented in 16S rRNA databases. Although there are no universally agreed-upon levels of similarity which differentiate taxonomic ranks, in general, 16S rRNA sequences of species from different genera exhibit similarities of <94% (6). On this basis, PUS3.42 (82.7% similar to *P. oralis*) appears to represent a new genus related to *Prevotella*. The sequence data obtained here will be used to design probes and primers to enable this phylotype to be sought in further clinical specimens.

Phylotypes PUS3.3 and PUS3.23 displayed a high degree of similarity to the known species *P. oris* and *P. endodontalis*. *P. oris* was cultured from the sample, but the RFLP profiles of the cultivated isolates and the clones were significantly different. *P. endodontalis* is a well-known organism frequently found in dentoalveolar abscesses but was not isolated from sample 3. This particular strain may not have been culturable under the particular conditions employed in the study, but *P. endodontalis* has been recovered by use of these culture media and incubation conditions in our laboratory in the past. One of the drawbacks of the molecular technique for this application is that it may detect dead bacteria which have been killed by the host immune system but whose DNA remains present in the sample. This may explain the detection of normally culturable organisms by the molecular method alone.

F. nucleatum was of interest in that it was recovered at a higher frequency from all samples by the molecular method than by culture. This was particularly apparent for sample 8, in which *F. nucleatum* made up only 0.2% of the culturable flora, while 80% of the clones displayed RFLPs matching that of the cultivated *F. nucleatum*. Either *F. nucleatum* may be underestimated by culture or differential lysis could cause *F. nucleatum* DNA to dominate the isolated DNA.

TABLE 5. Sequence similarity between partial 16S rRNA gene sequences from cultured strains and phylotypes and members of groups with matching RFLP profiles

Strain and clone	No. of bases compared	% Sequence similarity to:							
		PUS10.184	PUS10.121	PUS10.137	PUS10.241	PUS3.48	PUS3.79	PUS10.15	PUS10.73
P. micros	294	99.7	100						
10/126									
F. nucleatum				100	100				
10/104	260								
PUS3.3	243					100	100		
PUS10.40	255							99.6	100

The identity of PUS10.40 suggested by the phylogenetic analysis was surprising. This phylotype was found to be related to a group of gram-negative, aerobic bacteria none of which are part of the normal oral flora or have been reported to be associated with oral infections. The bacteria in this branch of the β-proteobacteria are a group of waterborne or soil organisms, some of which are plant pathogens, although *Burkholderia cepacia* has been implicated in human opportunistic infection (25). The phylotype detected here, which comprised around 30% of the clones from sample 10, may have acted as an exogenous opportunist, but it is difficult to see how the organism would have gained access to the root canal.

This study has demonstrated the potential of molecular methods for the characterization of the microflora associated with oral infections, avoiding the biases of cultural techniques. The procedures are obviously applicable to infections at all body sites and should lead to a more complete understanding of associations between bacteria and disease. Although the method described here is relatively time-consuming at present, the development of DNA probes from the sequence data should allow rapid detection of as yet unculturable bacteria.

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