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Has the use of molecular methods for the characterization of the human oral microbiome changed our understanding of the role of bacteria in the pathogenesis of periodontal disease?

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Abstract

Background: Only around half of oral bacteria can be grown in the laboratory using conventional culture methods. Molecular methods based on 16S rRNA gene sequence are now available and are being used to characterize the periodontal microbiota in its entirety.

Aim: This review describes the cultural characterization of the oral and periodontal microbiotas and explores the influence of the additional data now available from culture-independent molecular analyses on current thinking on the role of bacteria in periodontitis.

Results: Culture-independent molecular analysis of the periodontal microbiota has shown it to be far more diverse than previously thought. A number of species including some that have yet to be cultured are as strongly associated with disease as those organisms traditionally regarded as periodontal pathogens. Sequencing of bacterial genomes has revealed a high degree of intra-specific genetic diversity.

Conclusions: The use of molecular methods for the characterization of the periodontal microbiome has greatly expanded the range of bacterial species known to colonize this habitat. Understanding the interactions between the human host and its commensal bacterial community at the functional level is a priority.

Key words: 16S rRNA; microbiology; molecular ecology; periodontitis

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Periodontitis is an inflammatory disease in which bacteria play a central role. The microbiology of periodontal disease and the various factors affecting the composition of the periodontal microbiota have been extensively reviewed by others and are beyond the scope of this review. A recurring theme in the characterization of the periodontal microbiota has been the desire to associate specific organisms with disease in a way that implies causation. To date, there is no definitive evidence that demonstrates that periodontitis is an infectious disease in the classical sense, in that a susceptible individual could be exposed to a pathogen, which would then colonize the host and go on to cause the signs and symptoms of the disease via the production of specific virulence factors. Indeed, the species that have been associated with destructive disease are not exogenous pathogens but form part of the normal oral microbiota, although it is true that, in health, certain species may be present in

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only small numbers or be undetectable because of the limitations of sampling and detection methods. The first part of this review will discuss the history of the investigation of the periodontal microbiota to highlight how certain species came to be regarded as pathogens. It has long been recognized that bacterial culture methods do not reveal the true extent of the range of organisms that can be found in different habitats on Earth. In recent years, new methods have become available, based on the direct examination of nucleic acids from samples. The application of these methods to the characterization of the periodontal microbiota will also be reviewed, and finally, an attempt will be made to assess the impact of culture-independent microbiota characterization methods on our understanding of periodontal disease pathogenesis. Possible experimental approaches to take advantage of this new knowledge will also be proposed.

The nomenclature of oral bacteria has undergone considerable revision since the first studies describing the periodontal bacteria. For the purpose of clarity in this review, where it is possible to deduce the current names of species referred to in earlier work, the current validly published name will be used, with the name used in the original publication following in parentheses.

Cultural analysis of the periodontal microbiota

Oral bacteria were among the first microorganisms to be described on Earth, by Antony van Leeuwenhoek in his descriptions of animacules from scrapings from teeth in 1676 (Porter 1976). It was two centuries later, that following the development of agar-containing solid culture media for the growth of bacteria, which allowed the identification of many pathogens of man, that periodontitis came under study. Miller (1890) considered bacteria to be the cause of the suppuration associated with periodontitis but was disappointed in being able to culture only a small proportion of the morphotypes that could be seen microscopically. It was further noted that, almost without exception, the bacteria cultured from each patient were different, leading Miller to conclude that no specific bacterial species were responsible for the disease. A member of another group of microorganisms, the protozoa, was subsequently implicated. The amoeba ''*Endamoeba buccalis*'' was found to be consistently present in the purulent exudate from periodontal pockets but were not seen at healthy sites (Bass & Johns 1915). Ipecac, particularly one of its constituent alkaloids, emetin, was known to be a potent amoebicide and was reported to be an effective treatment for periodontitis (Bass & Johns 1915). Subsequent studies failed to confirm a causative association and it was concluded that ''*E. buccalis*'', now named *Entamoeba gingivalis*, and *Trichomonas tenax*, the other protozoon found in the mouth, were saprophytes feeding on food debris and bacteria, which would be likely to be abundant in periodontitis sufferers (Wantland et al. 1958).

Although the techniques of anaerobic culture had been established early in the history of medical microbiology, they were often not used in the early studies of the human-associated microbiota. For example, Medalia (1913) performed aerobic cultural studies on the subgingival plaque from 115 patients with periodontitis. The most common species detected was *Streptococcus pneumoniae*, with other streptococci and *Staphylococcus* and *Micrococcus* species, among the predominant organisms present, and were considered to be the cause of the infection.

The two examples quoted above are instructive, in that they demonstrate how easy it is to be convinced of causation based on evidence only of association and how all methods of bacterial community characterization introduce biases, which will affect the data generated. Indeed, because microscopic studies had identified a far more diverse microbiota, including spirochaetes, than had been seen from aerobic culture (Tunnicliff et al. 1936), anaerobic techniques were readopted. For example, Hemmens & Harrison (1942) used aerobic and anaerobic incubation and microscopy to compare the bacterial biota associated with suppurative lesions and healthy gingivae. The bacterial communities were essentially the same except that the numbers of ''*Bacteroides melaningenicum*'' and fusiform bacilli in the cultural analysis and spirochaetes, by microscopy, were increased at disease sites. Rosebury et al. (1950) performed detailed anaerobic studies on gingival scrapings from 35 subjects with a range of periodontal conditions including marginal gingivitis, acute ulcerative gingivitis, pericoronitis and periodontitis. A diverse community was revealed including representatives of the genera *Fusobacterium*, *Bacteroides*, *Actinomyces*, *Veillonella*, anaerobic cocci, anaerobic vibrios and anaerobic Gram-positive bacilli. Each sample had a distinctive microbiota and no associations between individual species and disease states were seen. The authors concluded that periodontal disease was caused by factors unknown but that, once initiated, the condition allowed the proliferation of the normal microbiota, further exacerbating the disease process.

It was clear from these studies that anaerobic bacteria were predominant at subgingival sites, particularly in disease, and that strict anaerobic precautions during the collection, transport, processing and incubation of samples were important. One major advance was the development of the glove box or anaerobic workstation. Originally developed for the cultivation of oral spirochaetes (Socransky et al. 1959, Rosebury & Reynolds 1964), Aranki et al. (1969) demonstrated a three- to fourfold increase in the recovery of obligate anaerobes from subgingival plaque when plating was carried out in the glove box, compared with a conventional anaerobic jar technique. Similarly, the Hungate roll tube technique was also found to give superior recovery of anaerobes from oral specimens than jars (Gordon et al. 1971).

Studies using strict anaerobic techniques to determine the microbiota associated with a type of aggressive periodontitis, then described as juvenile periodontitis or periodontosis, found a characteristic microbiota associated with affected sites with a significant proportion of the organisms present being Gram-negative facultatively anaerobic bacilli, in contrast to the predominance of obligate anaerobes seen in chronic periodontitis (Slots 1976, Newman & Socransky 1977). A group of strains among the Gram-negative facultative anaerobes seen in these studies were later identified as *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* (Tanner et al. 1979), an organism previously associated with a range of clinical infections including pulmonary, cardiovascular and soft tissue infections (Page & King 1966), and which was shown to produce a leukotoxin active against neutrophils (Baehni et al. 1979, Tsai et al. 1979). Subsequent studies demonstrated that *A. actinomycetemcomitans* could be found in small numbers in a minority of periodontally healthy subjects, but in higher numbers in

around half of adult periodontitis patients and was common in juvenile periodontitis (Slots et al. 1980). The presence of *A. actinomycetemcomitans* was shown to be predictive of a poor response to treatment (Christersson et al. 1985).

An important element of elucidating bacterial aetiology for periodontal disease is accurate diagnosis and determination of disease activity. Tanner et al. (1979) used clinical measurements and radiographs to identify sites that had suffered bone loss in the preceding 12 months. These sites were found to be colonized by predominantly Gram-negative microbiota, including *Porphyromonas gingivalis* (*Bacteroides asaccharolyticus*), *Fusobacterium nucleatum*, *Tannerella forsythia* (''fusiform'' *Bacteroides*) and *Campylobacter* species (anaerobic vibrios).

The most detailed cultural studies of the periodontal microbiota were performed by W. E. C. Moore and L. V. Holdeman Moore and their colleagues at the Virginia Polytechnic Institute, using a combination of roll tube and plate methods. In the course of a study of severe periodontitis in 21 adolescents or young adults, they isolated and identified 2723 isolates to 190 taxa at species or sub-species level (Moore et al. 1982b). The predominant species in the gingival crevice of the severe periodontitis sufferers were *F. nucleatum*, *Mogibacterium* (*Eubacterium*) *timidum*, *Eubacterium nodatum*, *Atopobium* (*Lactobacillus*) *minutum*, *Parvimonas micra* (*Peptostreptococcus micros*) and *Prevotella intermedia* (*Bacteroides intermedius*). *A. actinomycetemcomitans* was not seen on the non-selective media used but was detected by means of a selective medium in three of four subjects tested. Around half of the taxa detected could not be identified as belonging to a named species. In a similar study of the microbiota in chronic periodontitis (Moore et al. 1983), the same group of researchers found 171 taxa at species or sub-species level among 1900 isolates from 22 adults. The predominant species were similar to those seen in the study of severe periodontitis (Moore et al. 1982b) and included *F. nucleatum*, *P. micra*, *E. nodatum*, *P. intermedia* and *E. timidum*. *P. gingivalis* made up only relatively minor proportions of the microbiota in moderate and severe periodontitis and was neither detected in supragingival or subgingival samples collected from healthy subjects nor in a separate study of experimental gingivitis by the same group (Moore et al. 1982a). Strains originally isolated as part of these studies survive and are being subjected to 16S rRNA gene sequence analysis. It is a testament to the quality of the Moores' cultural techniques that many of their un-named taxa have been confirmed and named as novel species (Dewhirst et al. 2001, Downes et al. 2002, Downes et al. 2005, Downes et al. 2009, Downes et al. 2010) and that their collection includes representatives of many species hitherto thought to be unculturable (Dewhirst et al. 2010). Despite the quality of this work, there are many difficulties in using culture to characterize the periodontal microbiota. Anaerobic bacteria, which predominate in this habitat are typically slow-growing and nutritionally fastidious. Identification of isolates, particularly if attempted by means of conventional biochemical and physiological tests, is also difficult and time-consuming and requires the expertise of experienced microbiologists. These constraints place a severe limitation on the number of samples that can be processed in studies on a reasonable timeframe. It was therefore clear that alternative methods were required to provide reliable and high-throughput diagnostic tools for application in the understanding of the role of bacteria in periodontal disease.

Molecular methods for periodontal bacterial detection

For the reasons mentioned above, investigators adopted DNA-based methods. One notable advance was the use of DNA probes specific for oral bacteria in a checkerboard format where DNA from 43 patient samples could be probed with 43 whole-genomic DNA probes (Socransky et al. 1994). This format had the advantage of allowing the presence or absence of species to be determined for much larger numbers of samples than could be processed by culture methods alone. Disadvantages of the method are that a pre-selection of target organisms is made, and the lack of specificity of whole-genomic probes when target species with widely varying DNA G+C contents are hybridized at the same temperature. The method was used to seek associations between 40 species identified previously in subgingival plaque by cultural studies (Socransky et al. 1998). 13,261 subgingival plaque samples were collected from 185 subjects (25 healthy, 160 with loss of attachment). Thirty-two of the 40 species were found in $>5\%$ of samples and were used in the analysis. Microbial complexes were clustered using unweighted linkage sorting of a similarity matrix created using the phi coefficient. In addition, community ordination was performed using principal components and correspondence analyses. All of the methods used clustered the species into five complexes. Of these, the red complex was comprised of *P. gingivalis*, *T. forsythia* and *Treponema denticola* and was significantly associated with advanced and active periodontitis, particularly pocket depth and bleeding on probing. In addition, all three species were individually associated with disease-related variables.

Although the use of DNA probes in the checkerboard format allowed large numbers of samples to be processed, enabling powerful statistical analyses, the choice of target organisms remained based on the earlier cultural studies, and thus suffered from the limitations of bacterial culture media.

Culture-independent methods for the characterization of the periodontal microbiota

From the earliest cultural analyses of the oral microbiota, it was apparent that not all of the bacteria seen under the microscope were able to grow on solid culture media incubated aerobically or anaerobically. This was the case both in terms of absolute numbers where, for example, Socransky et al. (1963) estimated that microscopic counts of bacteria in gingival crevice samples were twice those obtained by anaerobic viable counts and also diversity, where qualitative descriptions of bacterial size and shape revealed a number of morphotypes that could not be grown. This problem of ''unculturability'' is well known and it has been estimated that $<1\%$ of bacteria on earth readily grow in the laboratory (Hugenholtz et al. 1998). Methods for the culture-independent study of bacterial communities were developed following the emergence of molecular phylogeny, the use of macromolecular sequence data to construct evolutionary trees (Woese 1987). Ribosomal RNA has been used extensively for this purpose because of its ubiquity, being found in all cellular life, and the trade off between information content and size of the small sub-

unit, 16S, molecule. Two approaches were originally developed for the study of bacterial communities in the environment. In one, rRNA was purified directly from biomass and cDNA was synthesized by means of reverse transcriptase. Following polymerization of the second strand, the DNA was cloned and sequenced (Ward et al. 1990). The second, and subsequently far more widely used method, was to amplify 16S rRNA genes directly from DNA extracted from samples, taking advantage of the highly conserved regions found in the molecule. Amplicons were then cloned and sequenced (Giovannoni et al. 1990). The application of these methods to the study of the microbial ecology of environmental samples revealed enormous bacterial diversity including numerous lineages at the phylum level without cultivable representatives (DeLong & Pace 2001).

Dymock et al. (1996) applied the technique to pus aspirated from dentoalveolar abscesses and identified previously undescribed lineages within the phyla *Bacteroidetes* and *Firmicutes* and also found that the numbers of cultivable organisms including *F. nucleatum* and *Porphyromonas endodontalis* were detected at higher levels by the molecular method than culture. Among 2522 cloned 16S rRNA genes from samples of subgingival plaque collected from 31 healthy or periodontally diseased subjects, Paster et al. (2001) found representatives of nine phyla divisions (current taxonomic designation in brackets): OP11 (SR1), TM7, *Deferribacteres* (*Synergistetes*), *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes*. Subsequent studies have confirmed that these are the predominant phyla found in the human mouth (Hutter et al. 2003, Kumar et al. 2005, de Lillo et al. 2006). The members of the red cluster, *P. gingivalis*, *T. denticola* and *T. forsythia* (Socransky et al. 1998), were detected but less frequently than in previous, cultural studies. In total, 347 taxa at species level were detected, demonstrating that the subgingival microbiota was far more species rich than previously thought, and that undescribed species were frequently detected and numerous in disease-associated samples.

A strength of the 16S rRNA gene PCR/cloning/sequencing technique is that the PCR primers can be designed to be specific for particular groups of interest. This has been used to explore the diversity of *Treponema* (Moter et al. 2006), *Eubacterium* and related taxa (Spratt et al. 1999), *Porphyromonas and Tannerella* (de Lillo et al. 2004) and *Synergistetes* (Vartoukian et al. 2009). All of these studies revealed greater diversity among the target groups than had been seen previously using universal primers alone.

These studies using universal and group-specific primers have built on the body of work available from culture-based studies and led to the development of the Human Oral Microbiome Database (HOMD) and associated website (http://www.homd.org), a list of all bacterial species found in the human mouth.

Composition of the periodontal microbiota

The compilation of the HOMD database has allowed the most complete description of the periodontal microbiota to date. The genera found at this habitat are listed in Table 1. They include representatives of the domain *Archaea*, the methanogenic species *Methanobrevibacter oralis* and related phylotypes, whose numbers have been shown to be raised in periodontitis (Lepp et al. 2004). The vast majority of oral bacteria belong to the domain *Bacteria* and include representatives of 11 phyla/Divisions that can be reliably detected from human oral samples. Representatives of other phyla have been detected rarely, often only once, and more reports are required before they can be assumed to be members of the oral bacterial community. The phyla found are *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*,

Table 1. Phyla and genera found in the human mouth (Dewhirst et al. 2010)

Domain	Phylum	Genus
Archaea	*Euryarchaeota*	*Methanobrevibacter*
Bacteria	*Actinobacteria*	*Actinobaculum*
		Actinomyces
		Atopobium
		Bifidobacterium
		Corynebacterium
		Cryptobacterium
		Dermabacter
		Kocuria
		Microbacterium
		Mycobacterium
		Olsenella
		Olsenella
		Parascardovia
		Propionibacterium
		Rothia
		Scardovia
		Slackia
		Tropheryma
	Bacteroidetes	*Bergeyella*
		Capnocytophaga
		Porphyromonas
		Prevotella
		Tannerella
	Chloroflexi	*Chloroflexi*
	Firmicutes	*Abiotrophia*
		Anaerococcus
		Anaeroglobus
		Caryophanon
		Catonella
		Centipeda
		Dialister
		Enterococcus
		Eubacterium
		Filifactor
		Finegoldia
		Gemella
		Granulicatella
		Johnsonella
		Lactobacillus
		Leuconostoc
		Megasphaera
		Mitsuokella
		Mogibacterium
		Mycoplasma
		Paenibacillus
		Parvimonas
		Peptococcus
		Peptoniphilus
		Peptostreptococcus
		Peptostreptococcus
		Pseudoramibacter
		Schwartzia
		Selenomonas
		Shuttleworthia
		Staphylococcus
		Streptococcus
		Veillonella
	Tenericutes	*Bulleidia*
		Solobacterium
		Erysipelothrix
	Fusobacteria	*Fusobacterium*
		Leptotrichia
	SR1	Uncultivated
	Proteobacteria	*Actinobacillus*
		Campylobacter
		Cardiobacterium
		Desulfobulbus
		Desulfomicrobium
		Desulfovibrio
		Eikenella
		Haemophilus
		Kingella
		Lautropia
		Neisseria
		Ralstonia
		Rhodocyclus
		Simonsiella
		Suttonella
	Spirochaetes	*Treponema*
	Synergistetes	*Jonquetella*
		Pyramidobacter
	TM7	Uncultivated

Firmicutes, *Fusobacteria*, *Proteobacteria*, *Spirochaetes*, *Synergistetes* and *Tenericutes* and the un-named Divisions TM7 and SR11. Members of the *Firmicutes* are the most frequently detected from periodontal samples; this phylum includes common oral genera such as *Streptococcus* and also a substantial number of as yet uncultured species, particularly within the family *Lachnospiraceae*. A consistent finding from molecular studies is that Gram-positive and not Gram-negative species are predominant at periodontal sites, contrary to the ''textbook'' view. The latter observation dates from the earliest studies where Gram-stained smears from material collection from deep periodontal pockets were examined, and Gram-negatives were seen to be predominant. These conflicting observations have been reconciled by the recognition that the anaerobic Gram-positive bacilli found in large numbers in subgingival plaque frequently stain as Gram-variable or even Gram-negative in older cultures (Kononen & Wade 2007). A predominant cultivable microbiota study that used strict anaerobiosis and extended incubation times has confirmed the predominance of Gram-positives (Uematsu & Hoshino 1992).

The periodontal microbiota is clearly highly diverse, with over 400 bacterial species detected (Paster et al. 2006). It is unlikely that each individual species plays a distinctive role within the community and the variability of the microbiota between individuals suggests that there is a high degree of functional redundancy. One way of dealing with the complexity of the community in experimental investigations will be to assign functional activities to each species on the basis of their phenotypic characteristics and genetic potential as deduced from their genome sequences. It is likely that the genome of at least one representative strain from all species of cultivable oral bacteria will be sequenced within the next few years. Experimental systems can then be designed to establish a core set of functional bacterial activities associated with health and the periodontal diseases.

Novel taxa and disease

Kumar et al. (2003) used PCR to determine the prevalence of a selection of species and phylotypes, including the novel taxa described by Paster et al. (2001), in periodontal health and disease. A significant association with chronic periodontitis was seen for *Synergistetes* (*Deferribacteres*) D084 and BH017, *Bacteroidetes* AU126, Division SR1 (OP11) X112, Division TM7 I025 and the named species *Anaeroglobus geminatus* (*Megasphaera* BB166), *Eubacterium saphenum*, *P. endodontalis*, *P. denticola* and *Cryptobacterium curtum*, in addition to *P. gingivalis*, *T. denticola* and *T. forsythia*. Culture-independent molecular analysis had thus lengthened the list of bacterial species associated with chronic periodontitis.

The weakness of using PCR as a detection method is its extreme sensitivity; the typical detection limit for a specific target using conventional PCR protocols being between 10 and 100 cells (Greisen et al. 1994). Comparing the distribution of predominant taxa by culture-independent analyses is therefore arguably more valuable. In a 16S rRNA gene cloning and sequencing study comparing the subgingival microbiota in 15 subjects with moderate to severe chronic periodontitis with 15 healthy controls, Kumar et al. (2005) found a number of taxa to be associated with periodontitis or health. Those associated with disease included *P. micra* (*Peptostreptococcus* BS044), *Peptostreptococcus stomatis* (*Peptostreptococcus* CK035), *Filifactor alocis*, *A. geminatus* (*Megasphaera* BB166), *Desulfobulbus* species, *Dialister* species and *Synergistetes* (*Deferribacteres*) species. *P. gingivalis*, *T. denticola* and *T. forsythia* were only found in small numbers and only *T. forsythia* was associated with disease. Members of the genera *Streptococcus* and *Veillonella* were found in large numbers in all samples and were significantly more frequently seen in health than disease. Other species associated with health included members of the genera *Abiotrophia*, *Campylobacter*, *Capnocytophaga*, *Gemella* and *Neisseria*. One general observation from this study was that the microbiota at healthy and diseased sites in periodontitis subjects showed greater similarities than healthy sites in healthy subjects and diseased sites in diseased subjects, i.e. the differences seen were most marked at subject – rather than site-level.

Thirteen of the species found to be associated with periodontitis by Paster et al. (2001) and Kumar et al. (2003) were incorporated into a checkerboard assay (Dahlen & Leonhardt 2006) and *P. tannerae*, *F. alocis* and *P. endodontalis* were found to be significantly associated with diseased sites. A molecular study of the microbiota associated with aggressive periodontitis found that *Selenomonas* species, many of them representatives of un-named were predominant, while *A. actinomycetemcomitans* was not detected (Faveri et al. 2008).

In a study of orally healthy subjects, Aas et al. (2005) did not find any representatives of the red complex, in contrast to the findings of (Ximenez-Fyvie et al. 2000) who used the checkerboard method with whole genomic probes to compare the microbiota. There are two possible explanations for this disparity. Firstly, as has already been mentioned, genomic probes used in the checkerboard format can suffer from low specificity due to unpredicted cross-reactivity with unculturable phylotypes. Thus, it is likely that a probe for *T. forsythia* would hybridize with *Tannerella* BU063, which is health-associated (Leys et al. 2002) and it is possible that probes for *P. gingivalis* would bind to other members of the genus *Porphyromonas*. Secondly, the PCR/cloning method only reveals the predominant organisms in a sample. Typically, 50–100 clones are examined from a sample, which is likely to contain around 10^6 bacterial cells, even from a healthy site.

There are additional problems in using 16S rRNA for the characterization of bacterial communities. The first is a practical one in that, for a number of genera, 16S rRNA gene sequence analysis does not reliably distinguish between species. This problem is often encountered in groups of species that are naturally competent, such as *Streptococcus* and *Neisseria* where the boundaries between species have been blurred by recombination (Hanage et al. 2005, Do et al. 2009). Multi-locus sequence typing approaches have been shown to be useful in clarifying the population biology of these groups (Maiden 2006) but are not readily applicable to large-scale community surveys. Other oral bacterial groups for whom 16S rRNA-based identifications are inadequate include the genera *Actinomyces* (Beighton) (Henssge et al. 2009), *Veillonella* (Beighton) (Arif et al. 2008), *Selenomonas* and *Porphyromonas catoniae* and related, as yet un-named species (unpublished data). The second pitfall

is the recognition, from comparison of genome sequences, of substantial intraspecific genomic variation. It is now realized that, for a given species, only a minority of genes are found in all members of the species (Tettelin et al. 2005), and that genes encoding virulence factors of importance in disease may only be found in a sub-set of strains. The relevance of species-level identification is therefore questionable. Moreover, virulence genes can be acquired by health-associated species. *Streptococcus salivarius* is regarded as one of the more benign human oral bacterial species, and has been proposed for use in probiotic products (Burton et al. 2005), but an invasive strain of this species caused a fatal meningitis, after being acquired from the oral microbiota of an anaesthetist who performed a lumbar puncture on the patient (Shewmaker et al. 2010).

The availability of 16S rRNA sequence data for the majority of members of the oral bacterial community has made it possible to design oligonucleotide probes to detect hundreds of bacterial species in a microarray format. For example, Colombo et al. (2009) used the HOMIM microarray, which includes probes for 300 species, and found that patients with refractory periodontitis had a distinct microbial profile compared with healthy subjects or patients with severe periodontitis.

High-throughput sequencing methods

The amplification, cloning and sequencing of 16S rRNA genes by the Sanger dideoxy method remain time-consuming, even with the use of automated DNA sequencers, and relatively few samples have been included in most studies. It has been demonstrated even from these limited studies, however, that the oral bacterial community is highly diverse and characterized by a flat frequency distribution with a long tail, suggesting substantial unseen species richness. This is typical of environmental habitats on Earth, and has been termed the ''rare biosphere'' (Sogin et al. 2006). High-throughput methods are therefore needed to perform the deep sequencing studies required to reveal those species present in low numbers in samples.

A major advance over conventional sequencing techniques has been the development of pyrosequencing methods based on the detection of incorporation of nucleotides during synthesis by coupling pyrophosphate release to a chemiluminescent reaction (Ronaghi 2001). This has proved amenable for use in high-volume parallel sequencing technologies (Rothberg & Leamon 2008), such as the 454 system (Roche, Bradford, CT, USA) where genomic DNA is fragmented and bound to beads, and then amplified within an emulsion. The beads are then placed in wells on a fibre-optic slide to which are added beads carrying the pyrosequencing reagents. Detection of nucleotide incorporation is accomplished by a CCD camera-based imaging system. Initial use of this system was limited to relatively short sequences of around 150 bases, which typically targeted a single variable region of the 16S rRNA gene. For example, (Keijser et al. 2008) determined the composition of the microbiota of saliva and plaque in 71 and 98 subjects respectively by analysis of the V6 region. 197,600 sequences were analysed and at a 97% sequence identity threshold, representatives of 5600 phylotypes were found in saliva and 10,000 in plaque, a level far greater than seen previously using cultured or 16S rRNA gene cloning/dideoxy sequencing methods. It has been subsequently been shown, however, that the 454 technology is prone to errors in the reading of hompolymeric tracts (regions of sequences consisting of repeated single bases), as well as the universal problem of the formation of chimeric sequences from PCR with mixed templates. Software is now available to address this problem (Quince et al. 2009) and should lead to more accurate estimates of total human oral bacterial species richness. An alternative high-throughput sequencing system, the Illumina platform, has also been used to characterize the oral microbiota (Lazarevic et al. 2009) in a study targeting the V5 region. At the 97% sequence identity level, >8000 phylotypes were detected including representatives of the Divisions BRC1, OP10 and OP3, not detected previously in the human mouth. The sequences analysed in this study were only 82 bases long, which obviously limits the phylogenetic information available from the data. The current version of the 454 platform (Titanium FLX) generates sequences of up to 500 bp, which will greatly improve the phylogenetic resolution of future studies. Other studies have used high-throughput methods to characterize the oral microbiota (Costello et al. 2009, Nasidze et al. 2009a, b) but, to date, none have specifically addressed research questions relating to periodontal disease pathogenesis.

All culture-independent methods, however, do suffer from biases and limitations. Each step of the analysis process from bacterial cell lysis, DNA extraction, PCR, cloning and sequencing can be subject to bias (von Wintzingerode et al. 1997). One consistent observation is that the proportions of members of the phylum *Actinobacteria*, formerly the high G+C Gram-positives are lower than those seen when cultured were used (Munson et al. 2002, Munson et al. 2004). This has been partly attributed to primer design, with all ''universal'' primers commonly used having significant mismatches with species from certain regions of the phylogenetic tree (Hamady & Knight, 2009), such as the *Bifidobacteriaceae* (Frank et al. 2008). The effect of G+C content also appears to be important. It has been suggested that in the PCR, Taq polymerase struggles to amplify G+C-rich sections of the template. It is well known that such regions can cause the polymerase to pause or terminate prematurely (Henke et al. 1997). PCR additives, which destabilize the polymerase-blocking secondary structures formed by G+C-rich regions, have been recommended (Baskaran et al. 1996), together with alternative polymerases to Taq but no data are yet available which demonstrate that they correct the bias against the *Actinobacteria* in molecular ecology studies.

The use of alternative primers, the filtering of 16S rRNA gene sequences from metagenomic data or direct sequence analysis of the 16S rRNA molecule itself will all doubtlessly result in new lineages being revealed among the *Archaea* and *Bacteria* found in the gingival crevice.

The availability of the high-throughput methods described above has the potential to revolutionize our knowledge of the oral and periodontal microbiota. A number of challenges remain however. Not the least of these is the problem of analysing large amounts of data. Indeed, many authors do not even attempt to analyse their sequences to species level but merely present their data as a summary at higher taxonomic levels. While this may be informative for studies of the bacterial communities

found in the environment where the function of the community may not vary within the local habitat, in the human mouth we have a community living at a wide variety of micro-habitats under selective pressure from the host, in the form of the immune system and ecological pressures from the environment and disease processes themselves. The human oral microbiome has been relatively well studied to date and it has been shown that closely related species can have very different relationships with oral ecology and disease states, as discussed above for *T. forsythia*, associated with periodontitis and its closest phylogenetic relative, the uncultured *Tannerella* BU063, associated with health. If data are only presented to even genus level, these associations would be obscured. New tools are clearly required to allow handling of high volumes of data and enable identification to species level as is currently possible via the Human Oral Microbiome database (Dewhirst et al. 2010).

Conclusions – implications of molecular analyses of bacterial communities for pathogenesis of periodontal disease

The culture-independent molecular studies described in this review have added to our knowledge regarding the composition of the bacterial communities associated with periodontal disease. The periodontal microbiota is far more diverse than thought previously and as yet uncultivated organisms have been associated with disease. The new high-throughput methodologies are likely to further increase the number of bacterial species known to be oral commensals, as the rare biosphere is explored. But has our increased knowledge enhanced our understanding of the disease process itself? Arguably, it has not but has merely expanded the range of bacterial species known to be present at this habitat, which was already known to be a highly diverse community from culture-based studies. What has been achieved is a more complete understanding of the bacterial species found at diseased sites and which are involved in the interaction with the human host that results in disease. As mentioned in the Introduction, there has long been a debate as to whether specific oral bacteria cause periodontitis, in the manner of an infectious disease, or if host factors are responsible.

Recent studies have demonstrated that host susceptibility is of primary importance with an as yet uncharacterized defect of the immune system, which causes defects in the regulation of osteoclast recruitment, differentiation and activation, causing affected individuals to mount an inappropriately aggressively inflammatory response to the normal microbiota (Taubman et al. 2007). Whether individual bacterial species are important remains debatable. In general, the prevalence of disease-associated species is more strongly related to pocket depth than the disease type (Riep et al. 2009). Furthermore, a systematic review of the value of microbial identification in the management of periodontal diseases (Listgarten & Loomer 2003), concluded that microbiological assessment could be useful in the management of periodontal patients that do not respond to conventional treatment, but that evidence for the value of microbiological testing as a prognostic indicator overall was lacking. For example, arguably the strongest evidence for the role of a specific organism in periodontal disease pathogenesis is for the JP2 clone of *A. actinomycetemcomitans*. This organism has a deletion in the promoter for the gene encoding the leukotoxin, causing strains to hyperproduce the leukotoxin (Brogan et al. 1994). The clone appears to have a tropism for a sub-group of humans of African descent (Haubek et al. 1997), with infection resulting in an aggressive periodontitis (Haubek et al. 2001), although the molecular basis for action of the leukotoxin in periodontitis remains to be elucidated. It is possible, then, that for individuals of this particular racial type, a test for the JP2 clone would be diagnostically useful, while for the remainder of the population, it would have limited applicability.

There remains a need, then, to determine the basis for the interaction between the commensal microbiota and the host that results in periodontal disease, at the molecular level. One criticism of culture-independent molecular analyses has been that sequence information only is obtained for them and that for investigations of virulence, cultivable organisms are required. Considerable efforts have been made therefore to determine why so many bacterial species cannot be cultured on conventional bacteriological media and to develop new cultural methods for their isolation. Some species, such as *T. forsythia*, have specific nutritional requirements. *T. forsythia* is unable to synthesize *N*-acetyl muramic acid (NAM), one of the principal components of cell wall peptidoglycan. It therefore grows extremely poorly alone but grows well in co-culture with other species such as *F. nucleatum* or in media supplemented with NAM (Wyss 1989). A requirement for the presence of other bacteria has been a common finding in the investigation of bacterial cultivability. The domestication and eventual culture of a representative strain of a previously uncultivated lineage of the phylum *Synergistetes* were achieved after prolonged mixed serial batch culture of subgingival plaque *in vitro* (Vartoukian et al. 2010), which enabled the genome of the strain to be sequenced. Similar co-culture approaches have been successful in improving the cultivability of marine bacteria and may be applicable to the study of the periodontal microbiota (Kaeberlein et al. 2002, D'Onofrio et al. 2010).

The majority of studies to date investigating the interaction between bacteria and host tissues and immune cells have used single organisms, typically those that have been implicated as pathogens. The host will always, however, be in contact with a highly diverse polymicrobial biofilm and the degree of resulting inflammation will be dependent on a vast number of bacterial–host interactions. Some progress using polymicrobial bacterial challenge has been made (e.g. Huang et al. 2010) but new models need to be developed that include a mix of species typical of the natural plaque biofilm. Advances in co-culture such as those described above have now made it possible to maintain stable dental plaque communities in vitro that include ''unculturable'' phylotypes. These could be adapted to study host–bacterial interactions.

At the same time, reductionist approaches should still be pursued but should systematically screen all currently cultivable species for their interactions with the host. In susceptible individuals, inflammation arises at an early stage of plaque maturation; it is possible that the gross changes in bacterial composition occur as a result of inflammation rather than being its cause. There may be other species, yet to be identified, which play a key role in inducing an inappropriate inflammatory

response in the host. Conversely, there are likely to be other species, perhaps analogous to *Bifidobacterium* species in the colon (Preising et al. 2010) that exert an anti-inflammatory effect on the oral tissues. Systematic screening should allow assignation of oral bacterial species to specific functional groups based on their interactions with host cells.

It is also possible that the factors responsible for harmful and beneficial interactions with the host are not confined to individual species and may be part of the non-core, peripheral genomes of oral bacteria. The high level of genetic variability found among strains of a species has already been mentioned. Thus, not all strains of a species may include the gene encoding the factor of interest or alternatively, species that do not normally produce the factor may acquire the gene by horizontal transfer. One culture-independent approach that can analyse the genetic potential of a microbial community independent of species-level identification is to analyse the metagenome. The metagenome is defined as all of the DNA found at a particular habitat and can be randomly sequenced or functionally screened for activities of interest (Streit & Schmitz 2004). For example, the metagenome in pooled faecal samples from 124 European individuals has been sequenced and 3.3 million non-redundant microbial genes were analysed (Qin et al. 2010). The total species estimates both for the entire cohort and individuals were substantially lower than those estimated previously from 16S rRNA-based analyses. This suggests that either the metagenomic technique suffers from biases that lead to an underestimation of diversity or that improved methods of computational analysis are required. The human oral metagenome is currently under investigation as part of the Human Microbiome Project (The NIH Human Microbiome Group 2009). Making sense of the vast volume of data will be a major challenge but new bioinformatic tools will undoubtedly allow the accumulation of much valuable data of relevance to periodontal disease pathogenesis.

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Clinical Relevance

Scientific rationale for the study: Only around half of oral bacteria can be grown in the laboratory using conventional culture methods. The aim of this review is to describe recent advances in the culture-independent molecular analysis of the periodontal microbiota.

Principal findings: The periodontal bacterial community comprises over 400 species and is highly variable between subjects. Many novel species are as strongly associated with disease as those cultivable species regarded as periodontal ''pathogens''.

Practical implications: The work reviewed here suggests that a wide range of bacterial species are associated with periodontitis, expanding the range of possible targets for antimicrobial and other therapies.