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Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets

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Introduction: Molecular ecological analysis based on 16S rRNA gene sequence analysis is well established for the characterisation of complex bacterial communities. However, 'universal' PCR primers can introduce biases into the analysis of the species composition of clone libraries because of mismatches between the primer and target organism sequences. In this study, three universal primer sets were compared for the analysis of the microflora in subgingival plaque.
Methods: Three subgingival plaque samples were collected from two subjects with localised severe chronic periodontitis. Half of each sample was cultured while DNA was extracted from the remaining half and 16S rDNA amplified with universal primer pairs 27F, 1525R (A); 27F, 1492R (B) and 530F, 1525R (C). Amplified genes were cloned, sequenced and identified by comparison with 16S rRNA databases.
Results: 137 taxa were identified among 177 isolates and 417 clones sequenced. Of these, 86 were detected only by the molecular technique whereas 26 were found only by culture. Sequences from 81 taxa did not correspond to those of named species and of these, 38 were not represented in the nucleotide databases. 16S RNA genes for these 38 taxa were sequenced and deposited with GenBank.
Conclusion: The use of three sets of universal primers allowed the identification of 38 novel bacterial phylotypes. There were marked differences in the composition of the libraries generated by the different primer sets. A combination of molecular and cultural techniques is recommended to maximise the coverage of detection of bacterial taxa in oral samples.

Key words: PCR, periodontitis, unculturable bacteria

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Periodontitis is an inflammatory condition affecting the supporting tissues of the teeth which, if left untreated, will lead to tooth loss. The periodontal pockets formed between the gingivae and the teeth as a result of the disease become colonised with a complex, predominantly anaerobic, microflora (19). More than 350 species have been isolated by culture and of these, a small group including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Actinobacillus actinomycetemcomitans* have been implicated as periodontal pathogens (7). However, it is now recognised that approximately 50% of the oral flora is unculturable. It is therefore likely that the diversity of the periodontal microflora is considerably greater than has been demonstrated by culture and that taxa important in disease aetiology are part of the unculturable component (31).

Molecular methods for the characterisation of complex bacterial communities are now well established. Polymerase chain reaction (PCR), cloning and sequencing of 16S rDNA have allowed the dissection of bacterial communities from a wide variety of sources including the environment and human health and disease (13, 25, 29). In the human oral cavity, as yet uncultured organisms have been found to constitute a major part of the microflora in health and

in a variety of oral infections (8, 15, 17, 20, 21, 23).

Molecular ecology studies rely on the use of universal PCR primers, which have inherent biases (24, 29). For any given target bacterial community, it is therefore important to understand the extent of the bias that may be introduced by the choice of particular primer sets.

The aim of this study was to perform a combined cultural and molecular analysis of the microflora in advanced periodontitis and to compare the effect of using three different universal primer pairs on the composition of the bacterial community revealed by molecular analysis.

Material and methods

Ethical approval for the study was granted by the Guy's Hospital Research Ethics Committee. Patients participating in the study gave their informed consent. Three subgingival plaque samples were collected from two patients with localised severe chronic periodontitis. In the first patient, a 47-year-old female, a sample was collected from an 8-mm pocket associated with the maxillary right first molar. From the second patient, a 50-year-old male, samples were collected from a 10-mm pocket associated with the mandibular left first molar and an inflamed site with no loss of attachment associated with the maxillary right canine.

After removal of supragingival plaque, subgingival samples were collected with a curette and placed in 2 ml normal saline that had been prereduced for 24 h in an anaerobic workstation (MACS 1000, Don Whitley Scientific, Shipley, UK) in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. Samples were taken immediately to the laboratory and placed inside an anaerobic workstation. The samples were then dispersed by repeated passage through at 25 G needle and split for cultural and molecular analysis.

Ten-fold dilutions of the samples were prepared in reduced transport medium (3). Dilutions of the sample up to 10^4-fold were plated onto Fastidious Anaerobe Agar (LabM, Bury, UK) + 5% horse blood plates (FAA) (three plates per dilution) and Blood Agar Base no. 2 (LabM) + 5% horse blood plates (BA, six plates per dilution). FAA plates and one triplicate set of BA plates were incubated anaerobically in an anaerobic workstation for 7 days at 37°C and the remaining set of BA plates incubated in air + 5% CO_2 for 3 days at 37°C. Following incubation, plates with between 30 and 300 colonies were counted. Fifteen isolates were selected at random from the BA plates incubated in air + CO_2. Twenty-five colonies were subcultured at random from both BA and FAA plates that had been incubated anaerobically. Random selection of colonies was ensured by use of the following procedure. The plates were first counted and then the total number of colonies divided by the number of isolates required. The reverse of the plate was then divided into zones so as to give approximately the required number of each colonies in each zone. One zone was then chosen at random, after which the plate was turned over and all the colonies in that zone subcultured, moving methodically from the left hand side of the zone to the right, until the required number of colonies were selected.

DNA was extracted from the sample by the method of Grimont & Grimont (11). 16S rRNA genes were amplified using the following pairs of primers: for the bacterial domain – primer set A: 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1525R (5′AAGGAGGTGWTCCARCC3′); primer set B: 27F and 1492R (5′TACGGYTACCTTGTTACGACTT3′) and for the bacterial and archaeal domains – primer set C: 530F (5′GTGCCAGCMGCCGCGG3′) and 1525R (18). Five replicate amplifications using Ready to Go PCR beads (Amersham Biosciences, Little Chalfont, UK) were set up for each sample/primer pair using 1 μl of extract, 1 μl each of the primers at a concentration of 3 μM and 22 μl sterile water. Amplifications were carried out on a UnoII Thermocycler (Biometra, Gottingen, Germany) with 20 cycles of 94°C for 60 s, 50°C for 30 s, and 72°C for 2 min. There was a final 5-min extension step at 72°C, after which the samples were kept at 4°C until purified.

The five replicate PCR products were pooled, purified by means of a spin column (Qiaquik, Qiagen, Crawley, UK) and then cloned into the pGEM-T Easy vector (Promega, Southampton, UK) according to the manufacturer's instructions. The vector was then transformed into *Escherichia coli* XL1 Blue MFR′ supercompetent cells (Stratagene, Amsterdam, the Netherlands) according to the manufacturer's instructions. A total of 200 white colonies were chosen at random and the presence of inserts checked by PCR using vector specific primers SP6 and T7 under the conditions described above. Aliquots were electrophoresed on a 1% agarose gel, stained with ethidium bromide and then checked for the presence of the appropriate size DNA fragment.

Fifty clones, selected sequentially, from each library together with amplified 16S rDNA from the isolates were then partially sequenced using the universal sequencing primers 357F (Libraries A and B) or 530F (Library C) to give at least 500 bp of sequence in each case. Sequencing was performed using a CEQ2000 automated DNA sequencer (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions. Additional sequencing was performed for some groups of organisms as required.

The libraries were checked for the presence of chimerae by means of the CHIMERA_CHECK programme made available by the Ribosomal Database Project II (5). Sequences were presumptively identified by means of the programme SEQUENCE_MATCH at the Ribosomal Database Project II and by BLAST interrogation of the GenBank database. From the phylogenetic position indicated by SEQUENCE_MATCH and BLAST, related sequences were selected from sequence databases and aligned by means of CLUSTAL X (28). Further analysis was performed using the PHYLIP suite of programmes (9), specifically construction of a distance matrix using DNADIST and tree construction by the neighbour-joining method by means of NEIGHBOR. Trees were viewed using TREEVIEW (22). The nomenclature used was that described for use in the second edition of Bergey's Manual of systematic bacteriology (10). Full sequencing was performed of the 16S rRNA genes of isolates and clones identified as novel from the partial sequence analysis. Primers 27F, 357F, 519R, 907R, 926F, 1100R, 1114F, 1492R and 1525R (18) were used to obtain triple coverage of each gene. The nucleotide accession numbers of 16S rRNA genes of novel taxa sequenced in this study are shown in Table 1.

Results

16S rRNA gene sequences from 177 isolates and 417 clones were presumptively identified by means of the RDPII SEQUENCE_MATCH programme and BLAST interrogation of the NCBI nucleotide database. Nine sequences were found to be chimeric and were not included in subsequent analyses. Identification of the remaining sequences was confirmed by alignment with related sequences and phylogenetic analysis. A level of 99% sequence identity was used as the cut-off for identification as a specific taxon. However, taxa belonging to some genera,

Table 1. Bacterial species and phylotypes detected from subgingival plaque (novel taxa sequenced in this study are shown in bold and representative strain/clone number and GenBank accession number are given)

Actinobacteria
- *Actinomyces israelii*
- *Actinomyces meyeri*
- *Actinomyces naeslundii*
- *Actinomyces oricola*
- *Actinomyces* B27SC
- *Actinobaculum* EL030
- *Olsenella uli*
- ***Actinobaculum*** P1 (strain P2P_19, AY207066)

Bacteroidetes
- *Bacteroidales* E2a
- *Bacteroidales* E2b
- *Bacteroidales* E3
- *Bacteroides* AU126
- *Capnocytophaga gingivalis*
- *Capnocytophaga granulosa*
- *Capnocytophaga sputigena*
- *Capnocytophaga* BB167
- *Capnocytophaga* BM058/BU084
- *Capnocytophaga* DS022
- *Prevotella buccae*
- *Prevotella dentalis*
- *Prevotella intermedia*
- *Prevotella melaninogenica*
- *Prevotella nigrescens*
- *Prevotella* B31FD
- *Prevotella* FM005
- *Prevotella* DA058
- *Porphyromonas endodontalis*
- *Porphyromonas gingivalis*
- ***Bacteroidales*** **P1 (clone P4PB_6, AY341819)**
- ***Capnocytophaga*** **P1 (clone P4GA_52, AY429468)**
- ***Capnocytophaga*** **P2 (strain P4P_12, AY429469)**
- ***Capnocytophaga*** **P4 (strain P4G_35, AF538853)**
- ***Porphyromonas*** **P1 (clone P2PB_52, AY207054)**
- ***Porphyromonas*** **P2 (clone P4GB_100, AY207057)**
- ***Prevotella*** **P2 (clone P4PB_83, AY207050)**
- ***Prevotella*** **P4 (strain P4P_53, AY944134)**
- ***Prevotella*** **P6 (clone P4PB_24, AY331415)**
- ***Prevotellaceae*** P1 (strain P4P_62, AY207061)

Chloroflexi
- *Chloroflexi* P1 (clone P2PB_23, AY331414)

Firmicutes
- *Anaeroglobus geminatus*
- *Butyrivibrio* DA074
- *Centipeda periodontii*
- *Desulfobulbus* R004/CH032
- *Desulfomicrobium orale*
- *Dialister invisus*
- *Dialister pneumosintes*
- *Eubacteriaceae* E2
- *Eubacterium brachy*
- *Eubacterium infirmum*
- *Eubacterium nodatum*
- *Eubacterium saburreum*
- *Eubacterium saphenum*
- *Eubacterium sulci*
- *Eubacterium* BB142
- *Eubacterium* BR088
- *Eubacterium* DA014
- *Eubacterium* DO016
- *Eubacterium* FX028
- *Filifactor alocis*
- *Firmicutes* E1
- *Gemella* 933–88
- *Granulicatella adiacens*
- *Lachnospiraceae* E1
- *Mogibacterium pumilum/neglectum*
- *Mogibacterium timidum*
- *Paenibacillus* sp.
- *Peptococcus* E1
- *Peptostreptococcus anaerobius*
- *Peptostreptococcus micros*
- *Selenomonas artemidis*
- *Selenomonas dianae*
- *Selenomonas flueggei*/AH132
- *Selenomonas infelix*/EY047
- *Selenomonas noxia*/CI002/EQ054
- *Selenomonas sputigena*/EW051/DD020
- *Selenomonas* CS015/P4
- *Selenomonas* EW076/DY027
- *Selenomonas* EW079/GAA14/DM071/EZ011
- *Selenomonas* EW084/DS071
- *Staphylococcus warneri*
- *Streptococcus anginosus*
- *Streptococcus constellatus*
- *Streptococcus gordonii*
- *Streptococcus infantis/mitis/oralis*
- *Streptococcus intermedius*
- *Streptococcus salivarius*
- *Streptococcus sanguis*
- *Streptococcus sinensis/cristatus*
- *Veillonella dispar*
- *Veillonella parvula*
- ***Abiotrophia*** **P1 (clone P4PA_155, AY207063)**
- ***Clostridiales*** **P1 (clone P4PA_66, AY207065)**
- ***Clostridiales*** **P3 (clone P4PB_122, AF538854)**
- ***Clostridiales*** **P4 (clone P4GC_38, AY207058)**
- ***Eubacteriaceae*** P2 (clone P2PC_29, AF538855)
- ***Eubacteriaceae*** P3 (clone P2PB_46, AF538856)
- ***Eubacteriaceae*** P4 (strain P4P_50, AY207060)
- ***Lachnospiraceae*** P1 (clone P4PC_12, AF538857)
- ***Lachnospiraceae*** P3 (clone P4PC_43, AF538858)
- ***Peptostreptococcus*** **P3 (strain P4P_31, AY207059)**
- ***Peptostreptococcus*** **P4 (clone P4PA_156, AF538859)**
- ***Schwartzia*** **P2 (clone P2PC_32, AF538860)**
- ***Selenomonas*** **P2 (clone P4PA_36, AF538861)**
- ***Selenomonas*** **P4 (clone P2PA_80, AY207052)**
- ***Selenomonas*** **P5 (clone P4PA_145, AY341820)**
- ***Syntrophomonadaceae*** P1 (clone P4PB_66, AY341821)
- ***Streptococcus*** **P2 (clone P2PA_41, AY207051)**
- ***Streptococcus*** **P3 (clone P4PA_13, AY207062)**
- ***Streptococcus*** **P4 (clone P4PA_30, AY207064)**

Proteobacteria
- *Campylobacter concisus*
- *Campylobacter gracilis*
- *Campylobacter rectus/showae*
- *Campylobacter* BB120
- *Cardiobacterium hominis*
- *Kingella* DE012
- *Neisseria subflava*
- *Neisseria* AP085/AP015
- ***Haemophilus*** **P1 (clone P4PC_39, AY331417)**
- ***Neisseria*** **P1 (clone P4PC_20, AY341823)**

Fusobacteria
- *Fusobacterium nucleatum*
- *Fusobacterium necrophorum*
- *Leptotrichia hofstadii*
- ***Leptotrichia*** **P1 (clone P2PB_51, AY207053)**

Synergistes
- *Synergistes* BA121
- *Synergistes* BH017
- *Synergistes* D006
- *Synergistes* W028

TM7
- TM7 I025
- **TM7 P1 (clone P4PB_40, AY331416)**

Spirochaetes
- *Treponema socranskii*
- *Treponema* sp. 3:E:AT013
- *Treponema* BZ013
- *Treponema* sp. VI:G:G47
- *Treponema* sp. I:G:T21
- *Treponema* sp. I:8:G57
- ***Treponema*** **P1 (clone P4GB_42, AY341822)**
- ***Treponema*** **P2 (clone P2PB_60, AY345159)**
- ***Treponema*** **P3 (clone P2PB_53, AY207055)**

notably *Selenomonas* and *Streptococcus*, could not be definitively identified at this level and a choice of possible taxa is given as the identification. In addition, the nucleotide databases contain a large number of sequences for taxa related to the *Streptococcus mitis/oralis* group that differ by only a small number of bases. For this group only, identifications are given as groups corresponding to named species and their associated, closely related, phylotypes.

Applying this identification procedure, 137 taxa belonging to nine phyla were identified (Table 1), only 56 of which were named species. Forty-three taxa were identified as phylotypes for which sequences have been deposited in sequence databases, and the remaining 38 were novel. Nineteen of these belonged to the phylum *Firmicutes* (Fig. 1) and 10 to the phylum *Bacteroidetes* (Fig. 2). The remainder belonged to the phyla *Spirochaetes* [3 taxa], *Proteobacteria* [2], *Actinobacteria* [1], *Chloroflexi* [1], *Fusobacteria* [1] and TM7 [1].

Fifty-one taxa were detected by culture and 111 by the molecular analysis. Twenty-six taxa were found by culture alone and 86 by the molecular technique alone. Figure 3 shows the distribution of phyla detected by the cultural and molecular analyses. Members of the

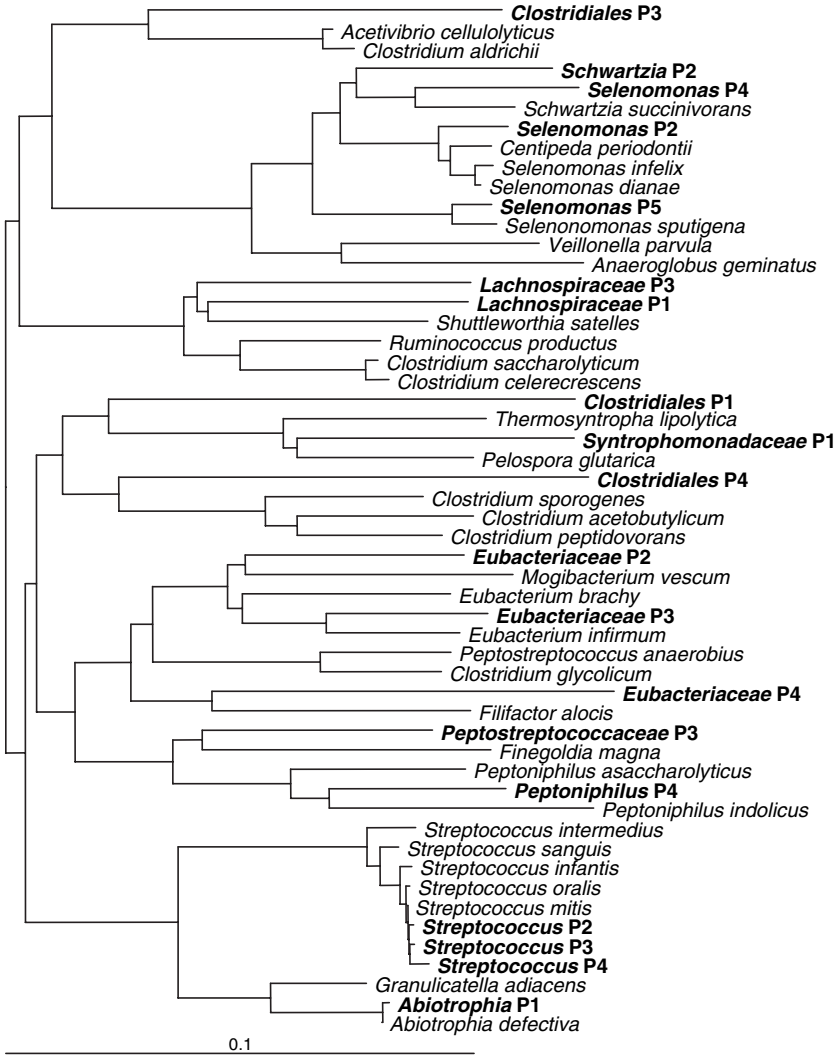

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons over 793 aligned bases showing relationship between novel taxa identified in the phylum *Firmicutes* and related species. Novel taxa detected in this study are shown in bold. Tree was constructed using the neighbour-joining method following distance analysis of aligned sequences. Scale bar shows number of nucleotide substitutions per site.

Firmicutes made up around 50% of the flora detected by culture but constituted 70% of the molecular clones. In contrast, the phyla *Actinobacteria* and *Bacteroidetes* were detected in greater numbers by culture than by the molecular approach.

Figure 4 shows the identification of the clones in each of the three libraries, grouped by phylum: 91% fell into three phyla – *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. Eight of the nine phyla were seen in Library B, compared to six in Library A and four in Library C. There were marked differences in the detection of members of the phyla in the three libraries; for example, in Libraries A and C, the *Firmicutes* made up 77% and 79% of the clones, respectively, but only 53% of the clones in Library B. By contrast, the *Bacteroidetes* made up only 9% and 5% of Libraries A and C, respectively, but 31% of Library B. *Proteobacteria* and *Synergistes* were most numerous in Library C whereas members of the *Spirochaetes* and *Fusobacteria* were not detected in this library. Representatives of candidate Division TM7 and the phyla *Actinobacteria* and *Chloroflexi* were only found in Library B.

Table 2a–c shows the composition of each library, with the predominant members of each library given in turn. Table 2b shows that although *P. gingivalis* was the predominant organism in Library B, it was not detected at all in Library C. Similarly, *Prevotella intermedia* made up 5.2% of the clones in Library B but was not detected in Library A and made up only 0.7% of Library C.

Further analysis of the differences between libraries was performed by the estimation of shared species statistics (4) using the programme ESTIMATES (6). The observed and estimated species richness of the libraries was: Library A: 54, 130; Library B: 63, 168; Library C: 52; 98. Thus Library B was the most species rich, followed by Libraries A and C. The observed and estimated shared species between each pair of libraries was as follows: A and B: 24, 61; A and C: 26, 134; B and C: 24, 92. Libraries A and C were therefore the most similar and A and B the least similar. However, comparison of the estimated species richness (14) of the three libraries showed that the 95% confidence limits of the total estimated number of taxa in each library overlapped, indicating

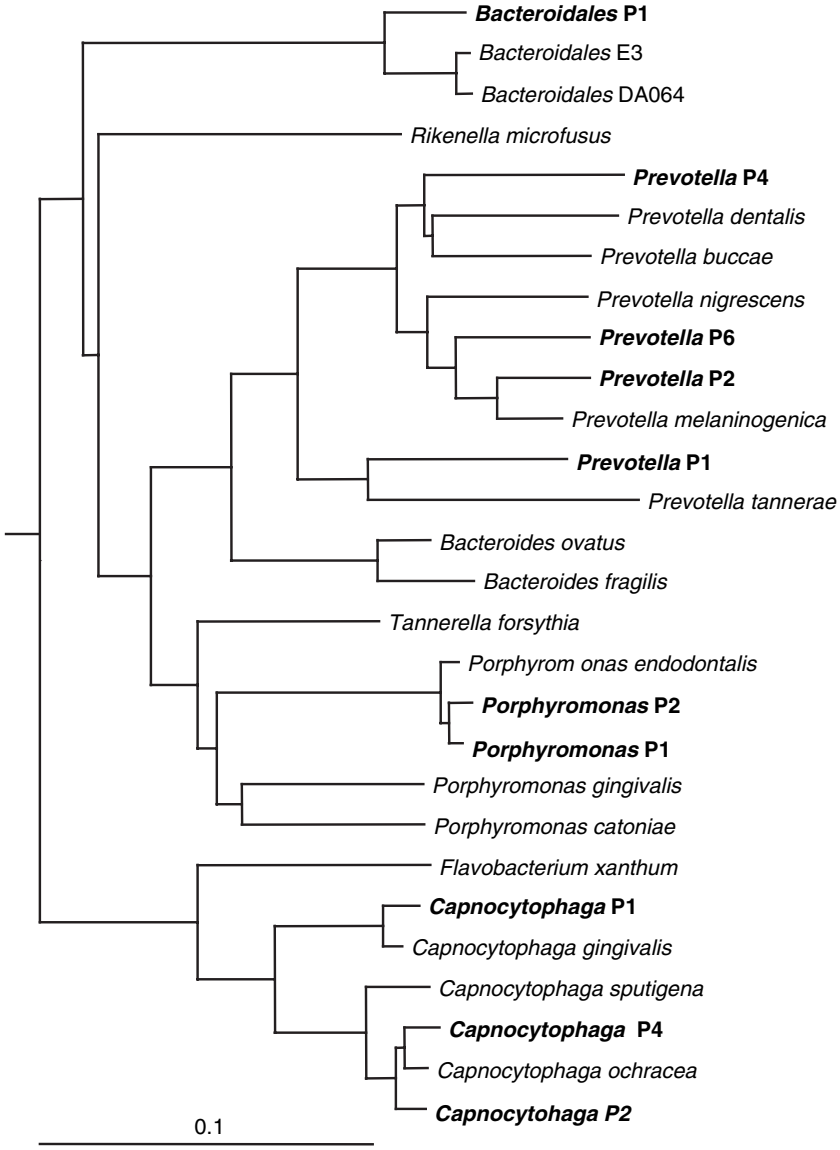

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence comparisons over 1276 aligned bases showing relationship between novel taxa identified in the phylum *Bacteroidetes* and related species. Novel taxa detected in this study are shown in bold. Tree was constructed using the neighbour-joining method following distance analysis of aligned sequences. Scale bar shows number of nucleotide substitutions per site.

no significant difference between them (Table 3).

Discussion

This study supports and adds to previous reports describing the diversity of the periodontal microflora (17, 23) and of the oral microflora in general (8, 20, 21). Overall, the range of phyla found in this study and that of Paster et al. (23) was greater than that found by Kroes et al. (17) who did not detect members of the *Spirochaetes*, TM7 or *Synergistes* (found in this study and by Paster et al.) or *Chloroflexi*, found only in this study, or OP11, found only by Paster et al. The reasons for these differences are unclear but are likely to be related to the different range of primer sets used in each study and variations in PCR conditions and cloning protocols. Clearly, much diversity from this habitat remains to be revealed, since, despite the large number of sequences representing novel phylotypes deposited with the sequence databases from earlier studies, a substantial number of additional novel taxa were identified here, predominantly in the phyla *Firmicutes* and *Bacteroidetes*.

It was interesting that, despite the wide range of taxa found, these did not include the established periodontal pathogens *A. actinomycetemcomitans* and *T. forsythia*. *A. actinomycetemcomitans* was also not detected by Kroes et al. (17) but is often present in this habitat as a relatively small proportion of the microflora and, in cultural studies, selective media are normally used to maximise detection of this species. The small number of samples studied here also make it likely that, by chance, the individuals and sites examined were not colonised by these two species at detectable levels.

The extensive comparison of the molecular and cultural analyses in this study revealed a pattern of differences in detection that was virtually identical to that previously described in similar analyses of endodontic infections (21) and dentinal caries (20). Most striking was the almost

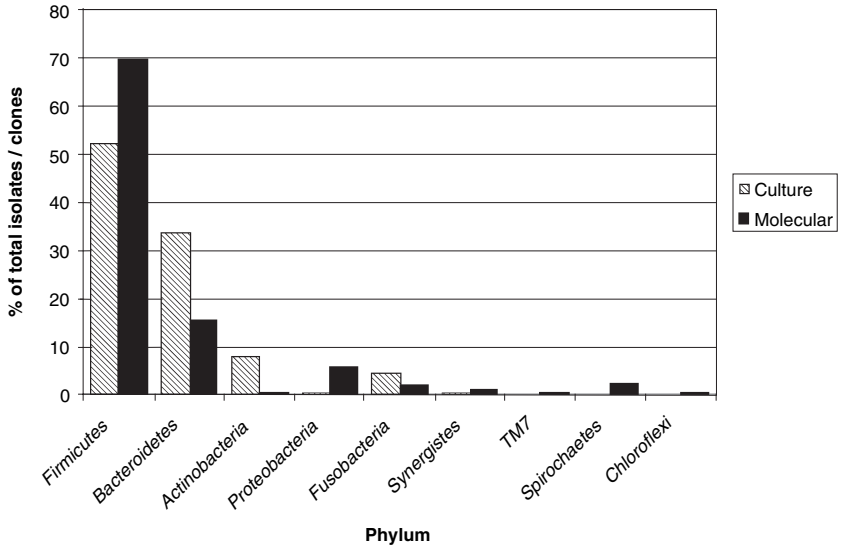

Fig. 3. Comparison of cultural and molecular analyses of the subgingival microflora by phylum.

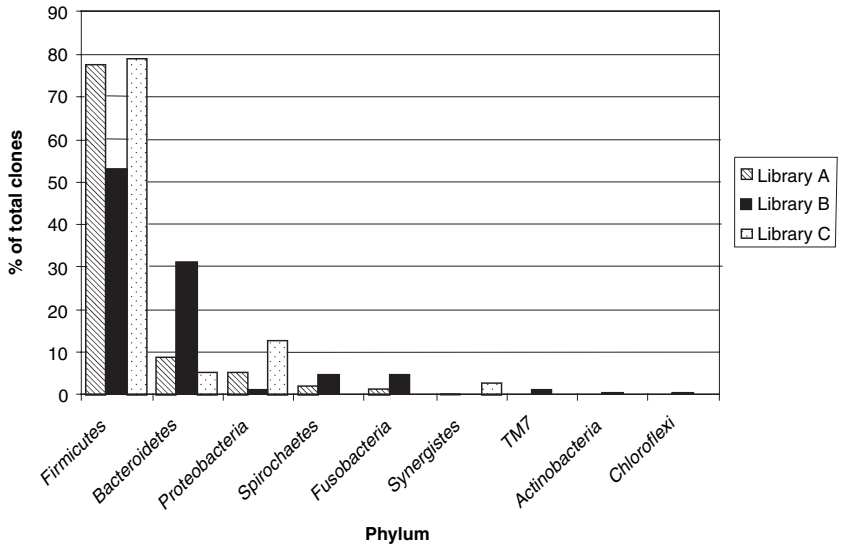

Fig. 4. Comparision of universal primer pairs for the detection of phyla in subgingival plaque samples.

complete failure to detect members of the *Actinobacteria* with the molecular method. Refinement of the methodology is urgently needed to ensure that members of this phylum, which includes important human oral pathogens such as *Actinomyces israelii* and other taxa implicated in oral disease such as *Slackia exigua* (24, 28), are not overlooked as PCR-based techniques are increasingly used in diagnosis. It is most likely that the failure to amplify the 16S rRNA genes of *Actinobacteria* relates to the high G + C content of their DNA. High G + C templates are known to dissociate poorly and, in addition, *Taq* polymerase frequently pauses or prematurely terminates when it encounters G + C rich regions (12). To overcome this, the supplementation of PCR reactions with betaine and DMSO to stabilise the template and in addition, the use of two enzymes *Klentaq*1 and *Pfu* DNA polymerase, as alternatives to *Taq* polymerase, have been proposed (1).

The problem of bias arising from the use of specific PCR primers is well known but poorly understood (16). The number of cycles used in the PCR appears to be important as the use of high numbers of cycles exacerbates biases (2, 26). In this study, 20 cycles were chosen to give a trade-off between maintaining the initial structure of the community in the amplified products and producing sufficient product for efficient cloning.

The comparison of the use of three different sets of universal primers revealed substantial differences between them in terms of both the overall distribution of phyla seen in each library and also the detection of individual species. The mechanism causing these differences is unclear and requires investigation. Whatever the cause, it would seem prudent to include multiple primer sets in future studies to maximise the coverage of the analysis of the communities. However, if just one primer set is to be used, on the basis of the analysis presented here, then Library B(27F/1492R) would appear to be the most useful. This detected the highest number of both phyla and individual taxa, although, in the case of the latter, there were no significant differences between the libraries. It might also be useful to assess the community structure revealed by different primer sets in pilot studies using a profiling technique such as denaturing gradient gel electrophoresis (DGGE) (30).

In conclusion, this study has identified 38 novel bacterial phylotypes from the subgingival environment. As in previous studies using similar methodology, the high G + C phylum, *Actinobacteria*, was under-represented in all clone libraries compared to culture. In addition, marked differences were found in the composition of clone libraries obtained using three different PCR primer sets. The construction of libraries from multiple primer sets would appear to the most appropriate way to maximise the coverage of detection of bacterial taxa present in oral samples. In addition, a number of taxa were detected only by culture. The use of a combination of cultural and molecular techniques is also recommended to maximise detection of bacterial taxa associated with this habitat.

Acknowledgments

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Table 2. Predominant taxa ranked by Libraries A (**a**), B (**b**) and C (**c**)

(**a**)	A	B	C
Streptococcus infantis/mitis/oralis	**13.1**	9.5	11.7
Streptococcus sinensis/cristatus	**10.1**	8.3	5.1
Filifactor alocis	**6.7**	5.2	12.6
Eubacterium saphenum	**6.2**	1.3	5.6
Selenomonas infelix/EY047	**3.6**	2.8	0.0
Eubacterium nodatum	**2.9**	1.4	0.7
Selenomonas sputigena/EW051/DD020	**2.9**	0.7	3.6
Streptococcus sanguis	**2.7**	3.3	0.0
Peptostreptococcus anaerobius	**2.3**	1.3	0.7
Porphyromonas gingivalis	**2.2**	10.5	0.0
Anaeroglobus geminatus	**2.1**	0.0	0.0
Clostridiales P1	**2.1**	0.0	1.5
(**b**)	A	**B**	C
Porphyromonas gingivalis	2.2	**10.5**	0.0
Streptococcus infantis/mitis/oralis	13.1	**9.5**	11.7
Streptococcus sinensis/cristatus	10.1	**8.3**	5.1
Prevotella intermedia	0.0	**5.2**	0.7
Filifactor alocis	6.7	**5.2**	12.6
Streptococcus sanguis	2.7	**3.3**	0.0
Fusobacterium nucleatum	1.8	**3.3**	0.0
Selenomonas infelix/EY047	3.6	**2.8**	0.0
Prevotella sp. oral clone DA058	0.0	**2.8**	0.7
Dialister invisus	0.7	**2.1**	2.2
Prevotella FM005	0.0	**2.1**	0.0
Streptococcus intermedius	0.0	**2.0**	0.0
(**c**)	A	B	**C**
Filifactor alocis	6.7	5.2	**12.6**
Streptococcus infantis/mitis/oralis	13.1	9.5	**11.7**
Eubacterium saphenum	6.2	1.3	**5.6**
Streptococcus sinensis/cristatus	10.1	8.3	**5.1**
Campylobacter rectus/showae	0.7	0.0	**5.0**
Peptococcus E1	0.0	0.6	**4.4**
Selenomonas sputigena/EW051/DD020	2.9	0.7	**3.6**
Mogibacterium pumilum/neglectum	1.6	1.4	**3.6**
Selenomonas dianae	0.7	0.0	**2.9**
Synergistes BH017	0.0	0.0	**2.3**
Eubacteriaceae E2	0.0	0.0	**2.2**
Dialister invisus	0.7	2.1	**2.2**
Veillonella parvula	0.9	1.4	2.1

Table 3. Statistical estimates of species richness in 16S rRNA gene libraries constructed with different universal primers

Library	No. of clones	No. of taxa	ACE	Chao1	Chao1_SD	95% confidence intervals
A	125	54	129.91	116.54	36.59	85.03–180.04
B	146	63	168.29	137.71	38.47	102.93–202.77
C	137	52	97.69	83.6	18.23	67.81–115.15

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