MOLECULAR ANALYSIS OF BACTERIA ASSOCIATED WITH CHRONIC PERIODONTITIS AND PERIODONTAL HEALTH

DISSERTATION

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By

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ABSTRACT

Chronic periodontitis is a disease of tooth-supporting tissues, affecting over half the population in the United States. The etiological role of bacteria is established, although contributions of individual species or groups of organisms are unclear. Molecular analysis of the plaque biofilm allows study of associations between health status and cultivated and uncultivated species. The purpose of the present investigation was to identify potential periodontal pathogens and beneficial bacteria using cultivation independent approaches. Plaque from deep and shallow sites of subjects with chronic periodontitis and age-matched healthy controls was studied using quantitative 16S clonal analysis. Several species and phylotypes of Peptostreptococcus, Filifactor, Megasphaera, Campylobacter, Selenomonas, Deferribacteres, Dialister, Tannerella, Streptococcus, Atopobium, Eubacterium, Treponema and Desulfobulbus were associated with periodontitis while Streptococcus,Veillonella, Campylobacter, Abiotrophia, Gemella, Capnocytophaga and Neisseria were associated with health. The stability of bacterial colonization in the subgingival crevice was examined using a similar approach. Subjects were either periodontally healthy over two years or demonstrated improvement or worsening of their periodontal status. The microbial stability of the stable group was significantly higher than that of the group demonstrating clinical change. *Veillonella oral*

clone X042 and uncultivated streptococci,were associated with health, while *F.alocis*, uncultivated treponemes and selenomonads were significantly associated with disease. This suggests that periodontal health is associated with a stable health compatible bacterial colonization and that change in clinical health is associated with a microbial shift. The prevalence of candidate species detected using the above approaches was studied in subgingival plaque from 66 subjects with periodontitis and 66 healthy controls. Species-specific ribosomal 16S primers for PCR amplification were developed for detection of 90 species. *Deferribacteres clones D084/BH017, Bacteroidetes clone AU126, Megasphaera clone BB166, OP11 clone X112, and TM7clone IO25, E.saphenum, P.endodontalis, P.denticola*, and *C.curtum* were associated with disease. *Deferribacteres clone W090, Bacteroidetes clone BU063, A.rimae* and *A.parvulum* were associated with health. In conclusion, the bacterial profile of health and disease contains large numbers of uncultivated bacteria. Robust associations are between between periodontitis and F.alocis, members of Deferribacteres, Megasphaera, Desulfobulbus, Campylobacter and Peptostreptococcus. *Veillonella oral clone X042, Bacteroidetes clone BU063*, streptococci and *Campylobacter gracilis* are associated with health.

Dedicated to Amma, Amli, Kumar, Taruni and Tanvi

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PUBLICATIONS

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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

Chronic periodontitis is a slowly progressing disease that affects the supporting structures of a tooth. Epidemiological studies estimate that over four million Americans over 45 years of age suffer from this disease. It is estimated that about one half of this population is affected by moderate periodontitis, that is, attachment loss of 4-6mm[1]. A third of the population aged between 55-64 years has advanced destruction. Both bacterial plaque and the host's response to the bacterial challenge contribute to loss of the attachment apparatus to the root surface, destruction of tooth-supporting bone and eventual tooth loss.

Bacteria colonize the oral cavity within a few hours after birth. Colonization of the gingival crevice occurs initially by bacterial interactions with the tooth and later by inter-bacterial interactions leading to the formation of an organized, cooperating community called the biofilm. Current evidence indicates that chronic periodontitis is a polymicrobial infection caused by biofilm-associated bacteria.

The bacterial etiology of chronic periodontitis has been studied for a number of years and our understanding of disease pathogenesis has undergone many changes. Advances in microbial detection and characterization techniques have contributed to changing

paradigms of disease etiology over the years. For example, initial studies indicated that periodontal diseases were caused by an increase in the amount of dental plaque. However, microscopic identification of the different morphotypes associated with periodontal health and disease suggested a role for specific bacteria.

Although technological advances over the years, particularly in the last few decades have improved our understanding of the bacterial etiology of periodontitis, we do not, at this time, have a complete picture of what causes periodontitis. This chapter will review the different methodologies used to explore the bacterial profile of periodontal health and disease and the consequent shifts in our understanding of the role played by bacteria in disease causation.

Evidence for a bacterial etiology

Tissue destruction in periodontitis is due to bacteria and bacterial products as well as the inflammatory cascade that is initiated in response to the bacterial challenge. There are many lines of evidence that suggest an important role for bacteria in disease causation. Correlation between prevalence of disease and the amount of plaque, the efficacy of antibiotics in ameliorating disease or improving outcomes of mechanical therapy, absence of disease in gnotobiotic animals and demonstration of an immune response to specific plaque bacteria provide evidence that bacteria are necessary in the pathogenesis of periodontitis.

Plaque levels and periodontitis: A positive correlation has been demonstrated between amount of plaque and incidence[2] and the severity of gingivitis[3]. Bone loss was also shown to be more severe in subjects with poor oral hygiene[4, 5]. Studies by the Aarhus and Gothenburg groups showed that the progress of periodontal destruction could be halted and reversed by twice monthly professional prophylaxis and surgery[6-10]. However, surgery alone in the absence of professional maintenance did not serve to reverse disease progression. This positive correlation between plaque levels and gingivitis or bone loss suggests that plaque levels may influence severity and progression of periodontal diseases.

Role of antibiotics: The effect of antibiotics on reducing the severity and progression of periodontitis, either alone or in conjunction with mechanical therapy, has been extensively studied. Antibiotics have been used to treat all forms of periodontitis including localized and generalized aggressive periodontitis [11-13] and adult periodontitis[14-16]. Using antibiotics and antimicrobials in conjunction with mechanical therapy has been shown to improve results of therapy significantly better than mechanical intervention alone, providing support for the infectious nature of the disease.

Animal studies: Studies on gnotobiotic animals have shown that human plaque bacteria induce alveolar bone loss in germ-free animals[17, 18]. Although germ-free rats form abundant amounts of calculus, gingival inflammation, pocket formation or destruction of alveolar bone was not seen[19, 20]. This suggests that presence of bacteria, not simply mechanical irritation, is essential in disease pathogenesis.

Host response: Bacterial infections induce an immune response in the host. More subjects with periodontitis exhibited serum antibody response to selected periodontal bacteria than healthy controls [21]. It has also been shown that there is a positive correlation between periodontal disease progression and immune response to selected plaque bacteria[22, 23]. Taken together, these studies indicate that there is a correlation between plaque bacteria and immune response in the host.

The above studies provide important insights into the etiopathogenesis of periodontal disease:

a. Periodontitis is infectious in nature.
b. Periodontitis does not occur in the absence of plaque
c. Severity and progression of disease shows a strong relationship to levels of plaque.

Search for pathogenic bacteria

While there is considerable evidence for the role of bacteria in periodontitis, the evidence for the etiological role played by specific organisms has not been as unequivocal. The identification of specific causative species, or periodontopathogens, has been hampered by some of the unique features of periodontal diseases. The foremost of these is that disease occurs in a site already colonized by a bacterial population. Thus, disease might be caused either by overgrowth of one or more species in the resident population, or by colonization by an exogenous pathogen. A second reason is the non-homogenous nature

of the biofilm. The bacterial profile of the biofilm adjacent to the root surface is different from the epithelial side. Different regions along the length of the root also exhibit different profiles[24-29]. The episodic nature of chronic periodontitis, with periods of remission and exacerbation, has further complicated the search since different species may be found in different phases of the disease process. Changes in disease classification have also contributed to the challenge of identifying periodontal pathogens, since microbiological data is interpreted in the light of clinical status.

The presence of bacteria in the oral cavity has been known since the time of Antonie von Leeuwenhoek, who, in 1699 described the presence of 'living animalcules' in dental plaque. However, the earliest studies identifying associations between bacteria and 'pyorrhea' developed in the late 19th century. The microbial etiology of periodontal diseases has been explored for over a hundred years and has evolved along with technological advances in bacterial identification and characterization. Initial studies used cultivation and microscopy for bacterial identification and Koch's postulates of disease causation to identify the pathogenic role of an organism. Although Koch's postulates were inviolate for nearly a century, the discovery of viral diseases and identification of bacterial genotypes made it difficult, if not impossible to apply these principles of disease causation. Socransky's modifications of Koch's postulates reflect the molecular approaches used for pathogen identification. The modified criteria are:

1. A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease.

2. Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease.

3. With resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur.

4. When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship.

5. The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms.

6. Efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located.

7. The sequence-based forms of evidence for microbial causation should be reproducible.

Chronic periodontitis is now seen as a polymicrobial infection caused by a consortium of bacteria[30]. Koch's postulates and Socransky's modification were targeted to identification of individual species. It is difficult to apply these postulates to heterogeneous, polymicrobial infections.

Conceptual challenges, for instance, definitions of clinical disease, temporal and spatial distributions of pathogenic bacteria, still exist and no study has been able to address all these issues. However, our understanding of the role of bacteria in periodontal disease causation has been strongly influenced by advances in bacterial identification and characterization. This section will focus on different approaches that have been used to

study the bacterial profile of periodontal health and disease. The various approaches will be reviewed in view of their impact on our understanding of the bacterial etiology of periodontitis.

Microscopy and cultivation: Microscopic identification and cultivation formed the basis of all early work on characterization of oral and gingival bacteria. Witzel (1882) and Miller (1890) identified and characterized different bacterial morphotypes and species found in caries and periodontitis[31]. Over the next 80 years, cultivation and phenotypic characterization were routinely used for bacterial identification. Based on this approach, Miller's hypothesis stated that the plaque mass was responsible for disease. He proposed that overgrowth of the normal oral flora occurred in response to changes in the host and that this led to periodontitis. This was known as the ***non-specific plaque hypothesis*** and was the prevailing paradigm for nearly a half century. This idea was further reinforced by Loe's *Experimental gingivitis in man[32],* which showed that lack of oral hygiene led to increase in amounts of plaque and gingivitis; this state could be reversed by reinstituting oral hygiene.

Direct visualization of the bacteria associated with health and disease using light and electron microscopy [33, 34] suggested a definite bacterial profile associated with health and a significant shift associated with alterations in health status. These studies showed that the healthy gingival crevice was colonized predominantly by gram-positive bacteria, this profile changed to a predominantly gram-negative one with the onset and progression of gingivitis. These and other such studies led to a change in the prevailing paradigm of a

non-specific infection. The role of specific species in disease causation began to be recognized and the ***specific plaque hypothesis*** was proposed by Loesche[35, 36].

Microscopic techniques provide valuable information on the general aspects of a bacterial community, since they can be used to detect shape, size and staining characteristics. However, lack of specificity is a major concern since morphotyping based on shape, size, motility and staining characteristics does not provide definitive identification. For example, several bacteria that were originally thought to be gram negative species (*Veillonella, Fusobacterium, Filifactor)* are now thought to be gram positive species based on their phylogenetic relationships[37]. Closer examination of cell wall characteristics and physiological behavior of *Filifactor* has revealed that it is in fact, a gram positive species[38]. It is possible that the traditional paradigm of a predominantly gram negative flora associated with disease may undergo drastic changes when more sophisticated methods are used to characterize bacteria.

Cultivation techniques, by their very nature, are selective for certain organisms. The 'great plate count anomaly' describes the discrepancy between microscopically observed cell counts and the number of visible colonies[39]. Two types of bacterial populations account for this: recognized species which have been selected out due to the cultivation conditions and unknown species for which cultivation conditions have not yet been established. Socransky[40, 41] showed that anaerobic cultivation techniques recover 10-20% of the total microbial counts from gingival debris and dental plaque. Slots et al found that it was not possible to characterize gram negative rods into individual species

based on culture characteristics[42]. Thus, studies that relied on culture and phenotypic characterization could not provide a complete picture of the periodontal microbial community. Further, certain species grow more luxuriantly than others under a given set of conditions, so it is possible that viable plate count or most-probable-number techniques provide inaccurate information on relative levels of various species that form a community.

Cultivation and microscopy provided us with the earliest evidence of specific bacterial morphotypes associated with disease. However, the selective nature of culturing and the non-specific nature of microscopic identification limit the use of these methods as comprehensive tools to accurately characterize the plaque biofilm.

Immunologic and enzymatic assays: The development of antibody-based detection systems enabled more accurate detection of targeted species. An integral step to all these assays was raising antibodies to each species of interest. This, again, necessitated growing the organism in culture before inoculating an animal and raising antibodies to the bacterial antigens. Using this method, many studies were undertaken to explore the prevalence of target species in the healthy and disease population and to follow the fluctuations in these species either naturally or in response to treatment[43-47]. A limitation with this approach is that the target organism has to be cultivated in order to raise antibodies against it making the method useful only for cultivated species. The cross reactivity of the antibodies can be tested only on cultivated species and cannot be confirmed against uncultivated or unknown species.

Antibody-based detection systems provided improved bacterial identification methods. However, these methods could be used to study only a limited list of bacterial species and comprehensive explorations of the plaque biofilm were not possible. These studies were also limited by the need to cultivate organisms and so could not be used to study uncultivated species.

DNA – DNA hybridization or checkerboard: A 'molecular' approach that was used in a large number of studies is DNA-DNA hybridization. This method detects bacteria based on hybridization of target species to labeled genomic DNA that has been previously attached to nylon membranes. Using this method, the levels of a limited number of species have been studied in adult periodontitis, periodontal health, refractory periodontitis and response to therapy [48-58]. The researchers used data from population-based studies to group the 40 species that were investigated into clusters or 'complexes' based on the relative levels of these species in health and disease. They found that three species, *Porphyromonas gingivalis, Tannerella forsythia (Bacteroides forsythus)* and *Treponema denticola,* showed a significantly higher prevalence in disease than in health. These species were grouped together to form the 'red complex'. Commercial chairside assays based on detection of these species were marketed as diagnostic tools. For nearly 20 years, members of the red complex were thought to be primary etiological agents and therapeutic intervention was directed to reducing or eradicating these species.
DNA-DNA hybridization provides a great advantage in that it allows simultaneous detection of multiple species from each sample. However, this process is not truly cultivation-independent, since it requires the cultivation of target species in order to

create genomic probes. As with antibody-based assays, specificity of the probe is an unknown variable, since cross reactivity can be verified only with cultivated species. In interpreting the results from these studies it must be remembered that selected species may not be representative of the total bacterial population. While it is possible that members of the red complex may be indicators of changes occurring in the biofilm in disease, there is no evidence to suggest that fluctuations of specific organisms reflects the changes occurring in a complex community.

Molecular methods: The use of molecular methods in exploring the bacterial constituents of plaque has challenged existing views on periodontal disease etiology. This section will focus on the impact of molecular approaches, especially DNA-based methods, in changing existing paradigms of bacterial pathogenesis in relation to periodontitis. Both open-ended, comprehensive bacterial surveys and targeted approaches that focus on certain species have been used to study the bacteria associated with periodontal disease and health.

*Directed DNA methods***:** These are closed-ended approaches that use short (15-40bp), synthetic nucleic acid sequences (oligonucleotides) specific to each species for bacterial detection. When these sequences are labeled with agents that enable their detection, they are called probes. The signal that is emitted when the probe hybridizes to its target DNA is measured to detect presence and levels of bacteria in community DNA. In situ hybridization, flow cytometry and reverse capture checkerboard are based on such an approach. These species-specific oligonucleotides can be used as primers to amplify

DNA in polymerase chain reaction (PCR). PCR has been used to study the relationship of cultivated and uncultivated species to health and disease [59-61]. These probes and primers have been used to quantitate the levels of selected bacterial species by monitoring PCR amplification in real time (real time PCR). While cultivation independent methods have provided us with a means to detect yet-to-be cultivated species, a main limitation with these methods is that they are restricted to detecting the presence and levels of previously known species.

Open-ended approaches: These approaches allow identification of all bacteria present in a population, even uncultivated and previously unknown species. Since the ribosomal gene is present in all free-living organisms and is highly conserved, evolutionary distance or phylogeny of all organisms can be computed based on similarities in 16S ribosomal gene sequences[62]. Cloning and sequencing the 16S ribosomal gene is a cultivation-independent approach that enables bacterial identification based on sequence homology. This approach might be considered the molecular counterpart of culturing in its ability to detect previously unsuspected organisms. In the last 5 years, this technology has resulted in a broad expansion of the spectrum of microorganisms regarded as important in periodontal diseases. This approach has been used to study the microbial population in different ecosystems, enabling the characterization of hitherto uncultivated microbial communities[63-68]. Using this approach, the diversity of different colonization niches in the oral cavity has been explored[69-72].

With the introduction of molecular methods to study the plaque biofilm, our understanding of oral bacteria has undergone significant changes, the most noteworthy being that the majority of the oral and periodontal flora is uncultivated. This might mean that data gathered using cultivation-based approaches provided us with an incomplete picture of the microbial community in periodontal health and disease.

In conclusion, while the bacterial etiology of periodontitis has been studied using sophisticated model systems and clinical study designs, the knowledge gained has been largely influenced by the available technology. Thus, exploring the bacterial population associated with periodontal health and disease using open-ended molecular approaches will provide a more representative view of the plaque biofilm. This is important in identifying candidate pathogenic and beneficial species for more targeted, detailed investigations.

The aim of the present study was to identify cultivated and uncultivated species associated with chronic periodontitis and periodontal health. The microbial flora associated with chronic periodontitis and periodontal health was investigated using 16S cloning and sequencing. The stability of the flora associated with change in clinical health status was also studied. Candidate pathogens and beneficial species detected by this approach were examined more closely in a larger sample using a targeted molecular approach

CHAPTER 2

IDENTIFICATION OF CANDIDATE PERIODONTAL PATHOGENS AND BENEFICIAL SPECIES BY QUANTITATIVE 16S CLONAL ANALYSIS

There is considerable evidence to show that bacterial plaque is the etiologic agent in chronic periodontitis. No single species has been implicated as a primary pathogen, and available evidence is consistent with a polymicrobial disease etiology. Nearly all studies on the bacterial etiology of periodontitis have used either culture-based or directed DNA approaches, targeting known species. The prevailing paradigm that implicates minor constituents of the subgingival community, the gram-negative bacteria *Porphyromonas gingivalis, Tanerella forsythensis* and *Treponema denticola* [30], as periodontopathogens is based on such approaches. However, culturing is not representative of the composition of a microbial community, since it is often too selective, especially for fastidious and yet-to-be cultivated species. Even culture-independent targeted approaches are limited to detecting the presence and levels of known species. Obviously cultivation will not detect uncultivated species, but the limitations of closed-ended molecular approaches such as PCR or hybridization assays such as checkerboard and microarrays are not as widely appreciated. Using these approaches it is possible to detect uncultivated species, but only if they have been previously characterized to allow primers or probes to be constructed. Perhaps more importantly, quantitative information is incomplete with these methods

since the total number of bacteria is not easily determined with a closed-ended approach. Thus, it is possible that pathogens remain undiscovered with such approaches. To advance our understanding of oral biofilm communities and disease processes, it is necessary to more comprehensively identify the microbiota in periodontal health and disease.

Open-ended molecular approaches, capable of detecting all bacteria in a sample, including uncultivated and previously unsuspected ones, are the most powerful methods available for exploring the microbial profile of any community. Recently cloning and sequencing of bacterial 16S rRNA genes have been employed to investigate the composition of environmental samples as well as samples from the human oral cavity. This culture-independent approach has revealed vastly greater diversity than was apparent with culturing [64, 70]. Investigations of oral bacteria employing these tools have used enrichment primers for rare taxa, for example, Spirochaetaceae and Bacteroidetes, or subtraction systems to eliminate predominant taxa such as Streptococcus, enabling selective amplification and identification of rare species. Using this approach, more than 700 orally-derived 16S sequences have been deposited in GenBank, less than half of which are from species that have been cultivated and characterized. To identify which of these many oral inhabitants are important in health and disease-associated biofilm communities, an adequately powered clinical study design and a quantitative, representational approach to ribosomal 16S cloning and sequencing that maintains the relative proportions of individual bacterial species is needed.

The purpose of this study was to achieve a culture-independent representational analysis of biofilms associated with chronic periodontitis and periodontal health, and to identify candidate pathogens and beneficial species or taxa. Since approximately half of oral bacteria are uncultivated, it seems likely that new associations would be revealed with this approach.

MATERIALS AND METHODS

Subject selection

Subjects for this institutionally approved study were recruited from the Dental Clinics at the College of Dentistry of the Ohio State University, and informed consent was obtained. Fifteen subjects with moderate to severe generalized chronic periodontitis were identified following clinical and radiographic examination. The subjects ranged in age from 42 to 80 years. A control group of 15 age and sex matched periodontally healthy individuals was also selected. Exclusion criteria for both groups included diabetes, antibiotic therapy in the previous three months, oral prophylactic procedures within the last three months, less than 20 teeth in the dentition, and a history of smoking.

Sample collection and DNA isolation

Subgingival plaque samples were collected on sterile endodontic paper points (Caulk-Dentsply) following isolation and supra gingival plaque removal. Plaque was collected and pooled from the mesial sulcus of every tooth for the healthy subjects. For the periodontitis group, sites for microbial sampling were selected based on probe depth measurements. Plaque from four non-adjacent proximal sites with probe depths of 6 mm

or more was collected and pooled (disease or deep-site samples). Samples were similarly acquired from four sites with probe depths of 3 mm or less and separately pooled (healthy or shallow-site samples). Samples were placed in 1.5 ml microcentrifuge tubes and frozen until further analysis. DNA was isolated using previously described methodology [73]. Briefly, bacteria was removed from the paper points by adding 750 μl of sampling buffer and vortexing for 1 minute. The paper points were then removed, the sample pelleted and the supernatant discarded. The pellet was suspended in 1% sodium dodecyl sulfate in TE, proteinase K was added and the samples were incubated overnight. DNA was isolated on glass beads and eluted in TE.

Amplification of 16s rDNA

Bacterial 16S rRNA genes were amplified from the community DNA with universal bacterial primers A17 (5'-GTT TGA TCC TGG CTC AG- 3') and 317 (5'AAG GAG GTG ATC CAG GC 3') (Biosynthesis, Lewisville, TX). PCR was performed by adding 1μl of community DNA to a reaction mixture (50μl final volume) containing 20 nmol of each primer, 40 nmol of deoxynucleotide triphosphates and 1U of Taq polymerase. The following cycling conditions were used: denaturation at 94oC for 1 min, annealing at 42oC for 2 min, and elongation at 72oC for 3 min. A final, 10-minute elongation at 72oC followed 22 cycles of amplification. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA).

Cloning and sequencing

The 16S amplicons generated by PCR were cloned into E.coli using a commercially available kit (TOPO TA cloning kit, Invitrogen, San Diego, CA). Competent TOP10 E.coli cells provided with the kit were transformed, plated onto Luria-Bertoni agar plates supplemented with ampicillin, and incubated overnight. Colonies were further selected for the presence of an insert with X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). The presence of inserts of the correct molecular size (≅ 1500bp) was confirmed by PCR amplification of the white colonies and gel electrophoresis of the amplicons on 1% agarose. DNA was stained with ethidium bromide and visualized under UV light (wavelength 320 nm) .The products were then purified with a Millipore kit (Millipore, Billerica, MA) and sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit) using an ABI 3700 instrument.

Sequence analysis

Partial sequences of 500-800 bp were obtained from each amplicon. The sequences generated were compared to the GenBank database to identify the closest relatives using a Time Logic DeCypher Tera Blast server hosted by the Ohio Supercomputer Center. Sequences with low homology to GenBank entries were screened for chimeras using the ChimeraCheck program of the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/). Twenty-three clones were identified as chimeric sequences and excluded from further analysis. Sequences were aligned and a similarity matrix constructed from the alignments using the method of Jukes and Cantor. Phylogentic trees were constructed using the neighbor joining method. MacVector

software was used to generate alignments, similarity matrices and in phylogenetic tree construction. A novel phylotype was defined as a sequence that differed from the closest GenBank entry by more than 2%. Sequence data for the whole 16S gene was obtained for novel sequences and submitted to GenBank.

Statistical analysis

Statistical analysis was carried out with JMP (SAS Institute Inc., Cary, NC). The microbial profile of periodontally healthy subjects was compared to that of healthy sites and deep pockets in subjects with periodontitis using Kruskal Wallis ANOVA. Within-subject comparisons between deep and shallow sites for individual species were made using Wilcoxon's signed rank test. Chi-squared analysis was used to test for the presence or absence of species in health and disease.

RESULTS

Plaque samples for the study were collected from 15 subjects with moderate to severe chronic periodontitis (separate deep and shallow site samples were collected from each subject) and from 15 age-matched, periodontally healthy control subjects (pooled samples from all teeth). The mean age of the experimental group was 63.6 yr (standard deviation [SD] 9.1), and the mean age of the control group was 60.2 yr (SD 11.3). The difference, as determined by a t-test, was not significant. The healthy group was 71% male, while the periodontitis group was 73% male. No significant difference was found by chi-squared analysis. The healthy group was 100% white, and the periodontitis group

was 87% white and 13% African-American. The sample size did not permit statistical comparisons by race.

Sequence data of 500 to 800 bp was obtained for 100 clones from each sample for a total of 4500 clones. Forty-three clones were less than 98% identical to current GenBank entries, and these clones were grouped into 7 novel phylotypes (GenBank accession numbers pending). A total of 274 species or phylotypes were identified. Table 1 lists these species in order of their ranking by overall prevalence and showing the mean prevalence in the three groups of samples.

Figure 1 shows the distribution of bacterial phyla in health and disease. Bacilli and Clostridia, two classes in the phylum Firmicutes, are displayed separately due to their high numbers. Both Clostridia and Deferribacteres showed a significant ($P<0.05$) association with periodontitis, and the Bacilli were significantly associated with periodontal health. Figure 2 shows the distribution of gram-positive and gram-negative anaerobes and facultative bacteria in relation to health status. Only gram-positive bacteria were significantly different among the groups.

Overall 59.9% of the clone population was made up of as-yet-uncultivated phylotypes. Figure 3 shows the relative prevalence of uncultivated phylotypes to cultivated species within each genus. The genera Deferribacteres, Megasphaera, Desulfobulbus and Lachnospira were composed entirely of uncultivated phylotypes. Uncultivated phylotypes were predominant within the genera Selenomonas, Veillonella and

Peptostreptococcus. Other genera such as Campylobacter, Gemella, Streptococcus and Neisseria were composed predominantly of named species.

Figure 4 shows the distribution of the 22 most common bacterial genera in relation to disease status. Table 2 lists species or phylotypes that showed an association with disease or health ($P < 0.1$). The ranking of these species indicates their relative prevalence among all clones.

DISCUSSION

The current paradigm of the microbial etiology of periodontitis implicates numerically minor gram negative anaerobic components of the plaque biofilm, such as P. gingivalis, T. forsythensis and T. denticola, as the primary etiologic agents. Although several lines of evidence are available to support an etiologic role for these species, the epidemiologic data linking these species to disease was obtained with closed-ended approaches that would not allow the detection and enumeration of previously unidentified species. The present study employed 16S PCR amplification using universal 16S primers of dental plaque samples, followed by cloning and sequencing to allow an open-ended and quantitative exploration of the bacterial populations present in periodontal health and disease. Using this approach an unexpected profile of health and disease-associated bacteria populations was observed.

Molecular approach

Subgingival bacterial popoulations have previously been explored using 16S cloning and sequencing. These studies have been qualitative studies exploring the diversity of subgingival bacterial populations and have included the use of primers targeted to specific, previously suspected groups of bacteria such as the Bacteroidetes [70, 74] Eubacterium [75], and even archaea [76], or subtraction systems to eliminate major species such as streptococci [77], and have used high cycle numbers to enrich for minor species. In the present, quantitative study, in order to retain a representative set of amplicons, a low PCR cycle number was employed to avoid plateau effects, and a set of broad, universal bacterial primers were used. One hundred clones were sequenced and identified from every sample to allow statistical comparisons to be made. Disease-associated samples were collected from the 4 deepest sites in subjects with established periodontitis. Control samples were collected from shallow sites in these same subjects and also from a separate, age-matched healthy control group. Including samples from completely healthy individuals as well as from sites that did not exhibit signs of disease in individuals with disease allowed questions regarding site-specific versus global ecological perturbation to be addressed.

The most numerous species by 16S clonal analysis belonged to the genera Selenomonas, Streptococcus, Veillonella, Campylobacter, and Peptostreptococcus (figure 4). These genera were all detected in a previous culture-based study of periodontal bacteria [78], although all but Streptococcus appeared to account for a relatively smaller fraction of total bacteria. Other major groups of bacteria detected in previous studies using DNA

hybridization included Fusobacterium, and using cultivation and DNA hybridization, Actinomyces [78, 79]. Both were rare in the present study. The greater sensitivity of cultivation as compared to molecular analyses for the detection of Actinobacteria has been previously reported [80]. To investigate this, the DNA isolation and amplification method was tested on Actinomyces viscosus in a mixture with other species, and A. viscosus was detected with comparable sensitivity (data not shown), suggesting that the bias might be attributed to over-representation with cultivation.

The genera Bacteroides and Porphyromonas were numerically minor, also consistent with earlier studies [50, 78], and spirochetes were also found in low numbers. Centrifugation, freezing, and long storage time before isolation of DNA have been suspected of contributing to loss of delicate, easily lysed organisms such as Spirochetes. However, the DNA isolation method was tested, both with and without centrifugation on both fresh and frozen samples, for recovery of DNA from Spirochetes, and no differences were detected (data not shown). The methodology used for DNA isolation in this study may have been slightly biased towards gram-negative species, since the protocol did not include disruption of cell wall by vigorous agitation. Nevertheless, large numbers of gram-positive bacteria were detected. Undoubtedly some bias is present with 16S cloning and sequencing of bacterial populations, due to differences in isolation of DNA from structurally varied bacteria, varied affinities for universal primers, and differences in copy number of ribosomal genes. For this study efforts were made to minimize bias, and compared to cultivation, with less than half of species detectable and many inaccuracies

inherent in phenotypic identification, molecular analysis offers a more comprehensive and accurate approach.

Overall 274 species or phylotypes of bacteria including 7 novel phylotypes were detected, and they belonged to 7 different phyla (table 1). Consistent with earlier observations [77, 81], approximately 60% of these species were uncultivated. Several of the most numerous genera, including Selenomonas, Veillonella, and Peptostreptococcus, were composed primarily of uncultivated species (figure 3). Distributions of several uncultivated bacteria were found to differ in subjects with health and periodontitis, and it appears that significant relationships may have been undetectable in previous studies using cultivation-based or closed-ended DNA approaches.

Only 0.5% chimeric sequences were detected in this study. Studies using similar approaches have found 1 to 15% of clones to be chimeric sequences [81]. For the current study, formation of chimeras was minimized by limiting PCR cycle number [82]. Colonies were also screened for inserts of the expected size by PCR and gel electrophoresis before sequencing, eliminating many potential chimeric sequences.

The large number of species observed necessitated grouping data into phyla and genera to obtain sufficient power for statistical analysis of all but the most numerous species. However, the data was analyzed at the level of species (alpha=0.1) to identify candidate species for subsequent investigation. Because of the non-normal distributions typically observed with bacterial counts, nonparametric statistics were employed for all analyses.

Phyla associated with periodontitis

The subgingival flora in both health and periodontitis was dominated by the phylum Firmicutes. The classes Clostridia and Bacilli of the Firmicutes together accounted for 75% of all clones, and were associated with opposite ends of the health spectrum: the Bacilli (most numerous genera were Streptococcus and Gemella) accounted for a greater fraction of the bacteria in healthy subjects; in contrast, the class Clostridia (most numerous genera were Peptostreptococcus, Veillonella, and Selenomonas) was more common in subjects with periodontitis. Several additional opposing patterns of association within phyla were observed, suggesting that analysis at the level of the phylum is not informative for disease classification.

Analysis at the level of genera showed several statistically significant associations with periodontitis and health. Surprisingly, many of these occurred among the gram-positives rather than the gram-negatives usually thought to be important in disease.

Genera and species associated with periodontitis

The taxonomy of the gram-positive anaerobic cocci (GPAC) commonly referred to as "peptostreptococci" is evolving, and some species previously classified as Peptostreptococcus have recently been reassigned to closely related genera [83, 84] such as Anaerococcus, Peptococcus, Micromonas and Peptonephilus [83]. In addition, several uncultivated peptostreptococci were detected in large numbers in the current study. Based on their phylogenetic similarity and evolving taxonomy, the peptostreptococci were

grouped together for this analysis. The association of the peptostreptococci with periodontitis was particularly robust, and they were far more numerous than the gram-negative anaerobes commonly associated with periodontitis. At the species level, Peptostreptococcus BS044 and CK035 were very numerous and were associated with disease (table 2). The selectivity of culturing and low specificity of chemical and phenotypic characterization may have prevented their identification as potential pathogens in previous studies.

GPAC have been isolated from a wide range of human infections, typically constituting one-fourth or more of anaerobic species from clinical specimens [84]. Most infections involving GPAC are polymicrobial, and appear to involve synergistic interactions with other bacteria [84]. Previous epidemiologic evidence has linked peptostreptococci with dental infections, although investigations have been limited to P. micros, a rare species in the current study (and not associated with disease). P.micros has been associated with odontogenic infections [85, 86], and is significantly higher in smokers, a population that has more extensive and severe periodontitis than non-smokers [87]. It is also more common around mobile teeth [88], and has been found at higher levels in epithelial-associated plaque as compared to unassociated plaque in the gingival sulcus [89]. Targeted DNA approaches have also found P.micros to be elevated in advanced chronic periodontitis [90] and more common in subjects with periodontitis [91]. Evidence regarding the mechanism of pathogenesis for GPAC-associated infections is limited. Peptostreptococci isolated from chronic skin ulcers have been shown to inhibit keratinocyte and fibroblast proliferation and wound repopulation in a tissue culture model

system [92]. P. micros demonstrates both adhesion to epithelium as well as coaggregation with other species such as P.gingivalis and F.nucleatum mediated by extracellular polysaccharides [93, 94]. These data suggest that peptostreptococci may play a role in preventing wound healing in chronic disease and may be important in the physical structure of a disease-associated biofilm. Further exploration of the role these bacteria play in periodontitis is needed.

The gram-positive rod Filifactor alocis is related to the peptostreptococci, and was also common in the samples (table 2), and significantly elevated in disease (figure 2). It has been previously associated with both chronic periodontitis [91] and endodontic lesions[95].

Several gram-negative bacteria were also associated with periodontitis, although they occurred in low numbers relative to the gram-positive, disease-associated species. The genus Megasphaera was elevated in periodontitis, and at the species level Megasphaera BB166, MCE3_141 and BS073 and were associated with disease. Megasphaera clone BB166 has been previously associated with chronic periodontitis [91]. Megasphaera have been reported as normal inhabitants of the gut and vagina [96], and M. elsdenii has been implicated in bacterial endocarditis in immunocompromised patients [97].

The genus Desulfobulbus was also associated with disease, and both Desulfobulbus CH031 and R004 were significantly associated with deep sites at the species level. Desulfobulbus species have been previously detected in the gingival sulcus [81, 91] and

the human gut [98]. Desulfobulbus are sulfate-reducing bacteria, and have been frequently detected in aquatic environmental samples.

Campylobacter sputorum sputorum and Campylobacter BB120 were strongly associated with disease. Taken as a whole the genus Campylobacter was associated with health, but this association was accounted for by the highly prevalent species C. gracilis and C. showae.

Many clones of Selenomonas were detected, most from the cultivable species S. sputigena, S. infelix and S. noxia. None of these were associated with disease, although S. noxia has been previously linked to active periodontitis [78]. The less numerous and uncultivated Selenomonas phylotypes D0-042, EY047 and AH132 were associated with disease, and, in contrast, Selenomonas DS051 was detected more frequently in healthy subjects.

Dialister pneumosintes and Dialister phylotype ME_134 were associated with periodontitis. D. pneumosintes has been previously linked to periodontitis [99, 100] and to endodontic infections [101]. Deferribacteres phylotypes W090 and BH007 were associated with periodontitis, and W090 has been previously linked to disease [91]. In addition, uncultivated phylotypes of Catonella, Streptococci, Atopobium, Eubacterium and Treponema were also significantly associated with disease (table 2). However, because of the large number of species examined, some associations are likely to occur by random chance, and these candidates require further investigation.

P. gingivalis and T. denticola, T. forsythia were rarely detected in the present study, and of these, only T. forsythia was associated with disease. Strong associations to disease have been observed for these species in many previous studies, but when quantitative results have been reported, they have comprised only a small fraction of the total bacteria. The sample size in the present study did not provide adequate power to detect association for minor species. More numerous bacteria did show strong associations with disease, however, indicating potentially important bacteria have been overlooked in previous studies due to technical challenges. What remains unclear at the present time is whether these newly identified and more numerous species play a more important role in pathogenesis than the less numerous previously implicated species.

Genera and species associated with health

Streptococcus and Veillonella were found in high numbers in all samples, and accounted for a significantly greater fraction of the microbial community in healthy subjects than in those with periodontitis. At the species level both S. sanguis and S. mutans were associated with periodontal health, as was the overall most abundant species, Veillonella X042. Both Streptococcus and Veillonella have been previously associated with periodontal health [78, 79]. Veillonella X042 is very closely related to V. parvula and V. dispar by 16S phylogeny, and may be part of an indistinguishable cluster [102]. The parallel relationship observed between levels of streptococci and Veillonella is not surprising in view of the fact that Veillonellae utilize short chain acids such as lactates

that are secreted by gram-positive facultatives such as streptococci [103], and it has been shown that Veillonellae will not colonize tooth surfaces without streptococci [104].

The microbial profile of periodontal health also included the less abundant genera Campylobacter, Abiotrophia, Capnocytophaga, Gemella and Neisseria. This confirms earlier studies linking Capnocytophaga [78, 79, 105] and Campylobacter gracilis [106] to health.

Levels of the genera Streptococci and Veillonella were more similar between shallow and deep sites in individuals with periodontitis than between healthy individuals and those with periodontitis. A similar phenomenon was observed for many health and disease-associated species (table 2): many more differences were observed between healthy and diseased subjects than were found between shallow and deep sites in individuals with disease. It appears that disease may involve a disruption in the microbial ecology of the entire dentition rather than a disease-site specific shift, and that transitions between health and chronic periodontitis are associated with shifts in the relative proportions of major bacteria.

Several issues regarding molecular epidemiologic approaches to the study of chronic bacterial diseases deserve mention. First, these studies can demonstrate association but do not establish causation; subsequent studies are needed. Second, interactions with the host are likely to be important and are poorly understood at the present time. Finally, the diversity in bacterial communities is just beginning to be explored. We have little

knowledge of the genetic heterogeneity in these communities beyond that occurring in ribosomal genes, so it is not clear if explorations should be conducted at the level of genus, species, or even virulence gene. Polymicrobial bacterial communities are complex and undergo interactions within the community that could be critical determinants. Bacterial profiles also vary among individual hosts, suggesting that periodontitis has a heterogeneous etiology. Because of this complexity, much larger sample sizes than those achievable with current technology may be required for a full understanding of chronic polymicrobial diseases.

In summary, the largest differences between health-associated and periodontitis associated biofilm communities were found among the gram-positive species. Peptostreptococci and Filifactor were elevated in periodontitis, and Streptococcus, Abiotrophia, and Gemella were elevated in health. Differences were also observed among the gram-negative bacteria: Veillonella, Campylobacter, and Capnocytophaga were higher in the plaque of healthy subjects, and Megasphaera and Desulfobulbus were elevated in periodontitis. Several species were also identified as candidates for further study, including many uncultivated phylotypes. These newly identified candidates outnumbered P. gingivalis and other species previously implicated as periodontopathogens, and it is not clear if newly identified and more numerous species may play a more important role in pathogenesis. Finally, more differences were found in the bacterial profile of the two subject groups than between deep and shallow sites within the same mouth. This suggests that chronic periodontitis is the result of a global

perturbation of the oral bacterial ecology rather than a disease-site specific microbial shift.

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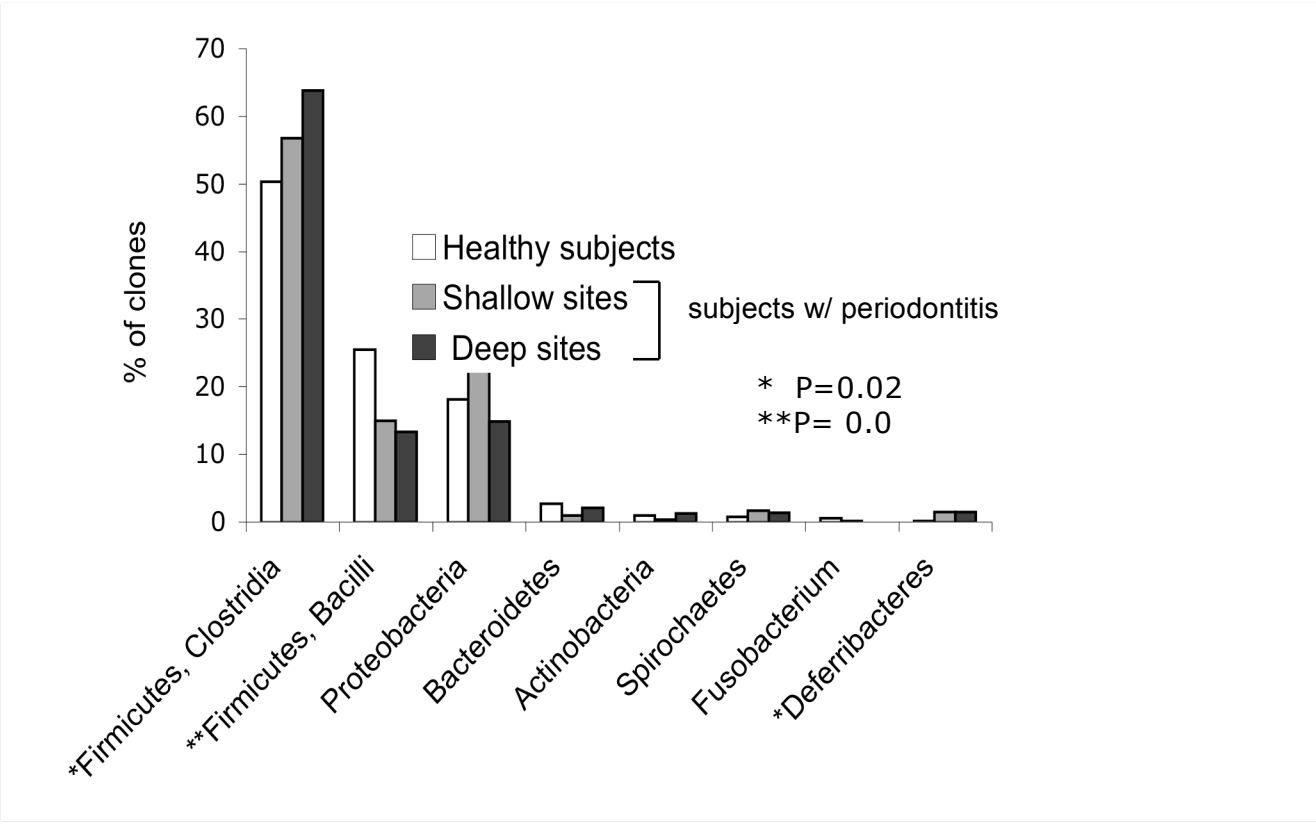

Figure1. Distribution of bacterial phyla in health and disease. Two classes of Firmicutes (Bacilli and Clostridia) are displayed individually due to their high prevalence.

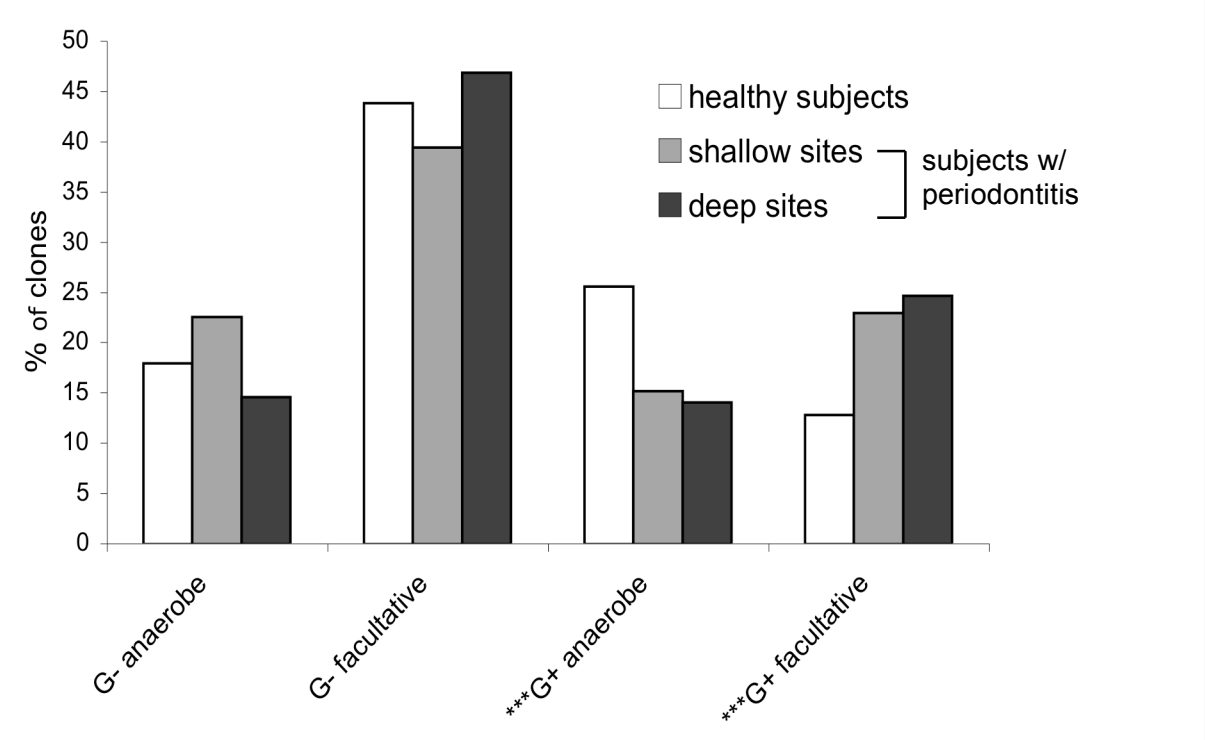

Figure2. Distribution of gram positive (G+) and gram negative (G-) anaerobes and facultative species in relation to disease status. The gram status of uncharacterized phylotypes was inferred from that of their closest neighbor. $P<0.005$ is indicated by asterisks(***).

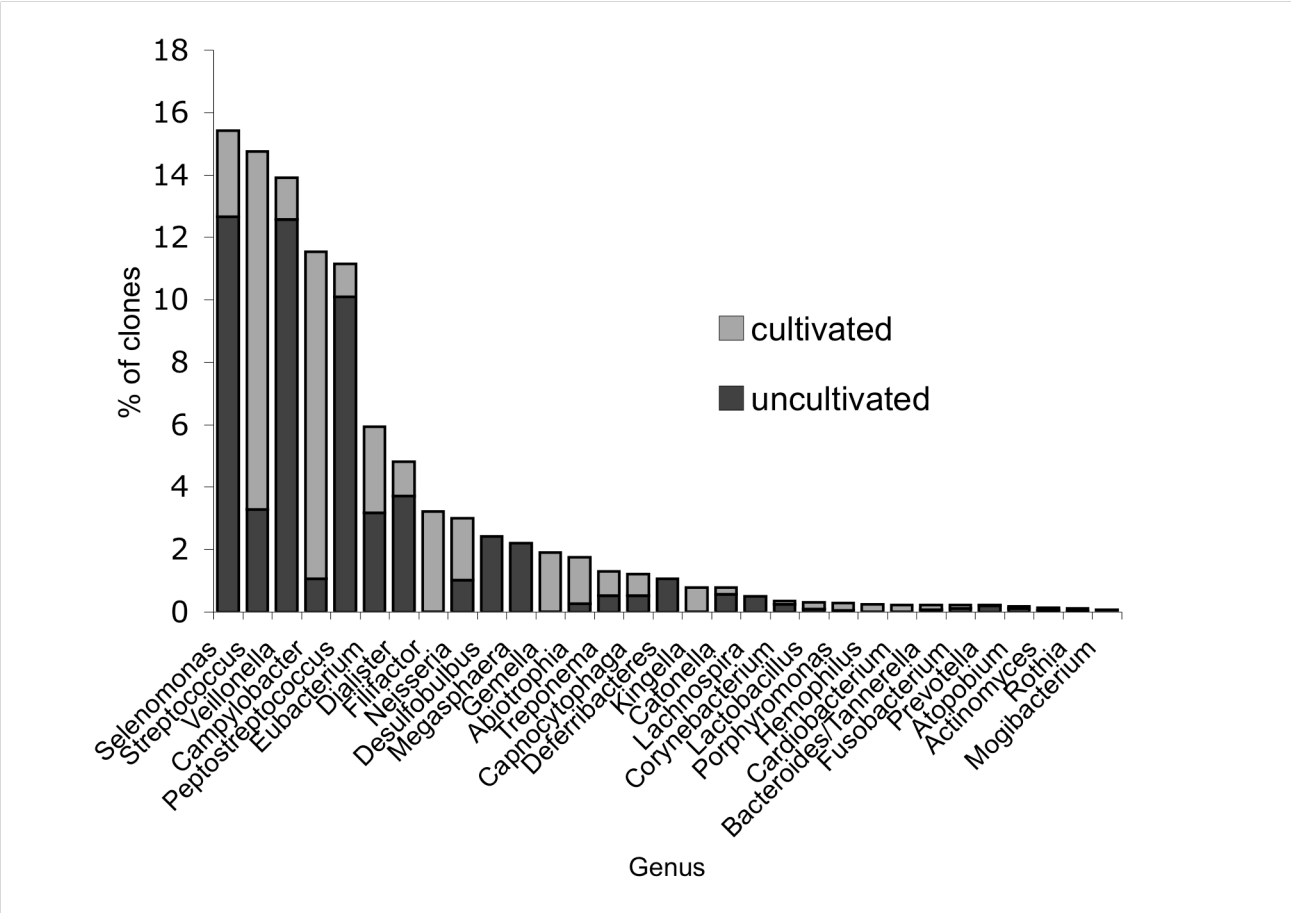

Figure 3. Distribution of cultivated and uncultivated bacteria by genus for all samples.

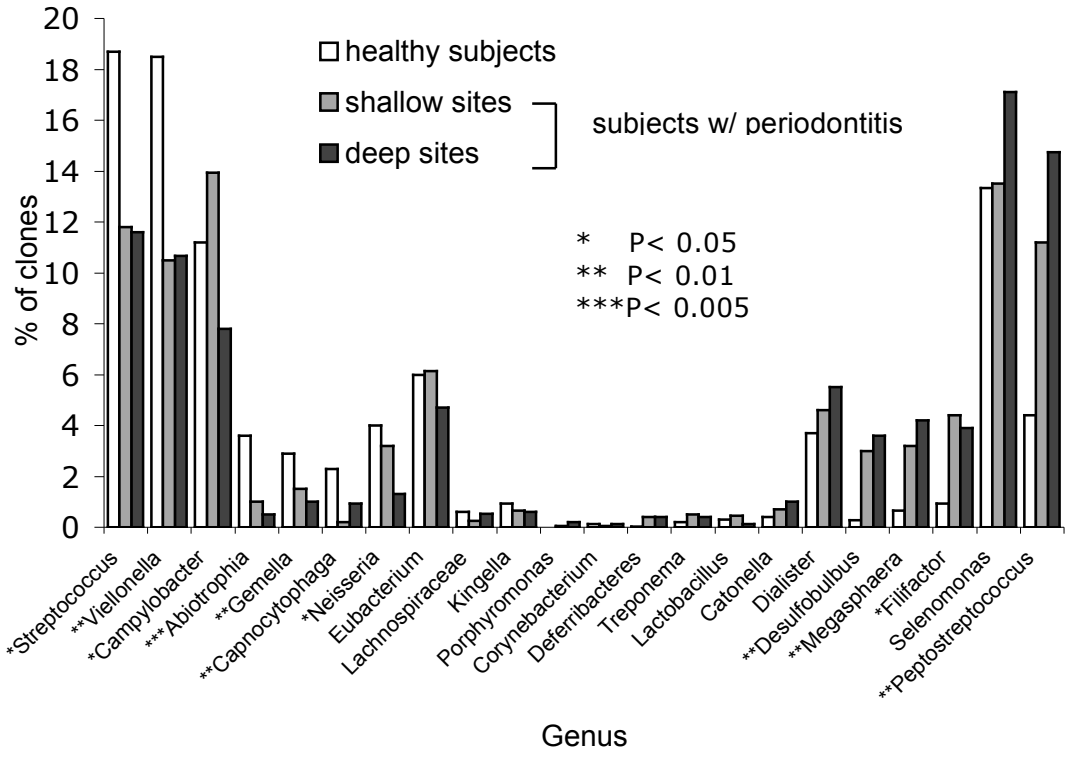

Figure 4. Distribution by health status for genera accounting for greater than 0.25% of total bacteria are shown. The genera are arranged in a gradient from those predominant in health shown on the left to those predominant in periodontitis shown on the right.

overall rank	species/phylotypes	% clones	mean prevalence ± SD: healthy subjects	shallow sites	deep sites
1	Veillonella sp. oral clone X042	7.38	13.1 ± 9.4	4.5 ± 3.3	4.6 ± 1.7
2	Campylobacter gracilis	6.71	8.1 ± 4.9	7.8 ± 3.9	4.2 ± 4.3
3	Peptostreptococcus sp. oral clone FG014	4.27	2.2 ± 2.7	5.2 ± 5.0	5.4 ± 7.9
4	Selenomonas sputigena/EW051a/DD020	4.02	3.4 ± 3.6	4.0 ± 3.3	4.7 ± 3.2
5	Viellonella sp. oral clone BU083	3.64	3.3 ± 3.9	3.5 ± 3.4	4.1 ± 2.5
6	Peptostreptococcus sp. oral clone BS044	3.11	0.9 ± 2.1	4.1 ± 6.9	4.4 ± 8.2
7	Filifactor alocis	3.07	0.9 ± 1.1	4.4 ± 3.9	3.9 ± 3.6
8	Streptococcus mitis	2.71	4.9 ± 5.2	1.8 ± 2.2	1.4 ± 1.8
9	Selenomonas infelix	2.51	2.0 ± 1.9	2.7 ± 2.1	2.9 ± 1.9
10	Selenomonas noxia/EQ054	2.18	2.9 ± 2.3	1.7 ± 1.8	2 ± 2.2
11	Dialister sp. E2_20	2.09	1.8 ± 1.8	1.9 ± 2.0	2.6 ± 2.5
12	Streptococcus gordonii	1.98	2.2 ± 2.7	1.9 ± 2.4	1.9 ± 3.6
13	Selenomonas dianae/AJ036/DY027	1.62	1.3 ± 1.3	1.3 ± 1.4	2.3 ± 2.1
14	Streptococcus oralis	1.56	1.7 ± 2.1	1.7 ± 2.0	1.3 ± 1.3
15	Peptostreptococcus sp. oral clone CK035	1.51	0.5 ± 0.8	0.8 ± 1.3	3.3 ± 4.7
16	Megasphaera sp. oral clone BB166	1.42	0.2 ± 0.4	2.1 ± 3.7	1.9 ± 2.7
17	Desulfobulbus sp. oral clone CH031	1.40	0.3 ± 0.6	2.1 ± 3.3	1.9 ± 2.8
18	Dialister sp. oral clone BS095	1.09	1.5 ± 1.9	0.8 ± 0.9	0.9 ± 1.2
19	Dialister pneumosintes	1.04	0.1 ± 0.4	1.7 ± 2.4	1.3 ± 1.3
20	Campylobacter sputorum sputorum	1.00	0.2 ± 0.4	1.9 ± 1.9	0.9 ± 1.1
21	Abiotrophia adiacens	0.96	2.1 ± 2.1	0.5 ± 1.0	0.3 ± 0.6
22	Neisseira meningitidis	0.93	1.4 ± 1.7	0.9 ± 1.2	0.5 ± 0.7
23	Streptococcus intermedius	0.91	1.3 ± 1.4	0.5 ± 1.1	0.9 ± 1.3
24	Desulfobulbus sp. oral clone R004	0.89	0.0 ± 0.0	0.9 ± 1.5	1.7 ± 2.5
25	Streptococcus pneumoniae	0.87	1.3 ± 1.2	0.8 ± 1.3	0.5 ± 0.8
26	Unidentified eubacterium	0.84	1.1 ± 1.5	1.1 ± 2.1	0.3 ± 0.5
27	Campylobacter sp. oral clone BB120	0.82	0.0 ± 0.0	1.3 ± 3.2	1.1 ± 2.4
28	Gemella morbillorum	0.80	0.9 ± 1.5	0.9 ± 1.2	0.6 ± 0.6
29	Firmicutes sp. oral clone A0068	0.73	0.6 ± 1.1	0.5 ± 0.7	1.1 ± 1.8
30	Eikenella corrodens	0.69	0.6 ± 1.3	0.8 ± 1.4	0.7 ± 1.1
31	Eubacterium saburreum	0.64	1.6 ± 1.5	0.1 ± 0.4	0.2 ± 0.4
32	Veillonella sp. oral clone AA050	0.64	0.5 ± 1.8	1.1 ± 3.1	0.3 ± 0.6
33	Campylobacter concisus	0.64	1.0 ± 1.1	0.5 ± 0.7	0.5 ± 0.9
34	Megasphaera micronuciformis (Anaerosphaera nuciformis)	0.60	0.3 ± 0.8	0.7 ± 1.1	0.7 ± 1.1
35	Peptostreptococcus sp. oral clone AJ062	0.60	0.1 ± 0.3	0.7 ± 1.1	1.1 ± 1.8
36	Selenomonas sp. oral clone D0042	0.58	0.1 ± 0.3	0.8 ± 1.3	0.9 ± 1.0
37	Veillonella atypica	0.58	0.7 ± 1.5	0.2 ± 0.4	0.9 ± 2.3
38	Campylobacter showae	0.58	1.1 ± 1.8	0.5 ± 0.8	0.1 ± 0.4
39	Campylobacter rectus	0.56	0.2 ± 0.4	0.9 ± 1.2	0.5 ± 0.8
40	Streptococcus hyointestinalis	0.53	0.6 ± 0.8	0.8 ± 1.6	0.2 ± 0.6
41	Streptococcus sp. oral strain H3-M2	0.53	1.2 ± 2.0	0.1 ± 0.3	0.3 ± 0.7
42	Gemella sp.	0.51	1.1 ± 1.4	0.3 ± 0.7	0.1 ± 0.3
43	Veillonella ratti	0.51	0.3 ± 0.6	0.8 ± 1.1	0.4 ± 1.1
44	Streptococcus sanguis	0.49	1.3 ± 2.2	0.1 ± 0.4	0.0 ± 0.0
45	Eubacterium yurii/A03MT	0.47	0.4 ± 0.6	0.9 ± 1.4	0.1 ± 0.4
46	Selenomonas sp. oral clone CS015	0.47	0.3 ± 0.9	0.5 ± 1.1	0.6 ± 0.9
47	Kingella oralis	0.47	0.7 ± 1.1	0.3 ± 0.6	0.4 ± 0.9
48	Eubacterium sp. sp. oral clone EI074	0.44	0.7 ± 1.0	0.1 ± 0.3	0.5 ± 1.8
49	Capnocytophaga gingivalis	0.42	0.9 ± 1.3	0.0 ± 0.0	0.4 ± 1.1
50	Deferribacteres sp. oral clone W090	0.42	0.0 ± 0.0	0.6 ± 1.0	0.7 ± 1.1
51	Centipeda periodontii	0.42	0.5 ± 0.8	0.3 ± 0.8	0.5 ± 0.9
52	Eubacterium sp. oral clone EW049	0.42	0.5 ± 1.1	0.6 ± 1.5	0.2 ± 0.6
53	Megasphaera sp. oral clone MCE3_141	0.42	0.0 ± 0.0	0.3 ± 0.6	1.0 ± 1.2
54	Selenomonas sp. oral clone EY047	0.42	0.7 ± 1.2	0.0 ± 0.0	0.6 ± 0.8
55	Neisseria elongata	0.42	0.3 ± 1.0	0.7 ± 2.1	0.3 ± 1.0
56	Gemella haemolysans	0.38	0.6 ± 0.9	0.3 ± 0.6	0.3 ± 0.7
57	Treponema socranskii subsp. socranskii	0.38	0.4 ± 1.3	0.3 ± 0.8	0.4 ± 0.6
58	Eubacteriaceae sp. oral clone MCE10_174 E2	0.36	0.1 ± 0.4	0.5 ± 0.9	0.4 ± 0.9
59	Peptostreptococcus sp.	0.36	0.4 ± 1.1	0.5 ± 0.9	0.1 ± 0.4
60	Streptococcus sp. oral strain 7A	0.33	0.2 ± 0.6	0.5 ± 0.9	0.3 ± 0.8
61	Catonella sp. oral clone EZ006	0.33	0.3 ± 0.6	0.3 ± 0.7	0.4 ± 0.8
62	Eubacterium brachy	0.33	0.2 ± 0.6	0.7 ± 1.3	0.1 ± 0.3
63	Johnsonella ignava	0.33	0.7 ± 0.8	0.1 ± 0.4	0.2 ± 0.4
64	Lachnospiraceae sp. oral clone MCE9_104 E2	0.33	0.4 ± 0.8	0.3 ± 0.6	0.3 ± 0.6
65	Neisseria sp. oral clone AP132	0.33	0.2 ± 0.4	0.5 ± 0.6	0.3 ± 1.0
66	Abiotrophia para adiacens	0.31	0.5 ± 0.9	0.3 ± 0.6	0.1 ± 0.3
67	Peptoniphilus ivorii (Peptostreptococcus ivoricus)	0.31	0.1 ± 0.5	0.7 ± 1.8	0.1 ± 0.3
68	Selenomonas sp. oral clone CS024	0.31	0.7 ± 1.3	0.1 ± 0.5	0.1 ± 0.4
69	Selenomonaslike sp. oral clone DM071	0.31	0.0 ± 0.0	0.4 ± 0.7	0.5 ± 1.2
70	Streptococcus pyogenes	0.29	0.0 ± 0.0	0.7 ± 1.3	0.2 ± 0.4
71	Selenomonas-like sp. oral strain FNA3	0.29	0.2 ± 0.4	0.3 ± 0.8	0.3 ± 0.7
72	Neisseria sp. oral clone AP085	0.29	0.7 ± 1.9	0.2 ± 0.6	0.0 ± 0.0
73	Streptococcus sp. oral clone BM 035	0.27	0.2 ± 0.6	0.1 ± 0.4	0.5 ± 1.3
74	Streptococcus sp. oral strain 12F	0.27	0.7 ± 1.4	0.0 ± 0.0	0.1 ± 0.4
75	Eubacterium saphenum	0.27	0.1 ± 0.4	0.3 ± 0.8	0.3 ± 0.8
76	Mitsuokella jalaludinii	0.27	0.1 ± 0.4	0.3 ± 0.8	0.4 ± 1.3
77	Firmicutes sp. oral clone F058	0.27	0.3 ± 0.6	0.3 ± 0.5	0.2 ± 0.6
78	Kingella denitrificans	0.27	0.3 ± 0.6	0.3 ± 0.6	0.2 ± 0.6
79	Veillonella like sp. oral clone 1A	0.27	0.3 ± 0.7	0.2 ± 0.6	0.3 ± 0.6
80	Streptococcus mutans	0.24	0.7 ± 1.2	0.1 ± 0.3	0.0 ± 0.0
81	Streptococcus suis	0.24	0.1 ± 0.3	0.3 ± 0.6	0.4 ± 0.8
82	Streptococcus sp. oral clone AA007	0.24	0.2 ± 0.8	0.1 ± 0.3	0.5 ± 1.1
83	Eubacterium sp. oral clone EH006	0.24	0.1 ± 0.4	0.2 ± 0.4	0.4 ± 0.9
84	Campylobacter sputorum	0.24	0.1 ± 0.3	0.6 ± 1.1	0.1 ± 0.3
85	Treponema socranskii subsp. buccale	0.24	0.2 ± 0.8	0.4 ± 0.9	0.1 ± 0.4
86	Porphyromonas gingivalis	0.22	0.0 ± 0.0	0.2 ± 0.4	0.5 ± 1.8
87	Capnocytophaga granulosa	0.22	0.4 ± 0.9	0.1 ± 0.4	0.1 ± 0.4
88	Capnocytophaga sp. sp. oral clone AH015	0.22	0.6 ± 1.7	0.1 ± 0.3	0.0 ± 0.0
89	Streptococcus sp. oral clone 2056B	0.22	0.1 ± 0.4	0.1 ± 0.4	0.4 ± 1.3
90	Dialister sp. oral clone MCE7_134	0.22	0.0 ± 0.0	0.1 ± 0.5	0.5 ± 0.9
91	Eubacterium sp. oral clone E1-K17	0.22	0.2 ± 0.4	0.3 ± 0.6	0.2 ± 0.6
92	Selenomonas sp. oral clone D027	0.22	0.1 ± 0.3	0.3 ± 0.8	0.3 ± 0.6

Table1. Species and phylotypes from three sample groups showing percentage of total clones and mean prevalence in each group arranged in order of decreasing overall prevalence

overall rank	species/phylotypes		mean prevalence ± SD		
		% clones	healthy subjects	shallow sites	deep sites
93	Alysiella filiformis	0.22	0.1 ± 0.3	0.3 ± 0.6	0.3 ± 0.7
94	Neisseria denitrificans	0.22	0.3 ± 0.9	0.1 ± 0.4	0.2 ± 0.6
95	Capnocytophaga sp. Oral strain S3	0.20	0.3 ± 0.5	0.0 ± 0.0	0.3 ± 1.3
96	Deferribacteres sp. oral clone BH017	0.20	0.1 ± 0.3	0.2 ± 0.4	0.3 ± 1.3
97	Streptococcus salivarius	0.20	0.1 ± 0.5	0.3 ± 0.6	0.2 ± 0.6
98	Streptococcus sinensis	0.20	0.3 ± 0.6	0.3 ± 0.8	0.0 ± 0.0
99	Catonella sp. oral clone BR063	0.20	0.0 ± 0.0	0.1 ± 0.4	0.5 ± 0.6
100	Catonella morbi	0.20	0.1 ± 0.4	0.3 ± 0.6	0.2 ± 0.4
101	Eubacterium sp. oral clone EW053	0.20	0.1 ± 0.3	0.3 ± 0.8	0.2 ± 0.6
102	Peptostreptococcus micros	0.20	0.5 ± 1.2	0.0 ± 0.0	0.1 ± 0.4
103	Selenomonas sp. oral clone EW076	0.20	0.2 ± 0.6	0.1 ± 0.4	0.3 ± 0.6
104	Selenomonas sp. oral clone EW079	0.20	0.1 ± 0.3	0.2 ± 0.6	0.3 ± 0.9
105	Selenomonas sp. oral clone CS023	0.20	0.1 ± 0.3	0.3 ± 1.0	0.3 ± 0.5
106	Streptococcus sp. oral clone 4093B	0.18	0.0 ± 0.0	0.1 ± 0.4	0.4 ± 1.1
107	Eubacterium sp. oral clone CK047	0.18	0.1 ± 0.3	0.1 ± 0.4	0.3 ± 0.7
108	Selenomonas sp. oral clone AA024	0.18	0.4 ± 0.9	0.0 ± 0.0	0.1 ± 0.4
109	Neisseria sp sp. oral clone AP060	0.18	0.2 ± 0.8	0.3 ± 0.6	0.0 ± 0.0
110	Selenomonas like sp. oral clone 4A	0.18	0.1 ± 0.4	0.3 ± 0.6	0.1 ± 0.4
111	Campylobacter like sp. oral clone 5A	0.18	0.2 ± 0.6	0.2 ± 0.6	0.1 ± 0.4
112	Prevotella sp. oral clone BR014	0.16	0.1 ± 0.4	0.3 ± 0.8	0.0 ± 0.0
113	Eubacterium sp. oral clone DO016	0.16	0.3 ± 1.0	0.1 ± 0.3	0.1 ± 0.3
114	Eubacterium sp. oral clone DA014	0.16	0.0 ± 0.0	0.1 ± 0.4	0.3 ± 0.8
115	Selenomonas sp. oral clone CS024	0.16	0.2 ± 0.6	0.1 ± 0.3	0.2 ± 0.4
116	Veillonella dispar	0.16	0.3 ± 0.7	0.2 ± 0.6	0.0 ± 0.0
117	Campylobacter curvus	0.16	0.3 ± 0.6	0.1 ± 0.5	0.1 ± 0.3
118	Tannerella forsythia (Bacteroides forsythus)	0.13	0.0 ± 0.0	0.1 ± 0.3	0.3 ± 0.7
119	Deferribacteres sp. oral clone BH007	0.13	0.0 ± 0.0	0.4 ± 0.7	0.0 ± 0.0
120	Lactobacillus lactis subsp. lactis	0.13	0.0 ± 0.0	0.3 ± 1.0	0.1 ± 0.3
121	Streptococcus agalactiae	0.13	0.3 ± 1.0	0.1 ± 0.3	0.0 ± 0.0
122	Streptococcus anginosus	0.13	0.1 ± 0.3	0.1 ± 0.3	0.3 ± 0.8
123	Streptococcus infantis	0.13	0.1 ± 0.4	0.1 ± 0.3	0.2 ± 0.8
124	Streptococcus sp. oral clone 3097C	0.13	0.2 ± 0.6	0.2 ± 0.4	0.0 ± 0.0
125	Streptococcus sp. oral clone BW009	0.13	0.0 ± 0.0	0.3 ± 0.8	0.1 ± 0.4
126	Streptococcus sp. oral strain 9F	0.13	0.0 ± 0.0	0.1 ± 0.3	0.3 ± 0.6
127	Selenomonaslike sp. sp. oral clone GAA14	0.13	0.1 ± 0.3	0.1 ± 0.5	0.2 ± 0.4
128	Abiotrophia like sp. oral clone 2A	0.13	0.2 ± 0.4	0.2 ± 0.6	0.0 ± 0.0
129	Atopobium sp. oral clone C019	0.11	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 1.0
130	Corynebacterium sp. oral clone DS081	0.11	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4
131	Corynebacterium sp. oral clone AK143	0.11	0.1 ± 0.4	0.0 ± 0.0	0.2 ± 0.4
132	Abiotrophia sp. oral clone P4PA_155 P1	0.11	0.3 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
133	Abiotrophia defectiva	0.11	0.3 ± 0.5	0.0 ± 0.0	0.1 ± 0.3
134	Gemella sanguinis	0.11	0.3 ± 0.8	0.0 ± 0.0	0.1 ± 0.3
135	Streptococcus oligofermentans	0.11	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4
136	Streptococcus sp. oral clone 2061A	0.11	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4
137	Eubacterium sp. oral clone DZ073	0.11	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4
138	megasphaera sp. oral clone BU057	0.11	0.1 ± 0.4	0.0 ± 0.0	0.2 ± 0.8
139	Peptostreptococcus anaerobius	0.11	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.9
140	Peptococcus sp. oral clone MCE10_265 E1	0.11	0.1 ± 0.4	0.1 ± 0.5	0.1 ± 0.3
141	Firmicutes sp. oral clone CK051	0.11	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4
142	Haemophilus segnis	0.11	0.2 ± 0.6	0.1 ± 0.3	0.1 ± 0.3
143	Treponema sp. V:19:D36	0.11	0.0 ± 0.0	0.1 ± 0.4	0.2 ± 0.6
144	Corynebacterium matruchotii	0.09	0.2 ± 0.4	0.0 ± 0.0	0.1 ± 0.3
145	Rothia dentocariosa	0.09	0.3 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
146	Deferribacteres sp. oral clone D084	0.09	0.0 ± 0.0	0.1 ± 0.5	0.1 ± 0.5
147	Streptococcus cristatus	0.09	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.3
148	Eubacterium clone vadinBB14	0.09	0.1 ± 0.5	0.1 ± 0.3	0.1 ± 0.3
149	Eubacterium sp. oral strain A35MT	0.09	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.4
150	Lachnospiraceae sp. oral clone MCE9_173 E4	0.09	0.1 ± 0.5	0.0 ± 0.0	0.1 ± 0.4
151	Megasphaera sp. oral clone BS073	0.09	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5
152	Selenomonas flueggei-like sp. clone AH132	0.09	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.4
153	Selenomonas sp. oral clone DS051	0.09	0.3 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
154	Selenomonas sp. oral clone DS071	0.09	0.0 ± 0.0	0.1 ± 0.4	0.1 ± 0.4
155	Anaeroglobus geminatus	0.09	0.0 ± 0.0	0.2 ± 0.4	0.1 ± 0.3
156	Lactobacillus cateneforme	0.09	0.1 ± 0.3	0.0 ± 0.0	0.2 ± 0.4
157	Firmicutes sp. oral clone MCE3_120E	0.09	0.0 ± 0.0	0.3 ± 0.7	0.0 ± 0.0
158	Fusobacterium nucleatum subsp nucleatum	0.09	0.1 ± 0.4	0.1 ± 0.4	0.0 ± 0.0
159	Burkholderia cepacia	0.09	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.3
160	Neisseira weaveri	0.09	0.1 ± 0.3	0.2 ± 0.6	0.0 ± 0.0
161	Niesseria flava	0.09	0.2 ± 0.6	0.1 ± 0.3	0.0 ± 0.0
162	Cardiobacterium sp. B	0.09	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.3
163	Treponema sp. Smibert-5	0.09	0.0 ± 0.0	0.1 ± 0.4	0.1 ± 0.4
164	Treponema sp. 6:H:D15A-4	0.09	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.4
165	Treponema sp. VI:G:G47	0.09	0.1 ± 0.3	0.2 ± 0.4	0.0 ± 0.0
166	Spirochaeta sp. Clone Nt17	0.09	0.0 ± 0.0	0.1 ± 0.4	0.1 ± 0.5
167	Eubacterium like sp. oral clone 3A	0.09	0.2 ± 0.4	0.1 ± 0.3	0.0 ± 0.0
168	Olsenella profusa	0.07	0.1 ± 0.4	0.0 ± 0.0	0.1 ± 0.3
169	Leptotrichia sp.	0.07	0.1 ± 0.4	0.0 ± 0.0	0.1 ± 0.3
170	Deferribacteres sp. oral clone W028	0.07	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.4
171	Lactococcus sp.	0.07	0.1 ± 0.3	0.1 ± 0.4	0.0 ± 0.0
172	Streptococcus sp. oral clone EK048	0.07	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
173	Streptococcus sp. oral clone DP009	0.07	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4
174	Streptococcus sp. oral clone FP064	0.07	0.1 ± 0.5	0.1 ± 0.3	0.0 ± 0.0
175	Streptococcus sp. oral clone KL-27-1-5	0.07	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.8
176	Streptococcus sp. oral strain B5SC	0.07	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
177	Streptococcus genomosp. C7	0.07	0.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
178	Dialister sp. oral strain GBA27	0.07	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
179	Eubacterium sp. oral clone BS091	0.07	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.4
180	Selenomonas lacticifix	0.07	0.1 ± 0.4	0.1 ± 0.3	0.0 ± 0.0
181	Selenomonas sp. oral clone CS002	0.07	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.4
182	Firmicutes sp. oral clone BB124	0.07	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3
183	Fusobacterium sp. oral clone BS019	0.07	0.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
184	Lautropia sp. oral clone AP009	0.07	0.0 ± 0.0	0.2 ± 0.6	0.0 ± 0.0

Table 1. continued.

overall rank	species/phylotypes		mean prevalence ± SD		
		% clones	healthy subjects	shallow sites	deep sites
185	Neisseria genomosp. P1 clone P4PC_20	0.07	0.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
186	Neisseria sp sp. oral clone AK105	0.07	0.1 ± 0.3	0.1 ± 0.5	0.0 ± 0.0
187	Cardiobacterium hominis HS-A	0.07	0.1 ± 0.4	0.0 ± 0.0	0.1 ± 0.3
188	Haemophilus parainfluenzae	0.07	0.1 ± 0.5	0.1 ± 0.3	0.0 ± 0.0
189	Actinomyces naeslundii	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.4
190	Bacteroides oral clone AU126	0.04	0.0 ± 0.0	0.1 ± 0.4	0.0 ± 0.0
191	Porphyromonas sp. oral clone DS033	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.5
192	Flexistipes sp. oral clone BB062	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
193	Deferribacteres sp. oral clone D006	0.04	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
194	Abiotrophia elegans	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
195	Lactobacillus sp. oral clone CX036	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
196	Marinococcus halophilus	0.04	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
197	Streptococcus equi	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
198	strep sp 3192A	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
199	Streptococcus sp. oral cloneCH016	0.04	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0
200	Streptococcus sp. oral clone FX003	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
201	Dialister sp. oral clone FY011	0.04	0.1 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
202	Eubacterium minutum	0.04	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
203	Uncult. equine intestinal eubacterium sp. CL11	0.04	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
204	Eubacterium sp. oral clone DN050	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
205	Megasphaera sp. oral clone CS025	0.04	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
206	Mogibacterium pumilum	0.04	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0
207	Selenomonas ruminantium	0.04	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
208	Selenomonas sp. oral clone EZ011	0.04	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0
209	Veillonella parvula	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
210	Fusobacterium sp. oral clone CY024	0.04	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
211	Neisseria lactamica	0.04	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
212	Neisseria perflava	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
213	Neisseria subflava	0.04	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0
214	Vogesella indigofera	0.04	0.1 ± 0.3	0.1 ± 0.3	0.0 ± 0.0
215	Campylobacter mucosalis	0.04	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
216	Cardiobacterium hominis HS-B	0.04	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
217	Haemophilus influenzae	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
218	Spirochaeta sp. Clone Nt25	0.04	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
219	Uncultured bacterial clone UB611	0.04	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
220	Anaerosphaera like sp. oral clone 6A	0.04	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
221	Slackia heliotrinreducens	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
222	Atopobium parvulum	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
223	Atopobium rimae	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
224	Actinomyces actinomyecetemcomitans	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
225	Actinomyces odontolyticus	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
226	Actinomyces oral strain C29KA	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
227	Actinomyces sp. oral clone DR002	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
228	Corynebacterium glutamicum	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
229	Rothia sp.	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
230	Bacteroidales sp. oral cloneMCE7_120E3	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
231	Capnocytophaga sp. ChDC OS44	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
232	Capnocytophaga sputigena	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
233	Capnocytophaga sp. oral clone DS022	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
234	Capnocytophaga sp. oral clone X089	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
235	Prevotella sp. oral clone AO009	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
236	Prevotella intermedia	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
237	Lactobacillus sp CLE-4	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
238	Lactobacillus sp Y10	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
239	Streptococcus constellatus	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
240	Streptococcus ferus	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
241	Streptococcus parasanguis	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
242	Uncultured bacterium ECS55	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
243	Dialister sp. ADV 04.01	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
244	Mogibacterium timidum (E. timidum)	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
245	Uncultured Eubacterium WFeA1-59	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
246	Uncult. Equine intestinal Eubacterium sp. PL35	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
247	Eubacterium sp. oral clone BE088	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
248	Eubacterium sp. oral clone BB142	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
249	Eubacterium sp. oral clone P2PC	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
250	Lachnospiraceae sp. oral clone MCE7_60	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
251	Lachnospiraceae sp. oral clone P4PC_12P1	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
252	Mogibacterium diversum	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
253	Peptoniphilus lacrimalis (Peptostreptococcus lacrimalis)	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
254	Veillonella sp. ADV 281.99	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
255	Zymophilus paucivorans	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
256	Acholeplasma palmae	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
257	Erysipelothrix rhusiopathiae	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
258	Firmicutes sp. oral clone CD4B11	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
259	Firmicutes sp. oral clone CH017	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
260	Firmicutes sp. oral clone A0069	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
261	Ehrlichia muris	0.02	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
262	Methylobacterium organophilum	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
263	Burkholderia sp.PJ431	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
264	Vitreoscilla stercoraria	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
265	Simonsiella steedae	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
266	Simonsiella muelleri	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
267	Campylobacter lari	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
268	Campylobacter fecalis	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
269	Brenneria (Erwinia) salicis	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
270	Serratia liquefaciens	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
271	Treponema sp. I:G:C1	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
272	Treponema vincentii	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
273	Uncultured Treponema clone RFS18	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
274	Firmicutes sp. oral strain FTB41	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
275	Neisseria like sp. oral clone 6A	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0

Table 1. Continued.

	overall rank	Species/phylotype	P value: levels: between sites[a]	P value: levels: between subjects[b]	P value: presence: between subjects[c]
Disease-associated	6	Peptostreptococcus sp. oral clone BS044		0.07	
	7	Filifactor alocis		0.04	
	15	Peptostreptococcus sp. oral clone CK035	0.06	0.05	0.06
	16	Megasphaera sp. oral clone BB166		0.01	0.009
	17	Desulfobulbus sp. oral clone CH031		0.03	
	19	Dialister pneumosintes		0.01	0.002
	20	Campylobacter sputorum sputorum		0.008	0.06
	24	Desulfobulbus sp. oral clone R004		0.006	0.001
	27	Campylobacter sp. oral clone BB120		0.03	0.008
	36	Selenomonas sp. oral clone D0042		0.01	0.002
	50	Deferribacteres sp. oral clone W090		0.03	0.008
	53	Megasphaera sp. oral clone MCE3_141		0.003	0.0003
	54	Selenomonas sp. oral clone EY047	0.031		
	90	Dialister sp. oral clone MCE7_134		0.06	0.01
	100	Catonella sp. oral clone BR063		0.01	0.002
	118	Tannerella forsythia (Bacteroides forsythus)			0.03
	119	Deferribacteres sp. oral clone BH007		0.01	
	123	Strep sp. oral strain 9F		0.05	0.01
	129	Atopobium sp. oral clone C019			0.03
	140	Peptostreptococcus anaerobius			0.03
	151	Eubacterium sp. oral strain A35MT			0.04
	153	Megasphaera sp. oral clone BS073		0.01	0.002
	154	Selenomonas flueggei-like sp. clone AH132			0.03
	165	Treponema sp. 6:H:D15A-4			0.04
	172	Streptococcus sp. oral clone DP009		0.04	0.008
Health-associated	1	Veillonella sp. oral clone X042		0.0008	
	2	Campylobacter gracilis	0.04	0.02	
	21	Abiotrophia adiacens		0.003	0.007
	31	Eubacterium saburreum		0.0009	0.005
	38	Campylobacter showae		0.05	0.02
	42	Gemella sp. strain 1754-94		0.009	0.002
	44	Streptococcus sanguis		0.01	0.002
	49	Capnocytophaga gingivalis		0.02	0.05
	81	Streptococcus mutans		0.02	0.003
	133	Abiotrophia sp. oral clone P4PA_155 P1			0.03
	145	Rothia dentocariosa			0.03
	150	Eubacterium sp. oral clone OH3A			0.04
	155	Selenomonas sp. oral clone DS051			0.03

[a] - comparison of levels between deep and shallow sites in subjects with periodontitis by Wilcoxon signed rank
[b] - comparison of levels between healthy and subjects with periodontitis by Kruskal Wallis ANOVA
[c] - comparison of presence/absence of species by Chi-square

Table 2. Species and phylotypes significantly associated with disease and health (P<0.1)

CHAPTER 3

COLONIZATION STABILITY OF SUBGINGIVAL BACTERIA IN CHRONIC PERIODONTITIS AND HEALTH

INTRODUCTION

The gingival sulcus contains an open, complex ecosystem formed by resident and transient bacteria, the majority of which are uncultivated. These bacteria interact with the host in complex ways that are not well understood. The role of bacteria in the etiology of chronic periodontitis is established and it is accepted that a consortium of bacteria is involved[30]. The contributions of individual species or groups of organisms have not been completely elucidated.

There are nearly 500 species of bacteria commonly found in the gingival sulcus[70] and it is thought that every individual carries only 150-200 species[107]. Studying the stability of this ecosystem in health as well as the shifts occurring in disease is important in advancing our understanding of the bacterial etiology of periodontitis. In order to understand microbial fluctuations within such a diverse community, it is necessary to study quantitative changes in all these species over time.

Stability of bacterial colonization has been studied in various niches in humans. Studies on flora of the gastrointestinal tract have revealed that adequate colonization with normal gut flora decreases susceptibility to infections by pathogens such as *Salmonella* or *C.difficile[108, 109].* One of the possible mechanisms for this colonization resistance is an ecological phenomenon called niche saturation, where the presence of a diverse ecosystem prevents colonization by exogenous species. Once these species reach a 'threshold dose' they are able to overcome colonization resistance. These studies have contributed to clinical management of gastrointestinal infections using probiotics or microbial replacement therapy[110].

Current perspectives on the microbial stability in periodontal health and disease have been gained either from culturing or from directed molecular approaches. Cultivation and microscopy have been used to survey the constituents of healthy or disease-associated bacterial plaque[33, 35, 36]. A limited list of target bacterial species e.g., *Porphyromonas gingivalis, Treponema denticola, Actinobacillus actinomycetemcomitans* and *Tannerella forsythia* have been examined in longitudinal studies. Change in levels of these bacteria associated with change in health status[78, 111-113] or in response to therapy[114, 115] have been studied. Although cultivation of bacteria can allow detection of previously unknown species[78], the bacterial profile is limited to cultivable species, and we have no knowledge of the colonization dynamics of the uncultivated majority. Even when targeted molecular methods capable of detecting uncultivated species are used, it is not yet technically feasible to study all species in each sample. We do not, at this time, have a

complete picture of the changes occurring in a complex microbial community. Thus; the natural fluctuations of the subgingival flora in health and disease are poorly understood.

16S rDNA cloning and sequencing has been used for bacterial identification and quantification in many naturally occurring microbial communities[96, 116]. This open-ended approach allows detection all bacteria in a sample, including uncultivated and previously unknown organisms. 16S sequence comparison provides a more accurate identification than phenotypic characterization. This comprehensive approach may be viewed as the molecular counterpart of culturing, in that it allows detection of previously unknown species. However, a great advantage to this method is that it can also be used to detect uncultivated species. When used under carefully controlled conditions, it can be quantitative.

The objective of the present study was to explore the stability of bacterial colonization in the gingival crevice and to identify microbial shifts associated with change in periodontal health using an open-ended, comprehensive molecular approach that allows the examination of the relationship between change in health status and the predominant subgingival flora, including cultivated and uncultivated species. To our knowledge, this is the first time this assay has been used to examine the stability of colonization in the gingival sulcus.

MATERIALS AND METHODS

Subject selection:

Subjects for this institutionally approved study were part of a larger patient population of 150 subjects who were monitored at 6-monthly intervals to study colonization stability of periodontal microflora. These subjects were recruited from four churches in Columbus, Ohio. For the purpose of this study twenty-four subjects were selected based on review of their periodontal examinations over two years. Subjects with evidence of periodontitis at baseline who showed no change in their disease status were excluded, leaving people who either maintained stable periodontal health for the duration of the study or showed clinical change in periodontal health status. Exclusion criteria included a history of smoking, diabetes and antibiotic therapy in the last three months. Inclusion criteria were age of 40 years or more and the presence of at least 20 teeth in the dentition.

Periodontal examination and sample collection

Each subject received a periodontal examination at baseline and after 24 months. Probe depths (PD), were recorded for six sites around each tooth at each visit. The numbers of sites with PD >4mm (deep sites) were determined from exam records. Subsequent increase or decrease in probe depth by ≥2mm at any site was considered indicative of change. Subjects who were periodontally healthy at initial presentation were considered stable healthy if ≤2 sites showed increase in probe depth at the two-year time point. Subgingival plaque samples were collected at each visit on sterile endodontic paper points (Caulk Dentsply). Plaque was collected and pooled from the mesial sulcus of every tooth in the mouth. Samples were placed in a 1.5 ml microcentrifuge tube and frozen.

DNA Amplification, cloning and sequencing

DNA was isolated from these samples and amplification, cloning and sequencing of the 16S rDNA was carried out using a protocol described earlier[73]. Briefly, PCR with broad range eubacterial primers was used to amplify the 16S ribosomal gene from the community DNA. The sequences of the primers have been previously reported. The amplicons were purified and ligated to a vector (pCR 2.1-TOPO). TOP10 *E.coli* cells were transformed with the ligation mixture, plated on Luria-Bertani medium supplemented with ampicillin and incubated overnight. Colonies were screened for the presence of inserts with X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). The presence of inserts of the correct molecular size (≅ 1500bp) was confirmed by PCR amplification of the white colonies and gel electrophoresis of the amplicons on 1% agarose. DNA was stained with ethidium bromide and visualized under UV light (wavelength 320 nm) .The products were then purified with a Millipore kit (Millipore, Billerica, MA) and sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit) using an ABI 3730 instrument.

Sequence analysis

Partial sequences of 900-1100 bp were obtained from each amplicon. The sequences generated were compared to the GenBank database to identify the closest relatives using a Time Logic DeCypher Tera Blast server hosted by the Ohio Supercomputer Center. Sequences with low homology to GenBank entries were screened for chimeras using the ChimeraCheck program of the Ribosomal Database Project II

(http://rdp.cme.msu.edu/html/). Eleven clones were identified as chimeric sequences and excluded from further analysis. Sequences were aligned and a similarity matrix constructed from the alignments using the method of Jukes and Cantor. Phylogentic trees were constructed using the neighbor joining method. MacVector software was used to generate alignments, similarity matrices and in phylogenetic tree construction.

Stability and modelling variability:

Microbial stability in each subject was computed as the number of bacterial clones that remained the same over 2 years. In order to do this, 100 clones from baseline were compared to 100 clones identified at the 2-year time point. All clones that were identical at both time points were summed. A computational model was constructed to estimate the random variability introduced by selecting just 100 clones from a sample for bacterial identification. Two sampling populations for the model were created with the 200 bacterial clones available from the subjects with the least and the most diverse flora (measured as the number of species detected over 2 years). One hundred clones were randomly selected from each of these populations using a random number generator. 50 pseudoreplicate datasets were generated and means and standard deviations were calculated to determine confidence intervals for sampling variability. The theoretical mean stability was calculated by averaging the microbial stability from the two sets of bootstraps. This value was used to adjust for methodological sampling error. Since the model suggested that 75% stability was the average resulting from sampling an invariant population, this value was assumed to represent total (100%) conservation of the microbial community. All observed values were adjusted to this upper limit.

Statistical analysis

Statistical analysis was carried out with JMP (SAS Institute Inc., Cary, NC). The microbial stability of periodontally healthy subjects was compared to that of those whose periodontal status improved or worsened using a t-test. Comparisons of the mean amounts of bacteria as well as change in levels (increase or decrease) between the three groups were made using the Kruskal Wallis test.

RESULTS

This retrospective study was designed to study the colonization stability of subgingival bacteria over 2 years in a group of twenty-four subjects. The subjects were divided into three groups based on their clinical periodontal changes over this time period –stable healthy, better or worse.

At initial presentation, the mean age of the stable healthy group was 54 yr (standard deviation [SD] 8.05), the worse group 48 yr (SD 8.7) and the better group 52.2 yr (SD 9.8). The difference was not significant by t-test. Males formed 85.7% of the better group, 14.2% of the worse group and 30% of the stable group. Although the results were significant ($P = 0.02$) by chi-square analysis, the microbial stability was not significantly different between males and the females in any group. The stable group was 72.3% white, while the better and worse groups were 79% and 84.3% respectively. The results were not statistically significant by chi-square analysis.

Sequence data was obtained for 100 bacterial clones from each subject at baseline and two years. Thus, a total of 4800 clones were identified for this study. A total of 260 species or phylotypes were identified. Table 3 lists these species in order of their overall prevalence and shows the mean prevalence in each sample group.

Figure 5 shows the change in periodontal status of the 24 subjects over 2 years. At initial presentation, the subjects were either periodontally healthy or had slight to moderate periodontitis. Periodontal stability was defined as the change in the number of sites with probe depths >4mm. By this criterion, 10 subjects demonstrated periodontal health for the duration of the study (stable healthy group). Seven subjects showed an increase in the number of deep sites (worse group) and for 3 of them this was initial disease. The subjects in the worse group were either periodontal healthy at presentation or had slight periodontitis in any site. The 7 subjects in the better group had slight to moderate periodontitis in any site and evidenced improvement in periodontal status at the end of 2 years.

Figure 6 shows the correlation between periodontal stability and the adjusted microbial stability. Microbial stability was calculated as the number of bacterial clones that remained the same over the two time points and adjusted based on the computed theoretical mean. The adjusted mean microbial stability of the stable healthy group was 75.5 (SD 18.7) and that of the unstable (better + worse) group 53.3(SD 16.6). This difference was statistically significant (P = 0.006) by t-test. The relationship of microbial stability to complexity of the sampling population was investigated. The mean number of

species detected in each subject in the stable healthy group was 39.05(SD 1.69), 38.7 (SD 2.02) in the better group and 41.28 (SD 2.02) in the worse group. This difference was not statistically significant by Kruskal Wallis analysis.

Figure 7 shows the levels and the amount of change of statistically significant species or phylotypes arranged according to periodontal status. The column 'levels' shows the P-values of species whose mean levels over two years were significantly different between the three groups. The mean levels of *Filifactor alocis, Selenomonas-like sp. oral clone CS015, Treponema sp. VI:G:G47, Deferribacteres sp. oral clone D084* as well as some numerically minor species belonging to the genera Neisseria and Streptococcus were significantly elevated in disease ($P<0.1$). The levels of other streptococcal species, *Kingella oralis* and *Alysiella filiformis* were significantly higher in health.
The column 'change' shows P- values of species whose levels changed (increased or decreased) significantly over two years between the three sample groups. *Veillonella sp. oral clone X042* showed an increase associated with improving periodontal health and a decrease associated with worsening health. Their levels were stable in the periodontally stable group. This difference was statistically significant ($P=0.04$) using Kruskal Wallis test. *Capnocytophaga gingivalis, Selenomonas sp. oral clone DS071,* and some streptococci also showed a change with improving periodontal health. An opposite relationship was seen with uncultivated phylotypes of the genus *Selenomonas*, *Dialister* and *Defferibacteres.*

DISCUSSION

Microbial and clinical stability

The 24 subjects fell into 3 groups based on their initial clinical status and disease progression in 2 years (Figure 5). One group of subjects (stable healthy) had no periodontal attachment loss at baseline and remained periodontally healthy during the 2 years. Subjects in the 'worse' group either had no disease or had slight periodontitis (attachment loss of 1-3mm) in any site at baseline, and subjects in the 'better' group had initial slight to moderate periodontitis, that is, 1-5mm of attachment loss at any site The 'better' and 'worse' groups were combined to form the 'unstable' group in order to increase power for the statistical analysis.

The mean microbial stability was significantly higher in the clinically stable samples than in the unstable group (P=0.006) suggesting that while periodontal health is associated with a stable bacterial colonization, improving or worsening periodontal health is associated with shifts in microbial population. The microbial stability of a single subject with stable moderate to severe periodontitis was analyzed but not included in the study. The adjusted microbial stability of stable, established disease was 73.3%, which was close to the mean of the stable healthy group (mean=74.7%). Taken together, these findings suggest that a highly stable microbial flora is associated with stable health and possibly established disease. What is not well understood are the triggers for microbial shifts and the extent to which behavioral factors such as change in oral hygiene patterns,

smoking, contact with new strains or species of bacteria or host-associated biological modulators influence bacterial colonization.

The microbial stability of the 24 subjects ranged from 12-73 % with a mean of 46.9%, suggesting that for most of the subjects nearly half of their flora was stable over two years. No subject exhibited complete microbial replacement during the time interval observed, and close to total conservation of the bacterial community was seen in a few healthy subjects. All subjects whose clinical status worsened showed a minimum of 33% species turnover. Our observations did not find a relationship between the diversity of the sampling population and either the microbial stability or the clinical status of the patient. This is contrary to the prevailing paradigm that periodontal health is associated with flora of low complexity, while a more diverse flora is seen in disease[32, 33, 35].

Species showing significant shifts

Nonparametric statistics were employed for all analyses because of the non-normal distributions typically observed with bacterial counts. The data was analyzed at the level of species (alpha=0.1) to identify candidate species for subsequent investigation. Both mean amounts as well as increases or decreases in levels (directional change) over two years were studied (Figure 7). The mean amounts allowed comparisons with a previous study using a similar approach, which was powered to examine differences between disease and health associated flora[117]. The previous study compared the bacterial flora associated with deep and shallow sites within the same subject to the pooled, whole-mouth flora of age-matched healthy controls using 16S cloning and sequencing. In the present study, pooled whole-mouth samples were analyzed for differences in microbial

stability between stable and changing clinical health. Studying change in levels allowed us to explore the relationship between shifts in the numerically dominant species with clinical change.

Mean levels of bacteria over two years were studied at the species levels and grouped into genera to improve power for statistical analysis. The distribution of the genera in the three sample groups was consistent with the previous study. The most numerous genera overall were *Streptococcus, Veillonella, Selenomonas, Campylobacter* and *Peptostreptococcus.* This is not inconsistent with previous observations using both cultivation[78] but more closely approaches observations made using molecular methods[117].

Candidate pathogens:

Filifactor alocis, a gram-positive rod, represented 1.5% of all clones. The mean levels of *F.alocis* were significantly higher in the disease-worse and disease-better groups than in the stable healthy groups. This is consistent with previous observations that presence[118-120] and levels[117] of *F.alocis* are associated with periodontitis and endodontic disease.

Three uncultivated phylotypes belonging to the genus Selenomonas were significantly associated with disease. In a previous study using a similar approach, nearly 87% of the

genus was uncultivated[117]. The selenomonads have been previously shown to be associated with disease[50, 78, 121].

Oral clone D084, from the phylum Deferribacteres, was significantly elevated in disease. This phylum has no known cultivated oral isolates and its members have not been widely studied in relation to periodontal health and disease. However, studies using molecular approaches have found a significant association between periodontitis and phylotypes belonging to this phylum[70, 117-119].

Dialister oral strain GBA27, which forms 2.3% of all clones, showed a significant change associated with worsening disease. This is consistent with our previous observations on members of these genus[117, 118].

Uncultivated phylotypes of the genus Treponema were found to be associated with disease and health. Treponemes, in particular *Treponema denticola,* have been associated with disease in many previous studies[52, 122, 123]. It has been observed that the genus contains many uncultivated and uncharacterized species[124, 125] many of which are disease-associated. One phylotype was found to be associated with health; however, this association seems unlikely in light of previous research.

Although several species showed health or disease association both in mean amounts as well as change in levels, this study was not powered to study the strength of association of numerically minor species. Due to the large number of species examined and the low

clone numbers of certain numerically minor species, it is expected that certain associations may have been detected due to random chance. These candidates require further investigation to establish their disease and health association. In this study, the genera *Porphyromonas, Bacteroides* and *Treponemes* were detected in low levels (<0.3% of all clones). Although their levels were higher in disease, this was not statistically significant.

Peptostreptococci did not show a significant association with disease in this study. It was surprising since our previous study reported a robust relationship between levels of peptostreptococci and periodontitis. However, it was interesting to note that 65% of the baseline flora of one subject in the 'worse' group was *Peptostreptococcus sp. oral clone FG014.* At the two-year time point, this dropped to 14%, while levels of *Peptostreptococcus sp. oral clone AJ062* increased to 22% of total flora. In the previous study, three subjects with chronic periodontitis demonstrated significantly higher levels of peptostreptococci in disease sites as compared to health. Both these observations appear consistent with the paradigm that periodontitis is a heterogenous disease, with different species or groups of species playing an important etiological role in different individuals.

Candidate beneficial species:

All subjects had high proportions of *Veillonella sp. oral clone X042* at baseline (9.54% ± 4.5 of total clones). This species showed an increase when the clinical status improved and a decrease when the clinical status worsened. This species was the most numerous in

a previous study using quantitative clonal analysis and was found significantly associated with healthy subjects[117]. This suggests that shifts in levels of *Veillonella sp. oral clone X042* may be indicative of change in periodontal health. Monitoring change in levels of this phylotypes may prove to be an important diagnostic tool in predicting disease progression. Exploring the role of this beneficial species in the plaque biofilm could have important implications for microbial replacement therapy or probiotics.

The hypothesis that shift from health to disease is associated with not only increase in levels of certain pathogenic species, but also decrease in levels of certain protective species is not novel or unique to the oral cavity. Studies on microbial stability in the gastrointestinal tract have shown that adequate colonization of the intestine with health-compatible bacteria has been shown to decrease susceptibility to infection with pathogenic organisms such as *Salmonella[110]*. When large numbers of resident bacteria saturate an ecological niche, it creates a resistance to colonization by exogenous pathogens. This 'colonization resistance' conferred by thre presence of beneficial species is considered an important barrier function of gut commensals. It is possible that the presence of beneficial genera, for example, *Veillonella sp. oral clone X042* confers a protection against pathogenic colonization in the gingival sulcus.

Higher mean amounts of *Streptococcus sp. oral clone BW009* and *oral strain 12F* were significantly associated with health. Cultivated species of streptococci have been associated with health in previous cultivation based approaches[51, 78]. The genus Streptococcus has many uncultivated phylotypes[70] and these have not been

characterized. It is possible that phenotypic characterization could not distinguish between the different phylotypes or species and so the taxonomic units used for bacterial classification in cultivation based studies are broader than molecular phylogenetic identification. Streptococci, which are normally present in high levels in supragingival plaque, were detected at higher levels in this study as compared to the previous study using the same approach. In contrast to the previous study, supragingival plaque was not removed prior to sampling subgingival plaque. This sampling strategy may have contributed to the high levels of streptococci observed. Another reason could be that the previous study used samples enriched from deep or shallow sites, while pooled whole-mouth samples were used in the present study.

The mean amounts of *Kingella oralis, Alysiella filiformis* and *Oribacterium sinus* were significantly higher in association with health. *Oribacterium sinus* also showed a significant health-associated change. The prevalence of *Kingella oralis* has been previously shown to be higher in aggressive periodontitis than in periodontal health[126]. The associations of *Alysiella filiformis* and *Oribacterium sinus* with periodontal health status have not been previously examined. *Oribacterium sinus* is a novel species identified from a sinus infection[127] and was previously classified under Lachnospiraceae. The low clone numbers of these species in this study may have precluded robust statistical inferences.

In interpreting these results it should be noted that plaque samples were pooled from the mesial sulcus of every tooth. This might have resulted in some dilution of the disease-

associated flora since only a fraction of the sites were affected by periodontitis. However, data from our previous study suggests that the bacterial profile is not significantly different between shallow and deep sites within the same mouth[117]. Another limitation of the study design is that bacterial levels were measured as proportions of the total bacteria rather than absolute counts, and total bacterial load may be a factor in disease.

In summary, the data show that over a two-year period clinically stable, periodontally healthy subjects exhibited higher microbial stability than subjects whose periodontal health status changed, either in the direction of improved health or increased disease. Based on these data, measures of microbial stability may be useful in clinical diagnosis, and further studies are warranted. Increases in levels of the uncultivated phylotype *Veillonella sp. oral clone X042*, a major component of the bacterial community, were associated with periodontal health, suggesting that it is an important beneficial species. *F. alocis* was found at high levels in subjects with disease, and a number of candidates found at low levels were identified. Closer examination of these candidates using targeted molecular approaches may further elucidate their role in the pathogenesis of periodontal diseases.

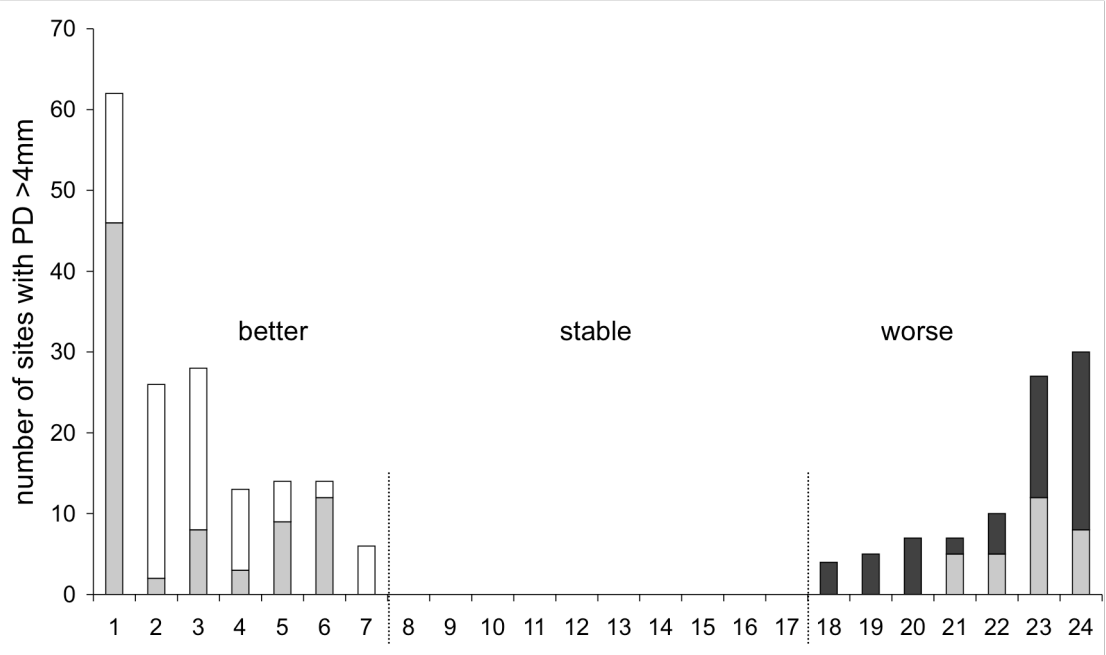

Figure 5. Clinical stability in 24 subjects over two years. In the better group, the grey and clear bars taken together indicate the number of sites with probe depths >4mm (deep sites) at baseline. In the worse group the grey bars indicate initial clinical status. The white bars represent decreases in the number of deep sites and the black bars represent increases in number of deep sites.

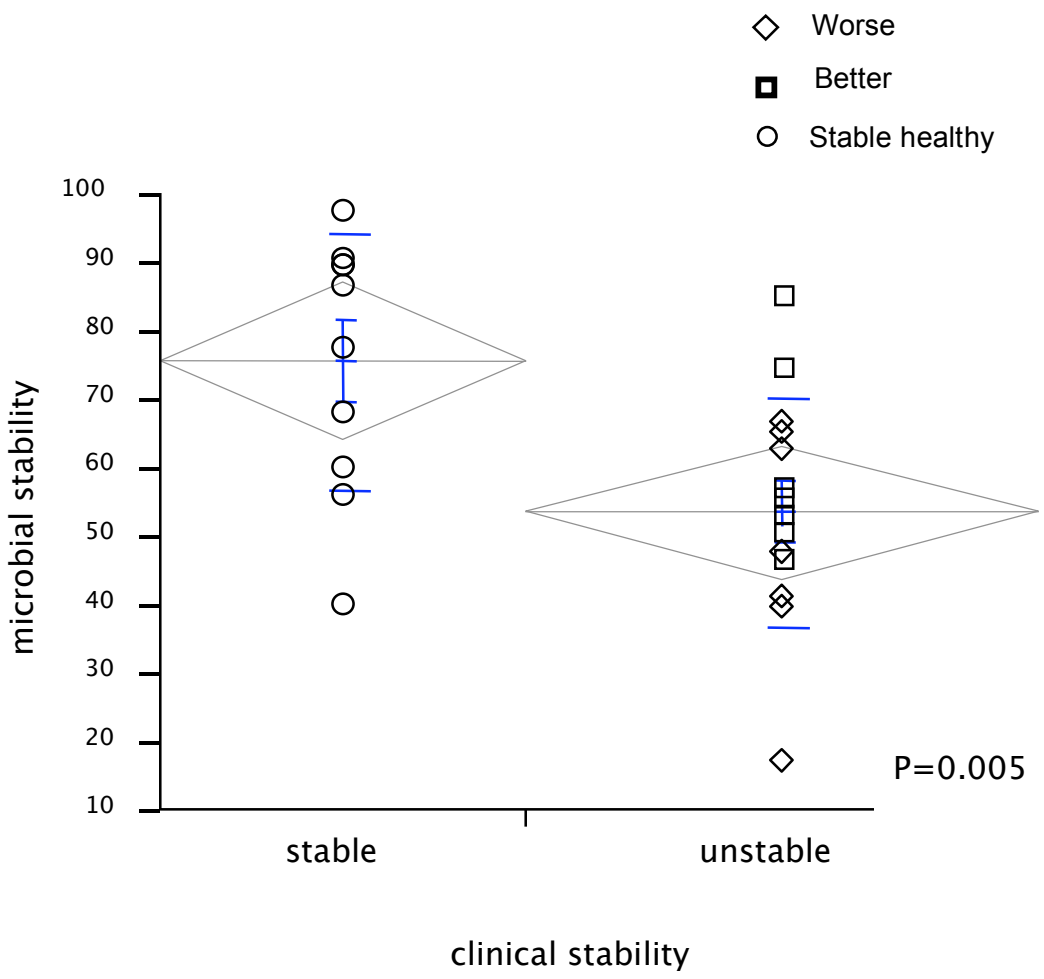

Figure 6. Correlation between clinical and microbial stability in 24 subjects over 2 years. Clinical stability was measured as the change in the number of deep sites (PD>4mm) over 2 years. Microbial stability was measured as the number of bacterial clones that remained the same over 2 years. The values were then adjusted for the inherent sampling variability due to the method. The better and worse groups were combined to form the unstable group. Mean diamonds illustrating the group mean (central line), standard deviations (small lines) and 95% confidence intervals (height of diamond) are shown for each group.

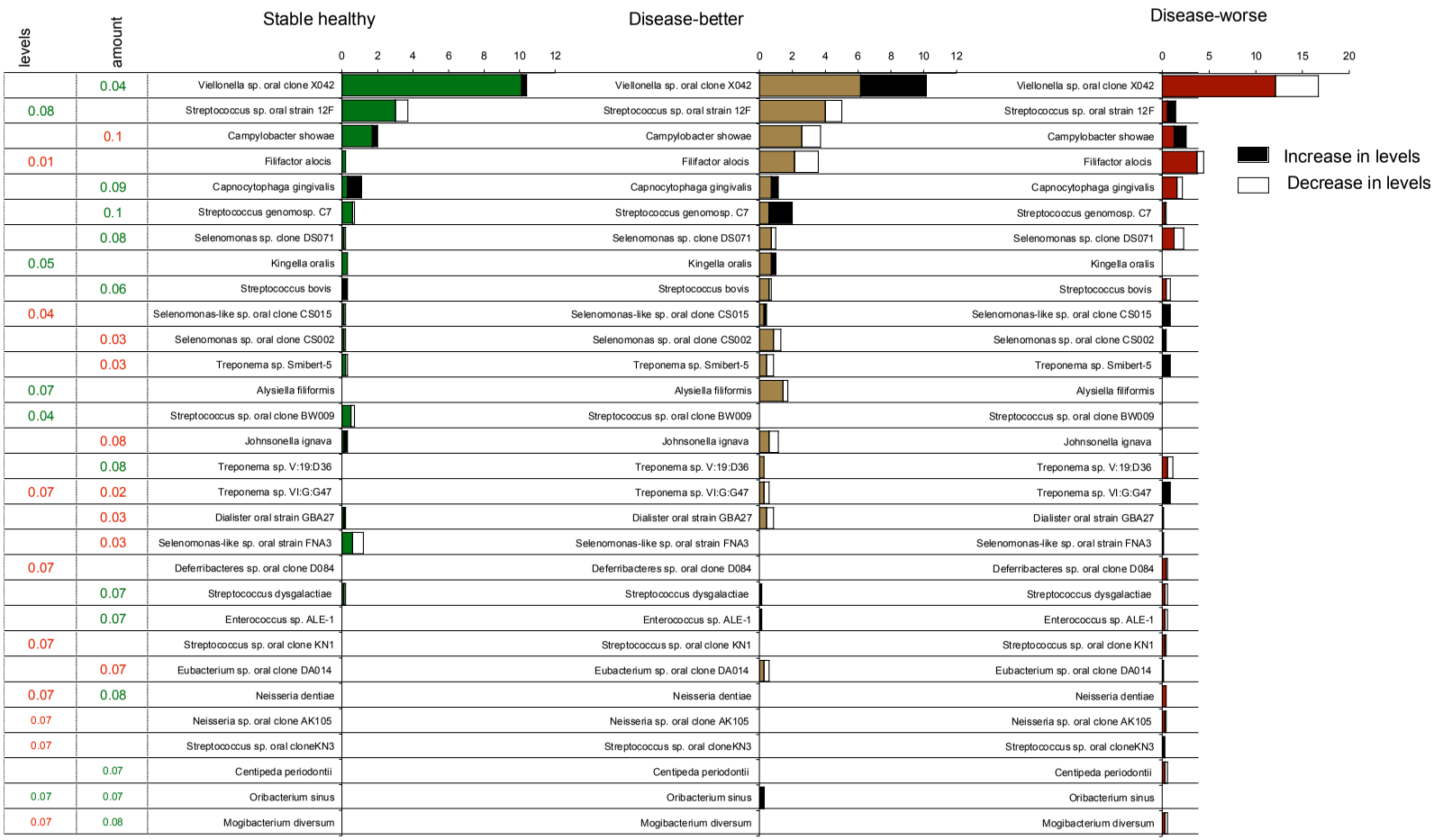

Figure 7. Distribution of species showing significant differences in the stable healthy, better and worse groups. Increases or decreases in mean levels at the two year time-point are represented by black and clear bars respectively. The colored bars together with the clear bars indicate mean bacterial levels at initial presentation. The P-values of species that demonstrated significant ($P \leq 0.1$) differences either in their mean levels or showed significant change over two years are indicated on the left. P values in green indicate health-association while red indicates disease-association.

Overall rank	Species/phylotype(s)	% Clones	Mean prevalence ± SD		
			Stable	Better	Worse
1	Viellonella sp. oral clone X042	9.52	20.5 ±4.04	16.29 ± 7.52	19.71 ±8.13
2	Campylobacter gracilis	3.65	8.9 ±4.29	8.43 ± 4.66	3.86 ±2.58
3	Streptococcus mitis	3.48	6.8 ±3.19	7.14 ± 3.18	7 ±2.24
4	Streptococcus gordonii	3.21	9 ±5.71	6.14 ± 1.82	3 ±1.74
5	Streptococcus oralis	2.75	5.4 ±2.3	6.86 ± 2.9	4.29 ±1.87
6	Streptococcus sanguinis	2.42	6.3 ±3.88	3.71 ± 2.41	2 ±2.09
7	Streptococcus sp. oral strain 12F	2.42	5.3 ±2.01	7 ± 3.89	2 ±1.66
8	Peptostreptococcus sp. oral clone FG014	2.17	4.2 ±2.1	4.57 ± 2.55	4.29 ±5.12
9	Selenomonas infelix	2.13	3.9 ±1.93	3.57 ± 1.37	5.43 ±2.4
10	Campylobacter showae	1.92	3.7 ±2.06	4 ± 2.88	3.86 ±2.75
11	Peptostreptococcus sp. oral clone AJ062	1.90	1.7 ±2.28	1 ± 1.4	9.57 ±17.04
12	Streptococcus genomosp. C8	1.88	4.5 ±3.99	3.43 ± 2.79	3 ±3.97
13	Streptococcus intermedius	1.81	3.8 ±1.74	3.29 ± 2.27	3.71 ±3.19
14	Gemella morbillorum	1.73	2.9 ±1.7	4.86 ± 3.5	2.86 ±1.65
15	Gemella hemolysans	1.63	3.8 ±1.62	3 ± 1.7	2.71 ±1.82
16	Streptococcus pneumoniae	1.63	3.6 ±2.12	2.71 ± 2.23	3.29 ±1.78
17	Filifactor alocis	1.48	0.4 ±0.41	2.86 ± 2.9	6.71 ±3.83
18	Granulicatella elegans	1.46	3.1 ±1.61	3.14 ± 1.4	2.43 ±1.37
19	Peptostreptococcus sp. oral clone BS044	1.46	2.6 ±1.75	3.43 ± 1.9	2.86 ±1.6
20	Veillonella atypica	1.42	3.2 ±2.06	2.43 ± 2.22	2.71 ±1.87
21	Streptococcus sp. oral clone 2056B	1.23	1.9 ±1.36	3.14 ± 2.31	2.57 ±2.02
22	Peptostreptococcus micros	1.19	3.7 ±2.83	1.43 ± 1.7	1.43 ±1.54
23	Neisseria elongata	1.17	2.8 ±3.73	2.71 ± 1.9	1.29 ±2.78
24	Streptococcus sp. oral strain 7A	1.08	1.7 ±1.5	2.14 ± 1.73	2.86 ±1.75
25	Selenomonas sputigena	0.98	1.6 ±1.36	2.29 ± 2.21	2.14 ±1.86
26	Streptococcus sp. oral clone FN051	0.96	1.3 ±1.5	3.29 ± 2.23	1.43 ±1.49
27	Capnocytophaga gingivalis	0.94	1.4 ±0.86	1.86 ± 1.21	2.57 ±1.54
28	Campylobacter concisus	0.90	2.6 ±2	1.14 ± 1.02	1.29 ±1.22
29	Selenomonas sp. oral clone EW051a	0.88	1.9 ±1.43	0.29 ± 0.53	3 ±2.18
30	Streptococcus anginosus	0.81	1 ±0.89	1.29 ± 1.01	2.86 ±1.28
31	Megasphaera sp. oral clone BB166	0.79	1.6 ±1.15	1.86 ± 1.54	1.29 ±1.01
32	Eikenella corrodens	0.73	1.5 ±0.97	1.17 ± 1.03	1.14 ±1.16
33	Dialister sp. oral clone E2_20	0.71	0.9 ±1.05	2.14 ± 1.64	1.43 ±1.07
34	Streptococcus genomosp. C7	0.67	1.1 ±1.19	2.57 ± 2.27	0.43 ±0.57
35	Neisseria sp. oral clone AP060	0.67	1.7 ±1.66	1.71 ± 1.46	0.43 ±0.81
36	Streptococcus salivarius	0.63	1.5 ±1.77	0.57 ± 0.61	1.57 ±1.36
37	Selenomonas sp. oral clone AA024	0.63	2.2 ±2.53	0.57 ± 0.73	0.57 ±0.83
38	Selenomonas sp. oral clone AJ036	0.63	1.2 ±0.82	1.29 ± 1.01	1.29 ±1.22
39	Streptococcus infantis	0.58	1.2 ±1.05	1 ± 1.34	1.29 ±1.01
40	Dialister sp. oral clone BS095	0.58	1.3 ±0.99	0.71 ± 0.93	1.43 ±0.83
41	Selenomoas noxia	0.56	1.3 ±1.18	1.43 ± 0.91	0.57 ±0.61
42	Gemella sanguinis	0.50	1.2 ±1.88	1.29 ± 1.28	0.43 ±0.43
43	Dialister pneumosintes	0.50	0.8 ±0.82	0.71 ± 0.84	1.57 ±1.13
44	Dialister invisus	0.50	1.4 ±1.3	1 ± 0.94	0.43 ±0.43
45	Selenomonas sp. oral clone DY027	0.48	0.9 ±1	0.86 ± 0.94	1.14 ±1.09
46	Selenomonas sp. oral clone DS051	0.48	0.7 ±0.67	2 ± 1.71	0.29 ±0.36
47	Selenomonas sp. oral clone IK004	0.46	0.6 ±0.66	1.29 ± 1.65	1 ±1.29
48	Veillonella sp. oral clone AA050	0.46	0.7 ±0.88	0.43 ± 0.43	1.71 ±1.88
49	Capnocytophaga granulosa	0.44	1.8 ±1.41	0.29 ± 0.53	0.14 ±0.27
50	Streptococcus sp. oral clone EK048	0.44	0.6 ±0.8	1.14 ± 1.4	1 ±0.78
51	Abiotrophia para-adiacens	0.42	1.2 ±1.1	0.71 ± 0.75	0.43 ±0.59
52	Abiotrophia sp. oral clone P4PA_155	0.42	0.9 ±0.89	0.86 ± 0.76	0.71 ±0.93
53	Streptococcus parasanguis	0.42	0.7 ±1.18	1.43 ± 1.86	0.43 ±0.58
54	Selenomonas sp. oral clone DS071	0.42	0.1 ±0.22	1.14 ± 1.16	1.57 ±1.31
55	Selenomonas sp. oral clone EQ054	0.42	1 ±1.24	0.86 ± 0.76	0.57 ±0.61
56	Neisseria meningitidis	0.42	1 ±0.83	0.43 ± 0.58	1 ±0.76
57	Campylobacter rectus	0.42	0.2 ±0.45	1.14 ± 1.02	1.43 ±1.07
58	Rothia dentocariosa	0.40	1.2 ±1.05	0.86 ± 0.85	0.14 ±0.27
59	Streptococcus cristatus	0.40	0.6 ±0.57	1 ± 0.94	0.86 ±0.94
60	Selenomonas dianae	0.40	1.2 ±0.94	0.14 ± 0.27	0.86 ±0.76
61	Capnocytophaga sp. oral clone AH015	0.38	0.5 ±0.44	1.14 ± 1.28	0.71 ±0.75
62	Streptococcus sinensis	0.38	0.6 ±0.57	0.57 ± 0.47	1.14 ±0.76
63	Kingella oralis	0.38	0.6 ±0.66	1.71 ± 1.23	0 ±0
64	Neisseria denitrificans	0.38	0.7 ±0.67	1.43 ± 1.27	0.14 ±0.27
65	Enterococcus faecalis	0.35	1.2 ±0.88	0.43 ± 0.58	0.29 ±0.36
66	Eubacterium brachy	0.35	0.6 ±0.73	0.14 ± 1.02	0.43 ±0.58
67	Capnocytophaga sputigena	0.33	0.6 ±0.66	1 ± 0.76	0.43 ±0.58
68	Eubacterium sp. oral clone EW053	0.33	0.6 ±0.98	0.86 ± 0.76	0.57 ±0.61
69	Selenomonas sp. oral clone EW084	0.33	0.9 ±0.128	0.43 ± 0.58	0.57 ±0.61
70	Eubacterium saphenum	0.31	0.6 ±0.8	0.14 ± 0.27	1.14 ±1.01
71	Porphyromonas gingivalis	0.29	0 ±0	0.43 ± 0.58	1.57 ±2.42
72	Streptococcus agalatiae	0.29	0.6 ±1.13	0.71 ± 0.84	0.43 ±0.58
73	Selenomonas sp. oral clone DO042	0.29	0.3 ±0.37	1 ± 0.94	0.57 ±0.82
74	Streptococcus bovis	0.27	0.3 ±0.37	1 ± 1.09	0.43 ±0.43
75	Streptococcus suis	0.27	0.6 ±0.47	0.14 ± 0.27	0.86 ±0.76
76	Peptostreptococcus sp. oral clone CK035	0.27	0.8 ±0.88	0.14 ± 0.27	0.57 ±0.83
77	Selenomonas-like sp. oral clone CS015	0.27	0.1 ±0.22	0.71 ± 0.74	1 ±0.76
78	Selenomonas sp. oral clone CS002	0.27	0.1 ±0.22	1.29 ± 1.28	0.43 ±0.58
79	Corynebacterium matruchotii	0.25	0.4 ±0.41	0.86 ± 0.85	0.29 ±0.53
80	Streptococcus sp. oral clone 4093B	0.25	0.4 ±0.7	0.57 ± 0.83	0.57 ±0.47
81	Streptococcus sp. oral clone FX003	0.25	0.5 ±0.64	0.29 ± 0.53	0.71 ±0.93
82	Eubacterium sp. oral clone E1-K17	0.25	0.6 ±0.66	0.43 ± 0.43	0.43 ±0.58
83	Treponema sp. Smibert-5	0.25	0.3 ±0.37	0.43 ± 0.8	0.86 ±0.94
84	Atopobium rimae	0.23	0.8 ±0.88	0 ± 0	0.43 ±0.58
85	Streptococcus pyogenes	0.23	0.5 ±0.79	0.57 ± 0.61	0.29 ±0.36
86	Desulfobulbus sp. oral clone CH031	0.23	0.5 ±0.79	0 ± 0	0.86 ±1.16
87	Peptostreptococcus sp. oral clone MDA2346-2	0.23	0 ±0	0.57 ± 0.61	1 ±1.16

Table 3. Species and phylotypes from three sample groups showing percentage of total clones and mean prevalence in each group arranged according to prevalence

overall rank	species/phylotype	% clones	mean prevalence ± SD		
			stable	better	worse
88	Selenomonas sp. oral clone CS024	0.23	0.7 ±0.81	0 ± 0	0.57 ±0.61
89	Selenomonas sp. oral clone GT010	0.23	0.9 ±1.39	0 ± 0	0.29 ±0.53
90	Selenomonas lacticifex	0.23	1.1 ±1.57	0 ± 0	0 ±0
91	Alysiella filiformis	0.23	0 ±0	1.57 ± 1.85	0 ±0
92	Lautropia sp. oral clone AP009	0.23	0.1 ±0.22	1.43 ± 1.27	0 ±0
93	Granucatella adiacens	0.21	0.5 ±0.64	0.43 ± 0.58	0.29 ±0.53
94	Gemella sp. strain 1754-94	0.21	0.2 ±0.31	0 ± 0	1.14 ±0.85
95	Streptococcus intestinalis	0.21	0.6 ±0.8	0.43 ± 0.58	0.14 ±0.27
96	Streptococcus mutans	0.21	0.4 ±0.7	0.29 ± 0.36	0.57 ±0.61
97	Streptococcus peroris	0.21	0.3 ±0.37	0.29 ± 0.53	0.71 ±0.74
98	Catonella sp. oral clone FL037	0.21	0.5 ±0.79	0 ± 0	0.71 ±0.93
99	Catonella morbi	0.21	0.2 ±0.31	0.71 ± 0.63	0.43 ±0.58
100	Selenomonas noxia-like sp. oral clone CI002	0.21	0.9 ±1.1	0 ± 0	0.14 ±0.27
101	Selenomonas sp. oral clone GI064	0.21	0 ±0	1.43 ± 1.98	0 ±0
102	Neisseria flava	0.21	0.5 ±0.79	0.29 ± 0.36	0.43 ±0.8
103	Campylobacter hominis	0.21	0.4 ±0.52	0.86 ± 0.85	0 ±0
104	Corynebacterium sp. oral clone AK153	0.19	0.4 ±0.52	0.71 ± 0.93	0 ±0
105	Streptococcus hyointestinalis	0.19	0 ±0	0.43 ± 0.58	0.86 ±0.94
106	Streptococcus sp. oral clone CH016	0.19	0.1 ±0.22	1 ± 0.76	0.14 ±0.27
107	Streptococcus sp. oral clone BM035	0.19	0.7 ±0.81	0 ± 0	0.29 ±0.53
108	Eubacterium saburreum	0.19	0.8 ±0.82	0.14 ± 0.27	0 ±0
109	Megasphaera micronuciformis	0.19	0.2 ±0.45	0.43 ± 0.58	0.57 ±0.83
110	Selenomonas sp. oral clone EW076	0.19	0.5 ±0.64	0.14 ± 0.27	0.43 ±0.43
111	Selenomonas sp. oral clone DD020	0.19	0.5 ±0.55	0.29 ± 0.53	0.29 ±0.53
112	Veillonella parvula	0.19	0.2 ±0.31	0.43 ± 0.58	0.57 ±0.61
113	Neisseria genomosp. P1 clone P4PC_20	0.19	0.7 ±1.14	0 ± 0	0.29 ±0.36
114	Neisseria pharyngis	0.19	0.6 ±0.66	0 ± 0	0.43 ±0.43
115	Deferribacteres sp. oral clone BH007	0.17	0 ±0	0.86 ± 0.94	0.29 ±0.36
116	Streptococcus didelphis	0.17	0.6 ±0.92	0.14 ± 0.27	0.14 ±0.27
117	Streptococcus oligofermentans	0.17	0.1 ±0.22	0.71 ± 0.63	0.29 ±0.36
118	Streptococcus sp. oral clone 3097C	0.17	0.1 ±0.22	0.29 ± 0.53	0.71 ±0.63
119	Streptococcus sp. oral clone AY020	0.17	0.5 ±0.55	0.29 ± 0.36	0.14 ±0.27
120	Streptococcus sp. oral clone BW009	0.17	0.8 ±0.82	0 ± 0	0 ±0
121	Catonella sp. oral clone EZ006	0.17	0.1 ±0.22	0.43 ± 0.8	0.57 ±0.83
122	Johnsonella ignava	0.17	0.4 ±0.7	0.57 ± 0.83	0 ±0
123	Selenomonas sp. oral clone EW079	0.17	0.3 ±0.49	0.29 ± 0.36	0.43 ±0.58
124	Veillonella dispar	0.17	0.3 ±0.37	0.29 ± 0.53	0.43 ±0.42
125	Treponema sp. VI:G:G47	0.17	0 ±0	0.29 ± 0.53	0.86 ±0.94
126	Treponema sp. V:19:D36	0.17	0 ±0	0.57 ± 0.72	0.57 ±0.83
127	Deferribacteres sp. oral clone BA121	0.15	0.4 ±0.7	0.43 ± 0.8	0 ±0
128	Gemella sp. oral strain C24KA	0.15	0.2 ±0.31	0.43 ± 0.58	0.29 ±0.36
129	Streptococcus sp. oral clone 2061A	0.15	0.1 ±0.22	0.14 ± 0.27	0.71 ±0.93
130	Streptococcus uberis	0.15	0.1 ±0.22	0.57 ± 0.61	0.29 ±0.36
131	Dialister sp. oral strain GBA27	0.15	0.3 ±0.37	0.43 ± 0.43	0.14 ±0.27
132	Eubacterium sp. oral clone EI074	0.15	0.1 ±0.22	0.57 ± 0.61	0.29 ±0.53
133	Eubacterium sp. oral clone DO008	0.15	0.1 ±0.22	0 ± 0	0.86 ±1.16
134	Eubacterium sp. oral clone IR009	0.15	0.4 ±0.7	0.14 ± 0.27	0.29 ±0.53
135	Megasphaera sp. oral clone BU057	0.15	0.3 ±0.49	0.29 ± 0.53	0.29 ±0.36
136	Selenomonas-like sp. oral strain FNA3	0.15	0.6 ±0.66	0 ± 0	0.14 ±0.27
137	Selenomonas-like sp. oral strain GAA14	0.15	0.2 ±0.31	0.14 ± 0.27	0.57 ±0.62
138	Firmicutes sp. oral clone CK051	0.15	0 ±0	0.43 ± 0.57	0.57 ±0.62
139	Neisseria mucosa	0.15	0.2 ±0.45	0.57 ± 1.07	0.14 ±0.27
140	Capnocytophaga sp. oral clone BR085	0.13	0.4 ±0.52	0.29 ± 0.36	0 ±0
141	Deferribacteres sp. oral clone W090	0.13	0 ±0	0.29 ± 0.53	0.57 ±0.83
142	Streptococcus sp. oral clone P4PA_13 P3	0.13	0.3 ±0.49	0 ± 0	0.43 ±0.43
143	Streptococcus sp. oral clone AA007	0.13	0.3 ±0.37	0.29 ± 0.53	0.14 ±0.28
144	Desulfobulbus sp. oral clone R004	0.13	0.1 ±0.22	0 ± 0	0.71 ±0.93
145	Eubacteriaceae oral clone MCE10_174 E2	0.13	0.2 ±0.45	0.57 ± 0.61	0 ±0
146	Megasphaera sp. oral clone BS073	0.13	0.6 ±0.98	0 ± 0	0 ±0
147	Selenomonas-like sp. oral clone DM071	0.13	0 ±0	0.71 ± 0.63	0.14 ±0.27
148	Selenomonas sp. oral clone P2PA_80 P4	0.13	0.3 ±0.37	0 ± 0	0.43 ±0.58
149	Veillonella ratti	0.13	0.2 ±0.31	0.43 ± 0.43	0.14 ±0.27
150	Firmicutes sp. oral clone AO068	0.13	0.4 ±0.41	0 ± 0	0.29 ±0.53
151	Haemophilus segnis	0.13	0.4 ±0.41	0.29 ± 0.53	0 ±0
152	Rothia mucilaginosa	0.10	0 ±0	0.43 ± 0.58	0.29 ±0.53
153	Tannerella forsythia	0.10	0.3 ±0.67	0 ± 0	0.29 ±0.53
154	Deferribacteres sp. oral clone D084	0.10	0 ±0	0 ± 0	0.71 ±0.93
155	Streptococcus sp. oral strain T1-E5	0.10	0.1 ±0.22	0.29 ± 0.36	0.29 ±0.53
156	Catonella sp. oral clone BR063	0.10	0.2 ±0.45	0 ± 0	0.43 ±0.8
157	Eubacterium sp. oral clone EW049	0.10	0.2 ±0.45	0 ± 0	0.43 ±0.59
158	Kingella denitrificans	0.10	0.1 ±0.22	0.29 ± 0.53	0.29 ±0.53
159	Treponema socranskii subsp. socranskii	0.10	0.1 ±0.22	0.57 ± 0.83	0 ±0
160	Treponema sp. 5:C:AT040	0.10	0 ±0	0 ± 0	0.71 ±1.33
161	Actinomyces naeslundii	0.08	0.2 ±0.31	0 ± 0	0.29 ±0.53
162	Capnocytophaga sp. oral strain S3	0.08	0.2 ±0.31	0 ± 0	0.29 ±0.53
163	Streptococcus dysgalactiae	0.08	0.1 ±0.22	0.14 ± 0.27	0.29 ±0.36
164	Streptococcus sp. oral clone BE024	0.08	0.2 ±0.31	0.14 ± 0.27	0.14 ±0.28
165	Streptococcus sp. oral clone PSH2	0.08	0.2 ±0.31	0.29 ± 0.53	0 ±0
166	Mogibacterium timidum	0.08	0.1 ±0.22	0 ± 0	0.43 ±0.58
167	Eubacterium sp. oral clone OH3A	0.08	0.1 ±0.22	0.14 ± 0.27	0.29 ±0.53
168	Lachnospiraceae sp. oral clone P4PC_12	0.08	0.3 ±0.49	0.14 ± 0.27	0 ±0
169	Selenomonas sp. oral clone EY047	0.08	0.1 ±0.22	0 ± 0	0.43 ±0.43
170	Fusobacterium sp. oral clone BS019	0.08	0.3 ±0.49	0.14 ± 0.27	0 ±0
171	Lautropia sp. oral clone FX006	0.08	0.3 ±0.49	0.14 ± 0.27	0 ±0
172	Neisseria perflava	0.08	0.3 ±0.37	0.14 ± 0.27	0 ±0
173	Neisseria sp. oral strain B33KA	0.08	0.4 ±0.62	0 ± 0	0 ±0
174	Neisseria sp. oral clone AP085	0.08	0.2 ±0.31	0.29 ± 0.53	0 ±0

Table 3. Continued

overall rank	species/phylotype	% clones	mean prevalence ± SD		
			stable	better	worse
175	Campylobacter sp. oral clone BB120	0.08	0.4 ±0.62	0 ± 0	0 ±0
176	Corynebacterium durum	0.06	0 ±0	0.14 ± 0.27	0.29 ±0.36
177	Bacteroides-like sp. oral clone AU126	0.06	0.2 ±0.31	0 ± 0	0.14 ±0.27
178	Deferribacteres sp. oral clone BH017	0.06	0 ±0	0 ± 0	0.43 ±0.8
179	Enterococcus hirae	0.06	0.1 ±0.22	0.29 ± 0.53	0 ±0
180	Enterococcus sp. ALE-1	0.06	0 ±0	0.14 ± 0.27	0.29 ±0.36
181	Streptococcus sp. clone KL-48-1-4	0.06	0.1 ±0.22	0 ± 0	0.29 ±0.53
182	Streptococcus sp. oral clone KN1	0.06	0 ±0	0 ± 0	0.43 ±0.58
183	Anaerococcus geminatus	0.06	0.2 ±0.31	0 ± 0	0.14 ±0.27
184	Dialister sp. ADV 04.01	0.06	0 ±0	0.14 ± 0.27	0.29 ±0.53
185	Eubacterium sp. oral clone DA014	0.06	0 ±0	0.29 ± 0.36	0.14 ±0.27
186	Eubacterium sp. oral clone BE088	0.06	0 ±0	0.29 ± 0.53	0.14 ±0.27
187	Eubacterium sp. oral strain A03MT	0.06	0.1 ±0.22	0 ± 0	0.29 ±0.53
188	Firmicutes sp. oral clone F058	0.06	0.2 ±0.31	0 ± 0	0.14 ±0.27
189	Fusobacterium sp. oral clone AJ050	0.06	0.1 ±0.22	0.29 ± 0.53	0 ±0
190	Fusobacterium nucleatum	0.06	0.3 ±0.37	0 ± 0	0 ±0
191	Hydrogenophaga flava	0.06	0.1 ±0.22	0.14 ± 0.27	0.14 ±0.27
192	Neisseria dentiae	0.06	0 ±0	0 ± 0	0.43 ±0.58
193	Neisseria sp. oral clone AK105	0.06	0 ±0	0 ± 0	0.43 ±0.58
194	Campylobacter curvus	0.06	0 ±0	0.14 ± 0.27	0.29 ±0.53
195	Campylobacter sputorum	0.06	0.3 ±0.49	0 ± 0	0 ±0
196	Cardiobacterium sp. A	0.06	0.3 ±0.49	0 ± 0	0 ±0
197	Actinomyces species	0.04	0 ±0	0 ± 0	0.29 ±0.36
198	Prevotella sp. oral clone DO022	0.04	0.1 ±0.22	0.14 ± 0.27	0 ±0
199	Prevotella genomosp. C1	0.04	0 ±0	0 ± 0	0.29 ±0.53
200	Porphyromonas-like sp. oral clone DA064	0.04	0 ±0	0 ± 0	0.29 ±0.53
201	Porphyromonas sp. oral clone DP023	0.04	0 ±0	0.29 ± 0.53	0 ±0
202	Porphyromonas gulae	0.04	0 ±0	0 ± 0	0.29 ±0.53
203	Capnocytophaga sp. P1 oral clone P4GA	0.04	0.2 ±0.45	0 ± 0	0 ±0
204	Deferribacteres sp. oral clone JV023	0.04	0.1 ±0.22	0 ± 0	0.14 ±0.27
205	Deferribacteres sp. oral clone BB062	0.04	0 ±0	0 ± 0	0.29 ±0.53
206	Abiotrophia sp. oral clone OH2A	0.04	0.1 ±0.22	0 ± 0	0.14 ±0.27
207	Carnobacterium sp. oral clone D35	0.04	0 ±0	0 ± 0	0.29 ±0.36
208	Streptococcus canis	0.04	0 ±0	0.14 ± 0.27	0.14 ±0.27
209	Streptococcus sp. oral clone P4PA_30 P4	0.04	0.1 ±0.22	0.14 ± 0.27	0 ±0
210	Streptococcus sp. oral clone KN3	0.04	0 ±0	0 ± 0	0.29 ±0.36
211	Streptococcus sp. oral strain T4-E3	0.04	0 ±0	0 ± 0	0.29 ±0.53
212	Centipeda periodontii	0.04	0 ±0	0 ± 0	0.29 ±0.36
213	Dialister sp. oral clone BS016	0.04	0.2 ±0.31	0 ± 0	0 ±0
214	Dialister sp. oral clone FY011	0.04	0.2 ±0.31	0 ± 0	0 ±0
215	Eubacterium sp. oral clone DZ073	0.04	0.1 ±0.22	0 ± 0	0.14 ±0.27
216	Eubacterium sp. oral strain A35MT	0.04	0.2 ±0.45	0 ± 0	0 ±0
217	Eubacteriaceae oral clone P2PB_46 P3	0.04	0 ±0	0.29 ± 0.53	0 ±0
218	Oribacterium sinus	0.04	0 ±0	0.29 ± 0.36	0 ±0
219	Lachnospiraceae oral clone MCE10_236 E5	0.04	0 ±0	0.14 ± 0.27	0.14 ±0.27
220	Lachnospiraceae genomospecies C1	0.04	0 ±0	0 ± 0	0.29 ±0.36
221	Mogibacterium diversum	0.04	0 ±0	0 ± 0	0.29 ±0.36
222	Peptococcus sp. oral clone MCE10_265 E1	0.04	0 ±0	0.14 ± 0.27	0.14 ±0.28
223	Propionispira arboris	0.04	0.1 ±0.22	0.14 ± 0.27	0 ±0
224	Selenomonas flueggei	0.04	0 ±0	0 ± 0	0.29 ±0.53
225	Fusobacterium sulci	0.04	0 ±0	0.29 ± 0.36	0 ±0
226	Leptotrichia sp. oral clone P2PB_51 P1	0.04	0.2 ±0.45	0 ± 0	0 ±0
227	Neisseria sp. oral clone AP132	0.04	0 ±0	0.14 ± 0.27	0.14 ±0.27
228	Haemophilus influenzae	0.04	0.1 ±0.2	0 ± 0	0.14 ±0.27
229	Treponema socranskii subsp. buccale	0.04	0 ±0	0.14 ± 0.27	0.14 ±0.27
230	Scardovia genomosp. C1	0.02	0.1 ±0.22	0 ± 0	0 ±0
231	Bacteroidales sp. oral clone MCE3_262 E2a	0.02	0 ±0	0.14 ± 0.27	0 ±0
232	Prevotella sp. oral clone F045	0.02	0.1 ±0.22	0 ± 0	0 ±0
233	Porphyromonas sp. oral clone AW032	0.02	0 ±0	0 ± 0	0.14 ±0.27
234	Capnocytophaga sp. oral clone BU084	0.02	0 ±0	0 ± 0	0.14 ±0.27
235	Capnocytophaga sp. LMG 12116	0.02	0.1 ±0.22	0 ± 0	0 ±0
236	Aerococcus christensenii	0.02	0 ±0	0 ± 0	0.14 ±0.27
237	Granulicatella balaenopterae	0.02	0 ±0	0.14 ± 0.27	0 ±0
238	Brevibacillus agri	0.02	0 ±0	0.14 ± 0.27	0 ±0
239	Enterococcus haemoperoxidus	0.02	0.1 ±0.22	0 ± 0	0 ±0
240	Gemella sp. oral strain A31S	0.02	0 ±0	0 ± 0	0.14 ±0.27
241	Streptococcus milleri	0.02	0.1 ±0.22	0 ± 0	0 ±0
242	Streptococcus sp. oral clone P2PA_41 P2	0.02	0.1 ±0.22	0 ± 0	0 ±0
243	Streptococcus sp. oral strain H3-M2	0.02	0 ±0	0 ± 0	0.14 ±0.27
244	Streptococcus sp. oral clone ES11	0.02	0 ±0	0.14 ± 0.27	0 ±0
245	Streptococcus sp. oral clone KN2	0.02	0 ±0	0 ± 0	0.14 ±0.27
246	Streptococcus sp. oral clone NJ9704	0.02	0.1 ±0.22	0 ± 0	0 ±0
247	Streptococcus sp. oral clone TW1	0.02	0 ±0	0.14 ± 0.27	0 ±0
248	Weissella minor	0.02	0 ±0	0 ± 0	0.14 ±0.27
249	Eubacterium yurii	0.02	0 ±0	0 ± 0	0.14 ±0.27
250	Filifactor villosus	0.02	0 ±0	0 ± 0	0.14 ±0.27
251	Pseudoramibacter alactolyticus	0.02	0 ±0	0 ± 0	0.14 ±0.27
252	Veillonella sp. oral clone OH1A	0.02	0.1 ±0.22	0 ± 0	0 ±0
253	Veillonella sp. oral clone BU083	0.02	0.1 ±0.22	0 ± 0	0 ±0
254	Kingella sp. oral clone DE012	0.02	0.1 ±0.22	0 ± 0	0 ±0
255	Neisseria cinerea	0.02	0 ±0	0.14 ± 0.27	0 ±0
256	Vogesella indigofera	0.02	0 ±0	0.14 ± 0.27	0 ±0
257	Simonsiella crassa	0.02	0 ±0	0 ± 0	0.14 ±0.27
258	Simonsiella steedae	0.02	0 ±0	0 ± 0	0.14 ±0.27
259	Haemophilus sp. oral clone BJ095	0.02	0 ±0	0.14 ± 0.27	0 ±0
260	Treponema clone RFS94	0.02	0 ±0	0 ± 0	0.14 ±0.27

Table 3. Continued

CHAPTER 4

NEW BACTERIAL SPECIES ASSOCIATED WITH CHRONIC PERIODONTITIS

INTRODUCTION

Chronic periodontitis is characterized by a bacterially-induced progressive loss of clinical attachment including destruction of periodontal ligament and adjacent supporting bone. In order to identify the bacterial pathogens responsible for periodontitis, a large number of investigations of known species have been conducted using both cultivation and molecular identification methods. *Tannerella forsythia* (*Bacteroides forsythus)* and *Porphyromonas gingivalis* are widely regarded as major periodontal pathogens, and evidence has implicated a number of other species in disease etiology. However, no single pathogen or group of pathogens has been clearly identified as the cause of periodontitis. A recent comprehensive investigation of the human subgingival oral flora based on ribosomal 16S cloning and sequencing showed 40 percent of the bacterial species present to be novel species or phylotypes [81]. Several other recent investigations using similar methodology have also shown the presence of high numbers of novel species in the oral cavity [81, 86, 128, 129]. Therefore, it seems likely that unrecognized periodontal pathogens remain to be identified.

The purpose of the present study was to evaluate the association of newly identified bacterial species or phylotypes with periodontitis. Targets for investigation included both

uncultivated phylotypes and characterized species that were not previously thought to be associated with periodontitis. In addition, species previously strongly linked to periodontitis were included for comparison.

MATERIALS AND METHODS

Study population.

Samples collected and stored from a previous study were available for re-analysis for this study. As previously described [130], subjects for this institutionally approved study were recruited from the dental clinics of the Ohio State University and informed consent was obtained. Exclusionary criteria were set to select the most and least periodontally healthy segments of the population on the basis of probing depths and attachment levels as previously described [130]. For each subject the mesial sulci of all teeth present were sampled with endodontic paper points, and samples from each individual were pooled. A set of samples from 66 subjects with chronic periodontitis and 66 age-matched controls was randomly selected for the present study. The same set of samples was used for all 39 species or phylotypes examined.

Detection of bacterial species and phylotypes.

We use the term "phylotype" rather than "species" to refer to novel clone sequences that differ by at least 2% in the ribosomal 16S gene from known species. Bacterial species and phylotypes were detected by PCR amplification of the 16S rDNA and the downstream intergenic spacer region (ISR). Inclusion of the ISR provided an additional check on the specificity of primers, since the length of this region varies among species.

DNA isolated from the plaque samples was first amplified with prokaryotic universal ribosomal 16S and 23S primers, as described previously [131]. Individual species were then identified by a second, nested amplification using species-specific 16S primers paired with a universal primer located in the 23S gene. Primer sequences are shown in table 4. DNA fragments were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under UV transillumination. A positive or negative score was assigned based on the presence of clear bands of the expected molecular size. All assays were repeated, and if the results were not in agreement, they were repeated again.

Primer specificity.

Species-specific primers in the 16S rRNA coding region were selected based on sequences available in GenBank (Table 4). Species specificity was confirmed by sequencing at least one PCR product from a clinical sample for each primer in an ABI Prism 310 automated sequencer as described previously [132], and comparing the sequence generated to those available in GenBank.

Data analysis.

Chi–square analysis was used to compare the prevalence of various species in healthy individuals and in individuals with chronic periodontitis. The alpha level was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons. Prevalence ratios and 95 percent confidence intervals were calculated for the presence of each species in the periodontally healthy group versus the group with chronic

periodontitis. Prevalence ratios and confidence intervals were calculated in the same manner as a relative risk [133].

RESULTS

The clinical status of the study population has been previously described [130]. In the subgroup examined for this study, the mean for the deepest PD was 3.8 mm (SD 0.8) in the periodontally healthy group, and 7.7 mm (SD 1.3) in the periodontitis group. The mean age was 47.9 years (SD 13.1) for the healthy group and 50.6 years (SD 16.2) for the group with periodontitis. This difference was not significant by t test. The healthy group was 79% white, 15% African American, 5% Asian-American, and 2% other racial groups. The group with periodontitis was 68% white, 21% African-American, 3% Asian-American, and 8% other racial groups. The racial distribution of the two groups was not significantly different by chi-square analysis. The periodontally healthy group was 68% female and 32% male, and the group with periodontitis was 41% female and 59% male. This difference was statistically significant by chi-square analysis (P=0.002). However, no statistically significant differences were seen in the presence of any species by sex or race by chi-square analysis, or by age by t-test.

Comparisons of the presence or absence of 39 species or phylotypes in periodontal health and in chronic periodontitis are shown in table 5 and in figure 8. Figure 9 and table 5 show the prevalence ratio for the distribution between subjects with and without periodontitis for each species or phylotype. Prevalence ratios greater than 1 indicate association with disease, and those less than 1 show association with health. Data for *P.*

gingivalis [130], and *T. forsythia* (*B. forsythus*) and clone BU063 [134] have been previously reported, and are included here for comparison.

DISCUSSION

Chronic periodontitis appears to have a complex bacterial etiology. Upwards of 400 species have been commonly detected in the gingival sulcus [81], and a number of different species have been previously associated by some measure with human periodontitis. Before the availability of DNA-based detection methods, investigations were limited to those species that could be cultivated on an artificial medium. The first molecular investigations of the microbiology of periodontitis were limited to molecular detection of species that had been previously identified by cultivation. Recently ribosomal 16S cloning and sequencing has been used to identify uncultivated bacteria in the oral cavity, and investigation of the disease-association of these new species is now possible. The purpose of the present investigation was to evaluate the association of newly identified bacterial species with chronic periodontitis. Newly identified targets for investigation included both uncultivated phylotypes and previously characterized species that were not thought to be common inhabitants of the gingival sulcus. Species and phylotypes were selected for testing based on their detection in a previous large-scale investigation of oral bacterial diversity by cloning and sequencing of bacterial 16S genes [81]. Samples from subjects with chronic periodontitis were compared to those from age-matched periodontally healthy subjects to identify species of bacteria that may play a role in determining periodontal health. The study was designed to determine the presence or absence of each species in the subgingival environment of the entire dentition for each

subject by sampling every tooth. This strategy was employed to avoid the bias introduced by sampling only selected sites, and to identify qualitative differences in the flora of periodontal health and disease. If periodontitis is caused by pathogens rather than commensal species that overgrow in the absence of oral hygiene, this strategy will allow them to be identified.

Five species or phylotypes more prevalent in periodontally healthy subjects than in subjects with periodontitis were identified: *Atopobium rimae*, *Atopobium parvulum*, *Corynebacterium matruchotii*, and two uncultivated phylotypes, clone W090 from the *Deferribacteres* phylum, and clone BU063 from the *Bacteroidetes* phylum. Data shown here for clone BU063 has been previously reported [134]. To our knowledge association with periodontal health has not been previously reported for the other 3 species/phylotypes. Both clone BU063 and W090 are very closely related to species or phylotypes (*T. forsythensis* and *Deferribacteres* clone D084/BH017, respectively) [81] that are strongly associated with periodontitis (Figure 8), suggesting that phylogeny is not necessarily a good predictor of disease association. The demonstration of a higher prevalence of certain bacterial species in the mouths of healthy subjects suggests that replacement of a pathogenic flora with a benign one may be therapeutically important, and deserves further study.

Several uncultivated phylotypes showed a very strong relationship to disease, suggesting that there may be previously unrecognized organisms that play an important role in the pathogenesis of periodontitis. Clones D084 and BH017 from the *Deferribacteres* group

(indistinguishable by our assay) and clone AU126 from the *Bacteroidetes* were among the most strongly associated with disease of any species tested, with strength of association comparable or greater to that of the organisms widely regarded as the major pathogens in chronic periodontitis, *P. gingivalis* and *T. forsythia* (figure 9). In addition, clone X112 from the OP11 phylum was strongly associated with periodontitis, and *Megasphaera* clone BB166 and clone IO25 from the TM7 group were associated with periodontitis at the P=0.05 level.

Named species more commonly found in subjects with chronic periodontitis than in healthy subjects applying a stringent threshold of P<0.002 included *Treponema denticola, Eubacterium saphenum, Porphyromonas endodontalis*, *P. gingivalis, T. forsythensis, Filifactor alocis, Prevotella denticola, Cryptobacterium curtum, Treponema medium, Treponema socranskii*, and *Actinomyces naeslundii*. Most of these species have been previously associated with periodontitis. Of these, *P. gingivalis, T. forsythensis*, and *T. denticola* have consistently been associated with periodontitis by previous investigators [50]. Associations with chronic periodontitis for several species were newly demonstrated in the present study, including *P. endodontalis, E. saphenum, P. denticola, T. medium, and C. curtum*. *P. endodontalis* has primarily been reported in symptomatic infections originating in the pulp chamber [135], but it has been detected in periodontal pockets and other oral sites [136]. *Eubacterium saphenum* has been isolated from periodontal pockets [137] and detected in infected root canal systems [138]. *P. denticola* [139, 140] and *T. medium* [141, 142] have been previously identified in deep periodontal pockets, although an association with disease has not been demonstrated.

Cryptobacterium curtum is a recently characterized species isolated from the gingival sulcus of a patient with periodontitis [143]. Limited evidence has demonstrated more frequent detection of *T. socranskii* in subjects with periodontitis, although the observed differences were not statistically significant [144, 145], and it has been seen more frequently in deeper pockets [146]. *Filifactor alocis* has been seen more commonly in sites with periodontitis than in healthy sites [78]. The strong association between the presence of *A. naeslundii* and periodontitis is somewhat surprising, since *A. naeslundii* and other *Actinomyces* species are more commonly found in the supragingival plaque than in the gingival sulcus [146]. However, both *A. naeslundii* 1 and 2 have previously been seen in higher numbers in the gingival sulcus of subjects with periodontitis as compared to healthy subjects [147].

Additional named species more commonly found in subjects with chronic periodontitis than in healthy subjects applying a less stringent threshold of $P<0.05$ include *Treponema lecithinolyticum, Peptostreptococcus micros, Selenomonas sputigena, Rothia dentocariosa, Eikenella corrodens,* and *Dialister* isolate GBA27. *T. lecithinolyicum* is a recently characterized species that has been associated both with chronic and aggressive periodontitis [148]. *R. dentocariosa* has been associated with gingival recession [78] and with pericoronitis [149]. *E. corrodens* has been associated with chronic periodontitis [147] and aggressive forms of periodontitis [150], and has been found in close association with other bacteria often found in this form of periodontitis [50, 151]. The presence of *P. micros* has been positively associated with periodontitis by several investigators [50, 147,

152, 153]. Both *Dialister* isolate GBA27 and *S. sputigena* have been detected in subjects with periodontitis [81].

Several species were nearly universally present in both health and disease states, including *Fusobacterium naviforme, Fusobacterium nucleatum, Gemella haemolysans, Gemella morbillorum*, and *Campylobacter rectus*. Little previous information is available about *F. naviforme or G. haemolysans* in the oral cavity. *F. nucleatum* has been frequently associated with periodontitis [147, 154, 155],and *G. morbillorum* and *C. rectus* have been found in higher numbers in the subgingival plaque of subjects with periodontitis as compared to healthy subjects [147]. These findings suggest that these ubiquitous species may be commensals that overgrow in periodontitis, in contrast to true pathogens that usually produce disease when present. Evidence suggests that *F. nucleatum* may play a role in disease by providing the anaerobic environment necessary for the growth of pathogens [156]. Several species, *A. naeslundii, S. sputigena, R. dentocariosa*, *E. corrodens* and clone IO25 from the *TM7* phylum were more prevalent in periodontitis than in health in the present study, but the prevalence in health was so high (table 5and figure 8), that they may also be regarded as commensal species.

The microbial etiology of chronic periodontitis appears to be complex, with a large number of species showing association with disease. The present investigation has expanded this list to include a number of uncultivated species recently identified by ribosomal sequence analysis. Multiple factors probably account for the observed complexity, including interdependence among bacterial species within the bacterial

community, and inter-individual variation in microbial etiology and host susceptibility. In addition to functional studies to elucidate mechanisms of pathogenesis, future studies that provide quantitative information on proportions of these newly identified species at sites of disease activity, and longitudinal studies elucidating the natural history of this chronic disease are needed. In addition, the bacterial species that are found in a healthy subgingival environment deserve further study.

ACKNOWLEDGEMENT

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Species/phylotype	Sequence
Actinobacillus actinomycetemcomitans	GAAGAAGAACTCAGAGATGGGTTT
Actinobaculum clone EL030	TCTTTCCACGGATTCTGCG
Actinomyces naeslundi II	TGGAGACGGGGTTTCCTCCTTTGG
Atopobium parvulum	GGGGAGTATTTCTTCCGTGCCGCA
Atopobium rimae	GTGGGAGAATACGTCTTCCGTG
Bacteroides sp. oral clone BU063	TGCGATATAGTGTAAGCTCTACAG
Bacteroides-like oral clone AU126	GAAATGCTATCGACCGACGGAGAG
Campylobacter gracilis	GAATGCGAAATTCGCTACC
Campylobacter rectus	TTGTTGCTTCGCTAGTCGAGGCAG
Capnocytophaga gingivalis	GCTGTTTGGCGCAAGCTGAGTGGC
Corynrbacterium matruchotii	CGGTTGTAGAGATACGTACCTCCC
Cryptobacterium curtum	GAGATGTCGCAGCCGAAAGG
Deferribacteres sp. oral clones D084 & BH017	GTAGGAGATGGAAACATTGACG
Deferribacters sp. oral clone W090	CTG AAA GG CGA CGA CGT ACT TTC
Desulfobulbus sp. oral clone R004	CCCATGAAAGTGGGTGGTGCCTTC
Dialister sp. oral strain GBA27	CAGAAATGCGGAGTTCTTCTTCG
Eikenella corrodens	GCAAGGAGCCCGCTTGCCACGGTA
Eubacterium saphenum	CCTCTGACGTACCCTTAA
Filifactor alocis	ACATACCAATGACAGCCTTTTAA
Fusobacteria nucleatum	TTCGGGGAAACCTAAAGACAGGTGG
Fusobacterium naviforme	GAGAGTCGCAAAGCTGTGAAGTGGA
Gemella haemolysans	CGAGAGTAAGCAAACCTCACA
Gemella morbillorum	CGAGAGTCAGCCAACCTCATA
Granulicattella adjacens	TACAACGAGCAGCGAACTCGCGAG
Megasphaera sp. oral clone BB166	CGGGTAGAGATACCTGGTTCTTCttcg
Oral clone IO25 from TM7	CGACCCCTCGAGTAATAAAGC
Oral clone X112 from OP11	TTGGCAGGAGTGTCGTAATCTAACGA
Peptostreptococcus micros	AACGAGAAGCGAGATAGAGATGTTA
Porphyromonas endodontalis	TTTAGATGATGGCAGATGAGAG
Porphyromonas gingivalis	CATCGGTAGTTGCTAACAGTTTTC
Prevotella denticola	GCGCGAGCCGCATCYAATCTTGAA
Prevotella oris	GATTTTGTGCAAACACGATCTAAT
Rothia dentocariosa	TGACATATACTGGACTGCGTCAGAG
Selenomonas sputigena	AGAGATAGCTTCCTCCCTTCGGG
Tannerella forsythia(Bacteroides forsythus)	TGCGATATAGTGTAAGCTCTACAG
Treponema denticola	CAAGAGCAATGACATAGAGATATGG
Treponema lecithinolyticum	CTTAAGTTCGTAGAGATACGGATG
Treponema medium	CATCTAGTAGAAGGTCTTAGAGAT
Treponema socranski	ATGTACACTGGGCGTGTGCG

Table 4. Primer sequences

	% prevalence				95% confidence interval	
Species or phylotype[1]	health	perio-dontitits	P value for chisquare	prevalence ratio	lower bound	upper bound
A. actinomycetemcomitans	30	21	0.232	0.7	0.4	1.1
A. naeslundii **	67	94	<0.0001	1.4	0.6	3.5
A. parvulum **	91	65	0.0004	0.7	0.5	1.0
A. rimae **	79	33	<0.0001	0.4	0.3	0.6
Actinobaculum clone EL030	58	44	0.117	0.8	0.5	1.1
Bacteroidetes clone AU126**	30	82	<0.0001	2.7	1.6	4.6
Bacteroidetes clone BU063**	55	12	<0.0001	0.2	0.1	0.4
C. curtum **	33	64	0.0005	1.9	1.3	2.8
C. gingivalis	45	36	0.288	0.8	0.6	1.1
C. gracilis	68	55	0.108	0.8	0.6	1.1
*C. matruchotii**	91	79	0.052	0.9	0.6	1.2
C. rectus	89	94	0.345	1.1	0.5	2.3
Deferribacteres clone D084/BH017 **	27	71	<0.0001	2.6	1.7	3.9
Deferribacteres clone W090*	88	73	0.029	0.8	0.6	1.2
Desulfobulbus clone R004	89	82	0.215	0.9	0.6	1.4
Dialister strain GBA27*	32	52	0.022	1.6	1.2	2.3
E. corrodens *	79	95	0.004	1.2	0.4	3.4
E. saphenum **	20	70	<0.0001	3.5	2.4	5.3
F. alocis **	29	59	0.0005	2.1	1.4	2.9
F. naviforme	94	92	0.730	1.0	0.5	1.8
F. nucleatum	97	100	0.154	1.0	na[2]	na[2]
G. adjacens	48	41	0.381	0.8	0.6	1.2
G. haemolysans	97	97	1.000	1.0	0.4	2.7
G. morbillorum	95	100	0.080	1.0	na[2]	na[2]
Megasphaera clone BB166*	24	48	0.004	2.0	1.4	2.8
OP11 clone X112**	45	80	<0.0001	1.8	1.1	2.9
P. denticola **	35	68	0.0001	2.0	1.3	2.9
P. endodontalis **	18	62	<0.0001	3.4	2.4	4.9
*P. gingivalis**	26	88	<0.0001	3.4	1.8	6.6
P. micros *	38	59	0.015	1.6	1.1	2.2
P. oris	47	53	0.486	1.1	0.8	1.6
R. dentocariosa *	70	89	0.005	1.3	0.7	2.5
*S. sputigena**	71	92	0.002	1.3	0.6	2.9
T. denticola **	17	62	<0.0001	3.7	2.6	5.3
T. forsythensis **	38	79	<0.0001	2.1	1.3	3.4
T. lecithinolyticum *	17	36	0.010	2.2	1.6	3.0
*T. medium**	53	88	<0.0001	1.7	0.9	3.1
T. socranskii **	59	95	<0.0001	1.6	0.5	4.8
TM7 clone IO25*	71	91	0.004	1.3	0.6	2.6

[1] P value for chisquare indicated by "*" for < 0.05 and "**" for <0.001
[2] Confidence interval could not be calculated due to 0 subjects without the species in the periodontitis group

Table 5. Prevalence of 39 species in chronic periodontitis and health

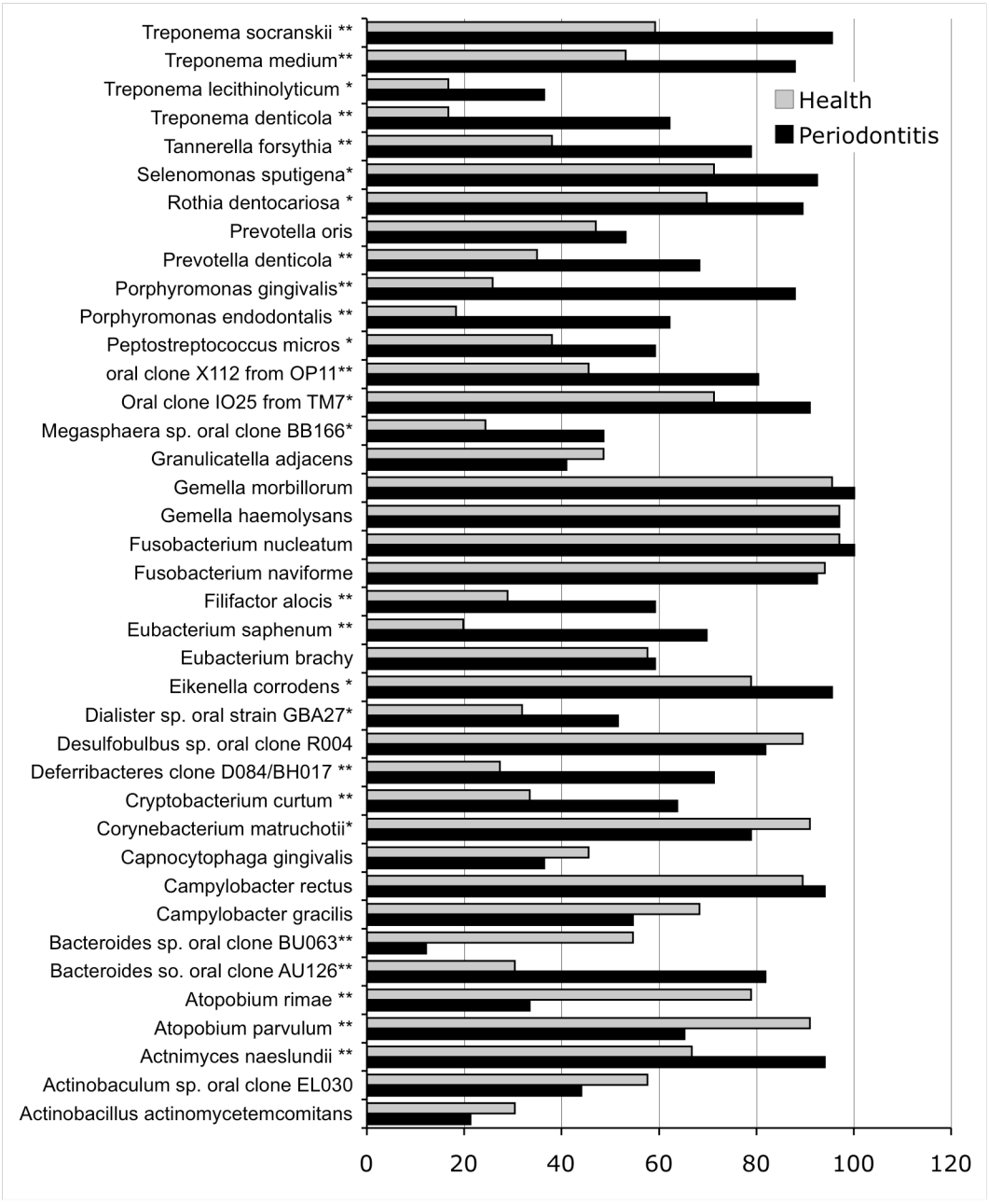

Figure 8. Prevalence of 39 bacterial species or phylotypes in 66 subjects with periodontitis and 66 healthy control subjects. Differences significant by chi-square analysis with $P<0.05$ are marked "*" after the species name; differences significant with $P<0.002$ are marked "**".

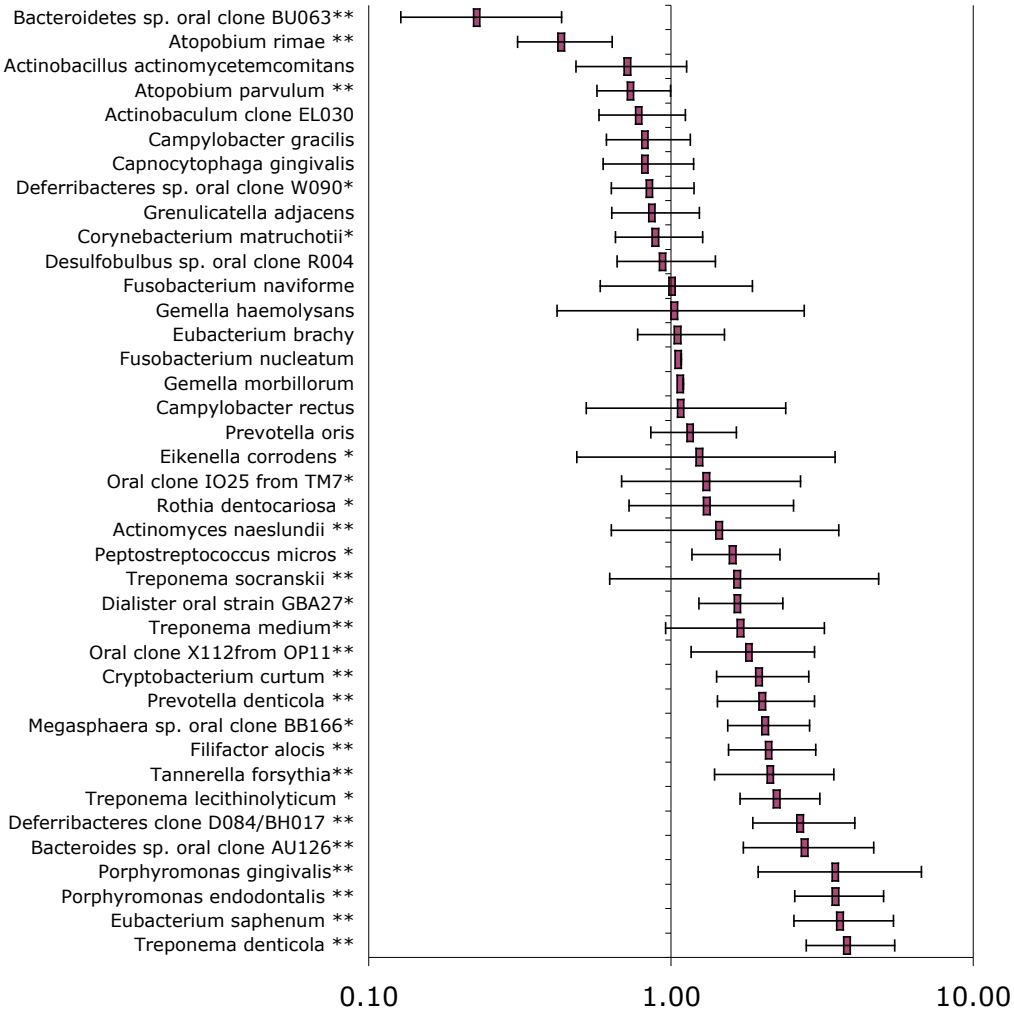

Figure 9. Prevalence ratios (calculated like a relative risk) for 39 bacterial species or phylotypes for 66 subjects with periodontitis and 66 healthy control subjects. 95 percent confidence intervals are shown as bars. The values to the left of 1 show species more common in health than in disease, and those to right of 1 show species more common in disease than in health. Confidence intervals could not be calculated for *F. nucleatum* or *G. morbillorum* since there were 0 subjects without the bacteria in the disease group.

CHAPTER 5

CANDIDATE PATHOGENS AND BENEFICIAL SPECIES IN CHRONIC PERIODONTITIS AND HEALTH

INTRODUCTION

Studies using quantitative ribosomal 16S cloning and sequencing have identified a number of candidate periodontal pathogens and beneficial species in chronic periodontitis and health, many of which are uncultivated. However, this approach cannot at the present time, study the bacterial profile of a large number of samples. Directed DNA approaches that target specific organisms provide a method to study large sample groups. We have previously published a study examining the relative prevalence of 39 species or phylotypes that were identified using a qualitative 16S clonal analysis. The objective of this study was to examine the association of 51 species or phylotypes with periodontal health and disease. These species were selected as candidates for study based on the findings of two studies using quantitative 16S cloning and sequencing.

MATERIALS AND METHODS

Study population. The population for this study has been previously described[61, 118] . Briefly, subjects for this institutionally approved study were recruited from the dental clinics of the Ohio State University and informed consent was obtained. 66 subjects with

chronic periodontitis and 66 age-matched controls were randomly selected from a sample set representing the most and least periodontally healthy segments of the population. The same set of samples was used for all species or phylotypes examined.

Detection of bacterial species and phylotypes. A nested 2-step PCR was used to detect the presence of target species or phylotypes. Broad range eubacterial ribosomal 16S and 23S primers were used in initial amplification of community DNA isolated from the plaque samples[73]. A second amplification using species-specific 16S primers and a universal primer located in the 23S gene was used to identify individual species. The primers for the second amplification were nested within the fragment generated from the initial amplification. Bacterial species and phylotypes were detected by PCR amplification of the 16S rDNA and the downstream intergenic spacer region (ISR). Inclusion of the ISR provided an additional check on the specificity of primers, since the length of this region varies among species. Primer sequences are shown in table 1. DNA fragments were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and viewed under UV transillumination. A positive or negative score was assigned based on the presence of clear bands of the expected molecular size. All assays were replicated.

Primer specificity. Species-specific primers in the 16S rRNA coding region were selected by visual inspection of closely related sequences available in GenBank (Table 1). Species specificity was confirmed by sequencing at least one PCR product from a

clinical sample for each primer in an ABI Prism 310 automated sequencer as described previously [132], and comparing the sequence generated to those available in GenBank.

Data analysis. Chi–square analysis was used to compare the prevalence of various species in healthy individuals and in individuals with chronic periodontitis. The alpha level was adjusted from 0.05 to 0.002 based on the Bonferroni correction for multiple comparisons. Prevalence ratios and 95 percent confidence intervals were calculated for the presence of each species in the periodontally healthy group versus the group with chronic periodontitis. Prevalence ratios and confidence intervals were calculated in the same manner as a relative risk [133].

RESULTS

The clinical status of the study population has been previously described. In the subgroup examined for this study, the mean for the deepest PD was 3.8 mm (SD 0.8) in the periodontally healthy group, and 7.7 mm (SD 1.3) in the periodontitis group. The mean age was 47.9 years (SD 13.1) for the healthy group and 50.6 years (SD 16.2) for the group with periodontitis. This difference was not significant by t test. The healthy group was 79% white, 15% African American, 5% Asian-American, and 2% other racial groups. The group with periodontitis was 68% white, 21% African-American, 3% Asian-American, and 8% other racial groups. The racial distribution of the two groups was not significantly different by chi-square analysis. The periodontally healthy group was 68% female and 32% male, and the group with periodontitis was 41% female and 59% male. This difference was statistically significant by chi-square analysis (P=0.002). However,

no statistically significant differences were seen in the presence of any species by sex or race by chi-square analysis, or by age by t-test.

Comparisons of the presence or absence of 51 target species or phylotypes in periodontal health and in chronic periodontitis are shown in table 6 and in figure 10. Figure 11 shows the prevalence ratio for the distribution between subjects with and without periodontitis for each species or phylotype. Prevalence ratios greater than 1 indicate association with disease, and those less than 1 show association with health. Figure 12 shows the data for 90 species examined so far. This includes the 39 species published earlier. Similarly, Figure 13 shows the prevalence ratio for all 90 species studied so far.

Discussion

Chronic periodontitis is a polymicrobial infection associated with a consortium of bacteria[30]. Although the etiological role of bacteria is well established, the involvement of specific species or groups of bacteria is not well elucidated. Quantitative 16S cloning and sequencing allows us to study the relationship of uncultivated and previously unsuspected species to health status. However, the approach is limited to detecting species that are form 3% or more of the population. The approach cannot, at this time, be used to study the microbial profile in large numbers of subjects. Targeted molecular approaches provide a sensitive, cultivation-independent method of studying specific species in large sample groups.

Prevotella nigrescens and *Prevotella intermedia* were found to be significantly more prevalent in periodontitis than in health. These closely related but genetically distinct species are indistinguishable from each other by phenotypic characterization. They have

previously been found in significantly higher levels in periodontitis than in health[100] and are associated with persistent bleeding on probing after therapy[157].

Johnsonella ignava, which belongs to the gram positive Clostridiae, was first isolated from subgingival plaque[158]. This species has been found significantly associated with periodontitis in our previous study[118].

Eubacterium saburreum, *Megasphaera sp. Oral clone MCE_141P1,* and *Catonella morbi,* all belonging to the class Clostridia, were significantly associated with periodontitis. This is consistent with our previous studies using 16S cloning and sequencing[117, 118].

Campylobacter sputorum, belonging to Proteobacteria, was found significantly associated with periodontitis[117, 118] and *Burkholderia cepacia* another member of Proteobacteria was significantly associated with peri apical infections(unpublished results).

Lachnospiraceaea oral clone MCE_141P1 was the only health-associated species detected in this study. To date, there is no report on the periodontal distribution of this phylotype.

In conclusion, as with the previous study using the same approach, more number of disease associated species than health associated were detected. Many of these were uncultivated phylotypes and novel, previously unsuspected species. Some of these species were found to be predominant members of the subgingival flora in our earlier studies. the subgingival flora appears to be more diverse than was previously suspected with many uncultivated phylotypes. The presence of a diverse disease associated flora provides further support of a polymicrobial etiology. Investigations on the acquisition,

colonization and interactions of these sepcies within the biofilm are important in improving our understanding of the bacterial etiology of periodontitis. Longitudinal studies monitoring the acquisition and stability of these candidate species and functional approaches that explore the role of these species in disease pathogenesis will further elucidate their role.

ACKNOWLEDGEMENTS

We wish to thank Erin Gross and Wang Zheng for carrying out the assays outlined above.

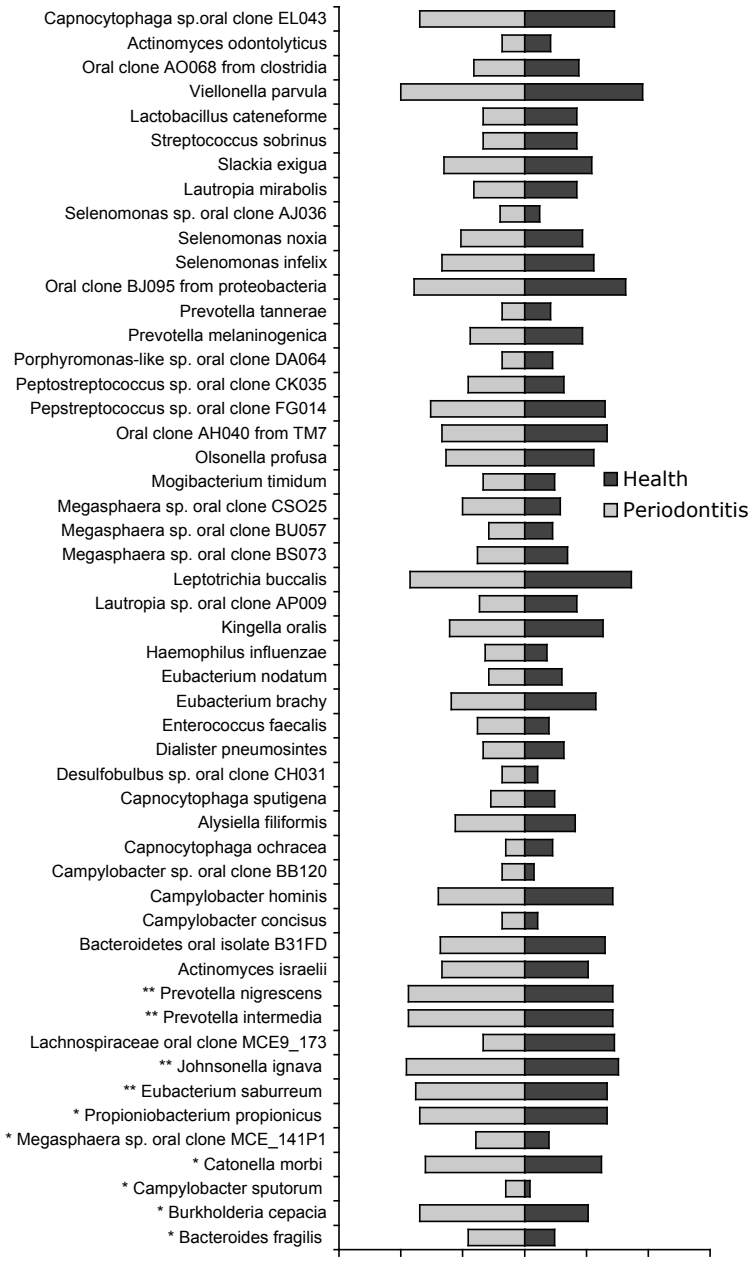

Figure 10. Prevalence of 51 bacterial species or phylotypes in 66 subjects with periodontitis and 66 healthy control subjects. Differences significant by chi-square analysis with $P<0.05$ are marked "*" after the species name; differences significant with $P<0.002$ are marked "**".

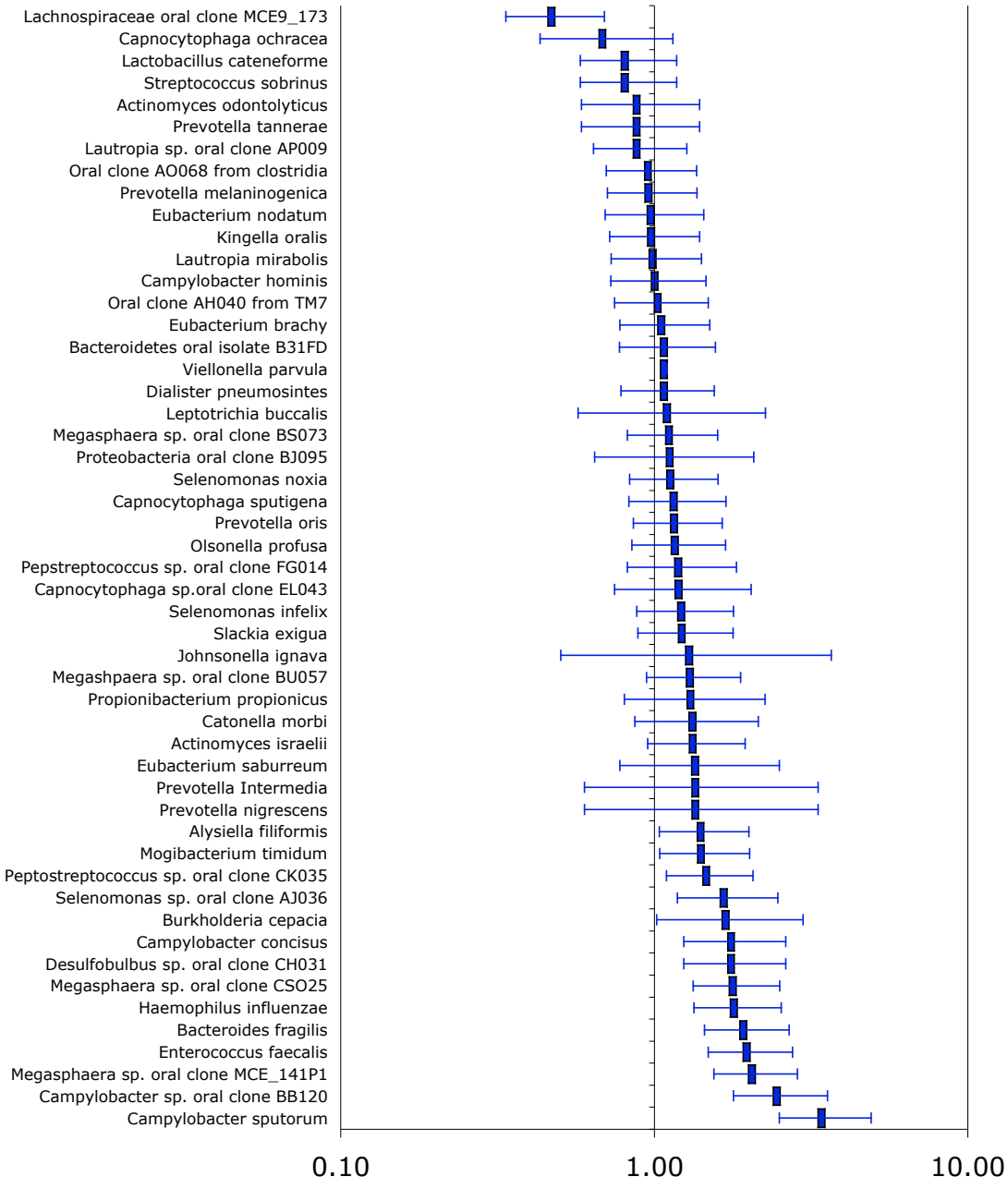

Figure 11. Prevalence ratios (calculated like a relative risk) for 51 bacterial species or phylotypes for 66 subjects with periodontitis and 66 healthy control subjects. 95 percent confidence intervals are shown as bars. The values to the left of 1 show species more common in health than in disease, and those to right of 1 show species more common in disease than in health. Confidence intervals could not be calculated for *V.parvula* since there were 0 subjects without the bacteria in the disease group

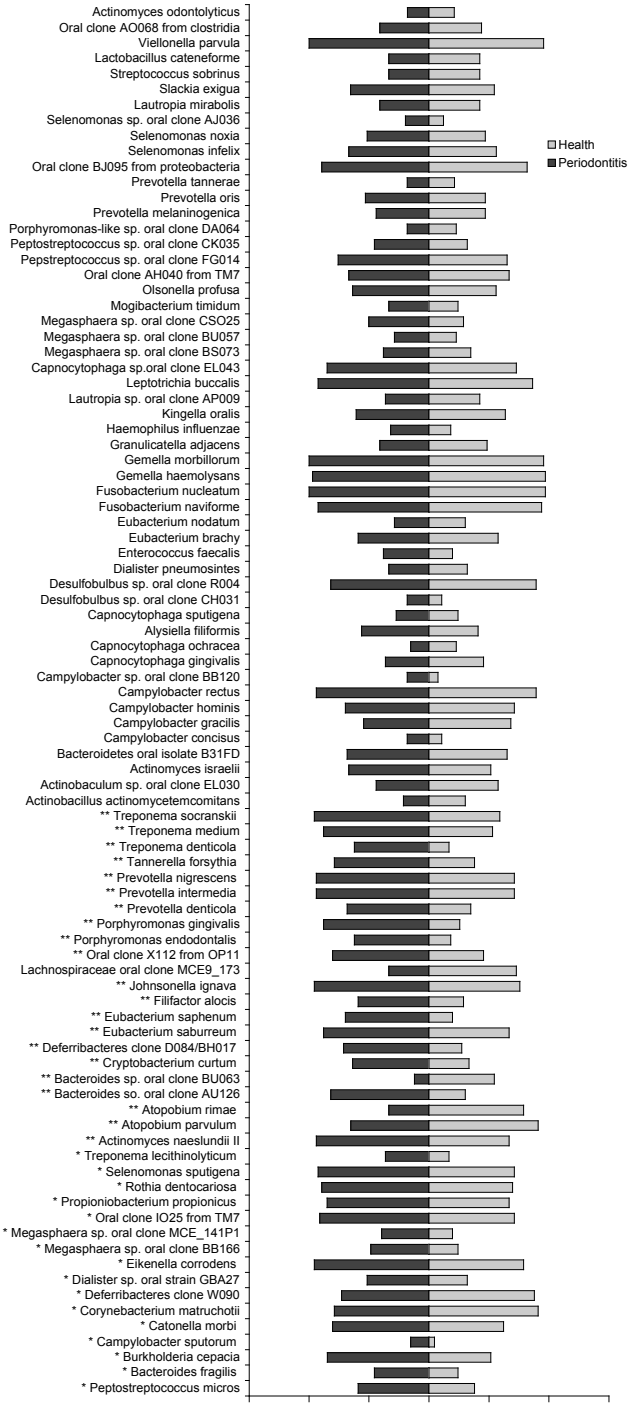

Figure 12. Prevalence of 90 bacterial species or phylotypes in 66 subjects with periodontitis and 66 healthy control subjects. Differences significant by chi-square analysis with $P<0.05$ are marked "*" after the species name; differences significant with $P<0.002$ are marked "**".

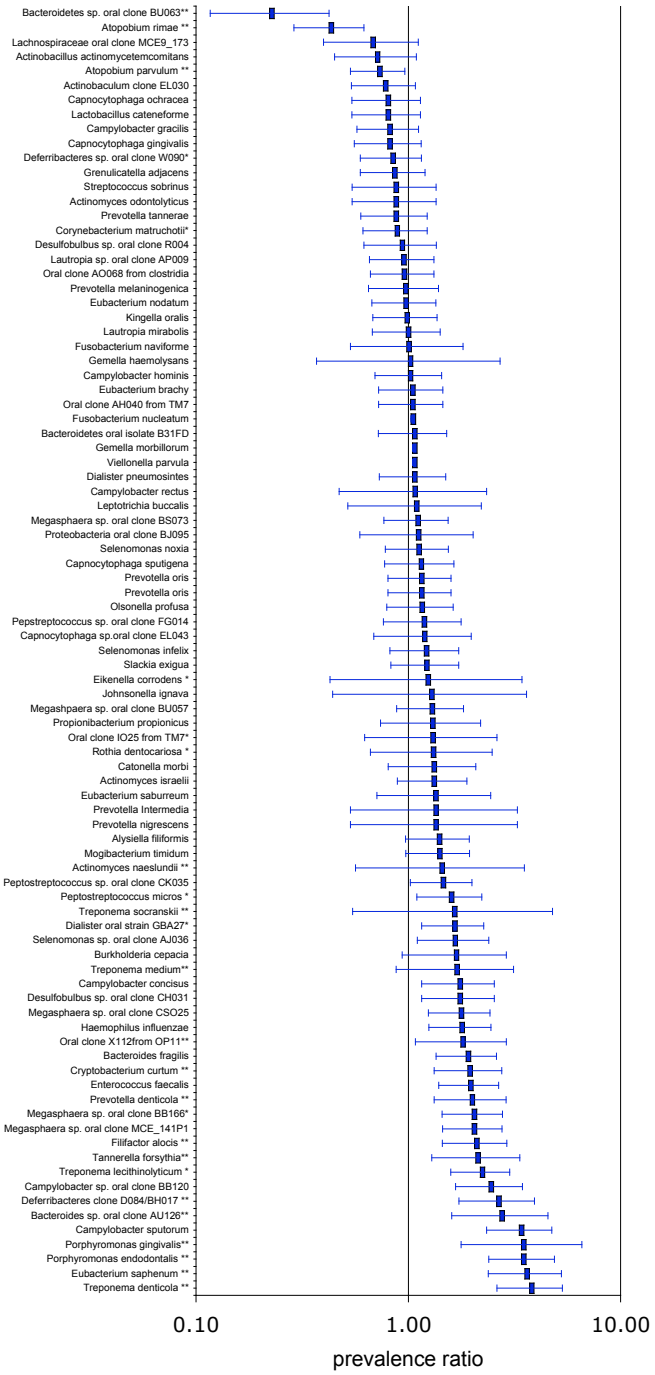

Figure 13. Prevalence ratios (calculated like a relative risk) for 90 bacterial species or phylotypes for 66 subjects with periodontitis and 66 healthy control subjects. 95 percent confidence intervals are shown as bars. The values to the left of 1 show species more common in health than in disease, and those to right of 1 show species more common in disease than in health.

Species/phylotype	Sequence
Actinomyces israelii	TGGGCCGGCTGCTCCTGGA
Actinomyces odontolyticus	GCA CGG CGG CAC TGC AGA GAT GTG GTG GCA
Alysiella filiformis	GCGGAAGGCTTTAGAGATAGAGC
Bacteoides fragilis	CATGTCAGTGAGCAATCACC
Bacteroidetes oral isolate B31FD	GCTCGTTGTCGGCCATTGTG
Burkholderia cepacia	GGTCGGAATCCTGCT
Campylobacter concisus	GGA ATA CTA AAT TAG TTA CCG T
Campylobacter sp. oral clone BB120	TCG GAA TGC TAA ACT AGC TAC CGC
Campylobacter sputorum	TAC TAA ACT GCT TGG GAA ACT ATC T
Capnocytophaga ochraceae	GTT TGG AGT AAT CTG AGT
Capnocytophaga sputigena	GCC ATT AGT TGC TAA CGA GTC AAG TCG A
Campylobacter hominis	TTACTTGAAAGCACTTTGGGT
Catonella morbii	GGTGCTGGGATGCATAAGCA
Dialister pneumosintes	CCT TGA CAT TGA TCG CAA TCC ATA GAA ATA T
Enterococcus faecalis	GTC GCT AGA CCG CGA GGT CAT GCA
Eubacterium brachy	GACCGGTCTTTAATAGGACCTT
Eubacterium nodatum	TGAAAGCTCGGTTAAAACTGAGCCC
Eubacterium saburreum	ACC GAT GAA AGG TGA GTA AAG TCA
Hemophilus influenzae	AAGCGAAGCTGCGAGGT
Johnsonella ignava	AAT CCT CTG CCC CTT GGG GCA C
Kingella oralis	TGG GCA ACA TGA TTG CTT
Lachnospiraceae oral clone MCE9_173	CGG ACG ATC CCG CAA CGG GGA
Lactobacillus cateneforme	ACGGAGCAGAGGGAGGCGAAGCC
Lautropia mirabolis	CTG AAG AGA TTT GGG GGT GCT
Lautropia sp. oral clone AP009	TTG GAG AGA TTC GAG GGT GCC
Leptotrichia buccalis	CTACGAATGCCTGTGAGAACA
Megasphaera oral clone BS073	AGC CTT GAC ATT GAG TGA AGG GC
Megasphaera oral clone BU057	AGC CTT GAC ATT GAT CGC AAG GA
Megasphaera oral clone CS025	AGC CTT GAC ATT GAG TGA AGG GC
Megasphaera oral clone MCE_141P1	AGC CTT GAC ATT GCT CGC AAC GG
Mogibacterium timidum	AGG ACT CTA GCG AGA CTG CCG AGG TCA
Capnocytophaga sp.oral clone EL043	GTTTGGTTTAAGGATTAA
Olsonella profusa	GTGGGCGGATTGTCCGT
Oral clone AH040 from TM7	TAGTTGGACTGCTTCGGTAAC
Oral clone AO068 from clostridia	TGTCGGTCTCCGAAGGAGA
Oral clone BJ065 from proteobacteria	AGGGTGGCGATGCCGCGA
Pepstreptococcus sp. oral clone FG014	GTT ATT GAG AAA TTG ATA AGT CCC TC
Peptostreptococcus sp. oral clone CK035	CGG ACA GGT GTT TAA TCA CAC CCT TCC TTC
Porphyromonas-like sp. oral clone DA064	TAACTAACCGCCGGCGAT
Prevotella intermedia	GACGTGGACCAAAGATTCATCGG
Prevotella melaninogenica	AGGAAGGATTTAGAGATAATGAC
Prevotella nigrecens	CGTTGGCCCTGCCTGCGG
Prevotella tannerae	CCA AGA GTG CGG AGT GCA GAG ATG CGC
Propionibacterium propionicus	GACATGGACTGGGAGTGCTC
Selenomoas infelix	AGC AGC GAA CCC GCG AGG GCA
Selenomonas noxia/DS051/EQ054/AA024/CS024	GGC AGC GAG AGA GTG ATC TTA
Selenomonas sp. oral clone AJ036	AGC AGC GAA CCC GCG AGG TTG
Slackia exigua	GCGCTAGGTGCGGGGGGACACGA
Streptococcus sobrinus	TTT TTC TTC GGA ACA TCG GAG
Viellonella parvula	AGACGGAAGCGAGATCGCGAGATG
Universal primer 785	GGATTAGATACCCTGGTAGTC
Universal primer 422	GGAGTATTTAGCCTT
Universal primer L189	GGTACTTAGATGTTTCAGTTC

Table 6. Primer sequences for 51 species

CHAPTER 6

CONCLUSIONS

To summarize, the majority of the bacteria in the gingival sulcus are uncultivated. It is possible that the quantitative data that has previously been gathered by cultivation, or by closed-ended molecular approaches based on a selection of known flora have given us a somewhat incomplete picture of the microbial community.

The disease-associated flora is largely uncultivated. These uncultivated phylotypes appear to be dominant members of the microbial community and show a robust association with disease. Certain cultivated species, which were previously not suspected as periodontal pathogens, have also shown a strong relationship to disease. the bacterial flora associated with periodontal health also has many uncultivated phylotypes that are found in high levels in subgingival plaque.

We found that periodontal stability was associated with microbial stability and that changes in flora were associated with changes in periodontal status, both for subjects who changed in the direction of health and those who changed in the direction of disease.

Bacteria most commonly regarded as periodontal pathogens were numerically minor and accounted for only a small fraction of the total flora. This doesn't necessarily mean they are not contributing to disease, but our data suggest that some dominant species that had been previously not been recognized are more strongly associated with disease and warrant a much closer look.

Species that have shown a robust association with disease are *Filifactor alocis, Deferribacteres oral clones D084 and BH017, Megasphaera sp. oral clone BB166, Desulfobulbus sp. oral clone R004* and *Dialister sp. oral strain GBA27.*

Certain species also showed a significant association with periodontal health. Of these, the most notable was *Veillonella sp. oral clone X042.* Although most subjects in our studies carried this phylotypes, the levels of this species were significantly higher in periodontally healthy subjects than in subjects with periodontitis. Further, the levels of this phylotype increased in association with improving periodontal health and decreased in association with worsening clinical health.

Further studies are required using targeted molecular approaches to study the levels of these bacteria in health and disease. Studying the fluctuations of these species in different phases of disease will further elucidate the role of these bacteria in the etiology of periodotitis.

Investigations of the levels and fluctuations of specific species or groups of organisms in association with health and disease have important implications for prediction of disease progression. The identification of beneficial bacteria and their association with improving clinical health may contribute to microbial replacement therapy or probiotics.

LIST OF REFERENCES

1. Brown, L.J. and H. Loe, *Prevalence, extent, severity and progression of periodontal disease.* Periodontol 2000, 1993. **2**: p. 57-71.

2. Arno, A., et al., *Incidence of gingivitis as related to sex, occupation, tobacco consumption, toothbrushing, and age.* Oral Surg Oral Med Oral Pathol, 1958. **11**(6): p. 587-95.

3. Ash MM Jr., G.B., Smith WA., *correlation between plaque and gingivitis.* J Periodontol, 1964. **35**: p. 424-429.

4. Schei, O., Waerhaug, J., Lovdal A., Arno A., *Alveolar bone loss as related to oral hygiene and age.* J Periodontol, 1959. **30**: p. 7-16.

5. Lovdal, A., A. Arno, and J. Waerhaug, *Incidence of clinical manifestations of periodontal disease in light of oral hygiene and calculus formation.* J Am Dent Assoc, 1958. **56**(1): p. 21-33.

6. Westfelt, E., et al., *Significance of frequency of professional tooth cleaning for healing following periodontal surgery.* J Clin Periodontol, 1983. **10**(2): p. 148-56.

7. Nyman, S., B. Rosling, and J. Lindhe, *Effect of professional tooth cleaning on healing after periodontal surgery.* J Clin Periodontol, 1975. **2**(2): p. 80-6.

8. Lindhe, J., et al., *Long-term effect of surgical/non-surgical treatment of periodontal disease.* J Clin Periodontol, 1984. **11**(7): p. 448-58.

9. Isidor, F., T. Karring, and R. Attstrom, *The effect of root planing as compared to that of surgical treatment.* J Clin Periodontol, 1984. **11**(10): p. 669-81.

10. Isidor, F., R. Attstrom, and T. Karring, *Regeneration of alveolar bone following surgical and non-surgical periodontal treatment.* J Clin Periodontol, 1985. **12**(8): p. 687-96.

11. Lindhe, J. and B. Liljenberg, *Treatment of localized juvenile periodontitis. Results after 5 years.* J Clin Periodontol, 1984. **11**(6): p. 399-410.

12. Kornman, K.S. and P.B. Robertson, *Clinical and microbiological evaluation of therapy for juvenile periodontitis.* J Periodontol, 1985. **56**(8): p. 443-6.

13. Christersson, L.A., et al., *Microbiological and clinical effects of surgical treatment of localized juvenile periodontitis.* J Clin Periodontol, 1985. **12**(6): p. 465-76.

14. Walker, C.B., et al., *A role for antibiotics in the treatment of refractory periodontitis.* J Periodontol, 1993. **64**(8 Suppl): p. 772-81.

15. Socransky, S.S. and A.D. Haffajee, *Effect of therapy on periodontal infections.* J Periodontol, 1993. **64**(8 Suppl): p. 754-9.

16. Haffajee, A.D., J.L. Dzink, and S.S. Socransky, *Effect of modified Widman flap surgery and systemic tetracycline on the subgingival microbiota of periodontal lesions.* J Clin Periodontol, 1988. **15**(4): p. 255-62.

17. Gibbons, R.J. and S.S. Socransky, *Enhancement of alveolar bone loss in gnotobiotic mice harbouring human gingival bacteria.* Arch Oral Biol, 1966. **11**(8): p. 847-8.

18. Crawford, J.M., M.A. Taubman, and D.J. Smith, *The natural history of periodontal bone loss in germfree and gnotobiotic rats infected with periodontopathic microorganisms.* J Periodontal Res, 1978. **13**(4): p. 316-25.

19. Gustafsson, B.E. and B. Krasse, *Dental calculus in germfree rats.* Acta Odontol Scand, 1962. **20**: p. 135-42.

20. Fitzgerald, R.J. and D.E. Mc, *Dental calculus in the germ-free rat.* Arch Oral Biol, 1960. **2**: p. 239-40.

21. Dongari-Bagtzoglou, A.I. and J.L. Ebersole, *Increased presence of interleukin-6 (IL-6) and IL-8 secreting fibroblast subpopulations in adult periodontitis.* J Periodontol, 1998. **69**(8): p. 899-910.

22. Ebersole, J.L., M.J. Steffen, and D. Cappelli, *Longitudinal human serum antibody responses to outer membrane antigens of Actinobacillus actinomycetemcomitans.* J Clin Periodontol, 1999. **26**(11): p. 732-41.

23. Taubman, M.A., et al., *Longitudinal monitoring of humoral antibody in subjects with destructive periodontal diseases.* J Periodontal Res, 1992. **27**(5): p. 511-21.

24. Noiri, Y. and S. Ebisu, *Identification of periodontal disease-associated bacteria in the "plaque-free zone".* J Periodontol, 2000. **71**(8): p. 1319-26.

25. Noiri, Y., L. Li, and S. Ebisu, *The localization of periodontal-disease-associated bacteria in human periodontal pockets.* J Dent Res, 2001. **80**(10): p. 1930-4.

26. Omar, A.A., et al., *Darkground microscopy of subgingival plaque from the top to the bottom of the periodontal pocket.* J Clin Periodontol, 1990. **17**(6): p. 364-70.

27. Friedman, M.T., et al., *The "plaque-free zone" in health and disease: a scanning electron microscope study.* J Periodontol, 1992. **63**(11): p. 890-6.

28. Vrahopoulos, T.P., P.M. Barber, and H.N. Newman, *The apical border plaque in severe periodontitis. An ultrastructural study.* J Periodontol, 1995. **66**(2): p. 113-24.

29. Babaahmady, K.G., et al., *Variations in the predominant cultivable microflora of dental plaque at defined subsites on approximal tooth surfaces in children.* Arch Oral Biol, 1997. **42**(2): p. 101-11.

30. Nishihara, T. and T. Koseki, *Microbial etiology of periodontitis.* Periodontol 2000, 2004. **36**: p. 14-26.

31. Gold, S.I., *Paeeriodontics. The past. Microbiology. Part (III).* J Clin Periodontol, 1985. **12**(4): p. 257-69.

32. Loe, H., E. Theilade, and S.B. Jensen, *Experimental Gingivitis In Man.* J Periodontol, 1965. **36**: p. 177-87.

33. Listgarten, M.A., *Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study.* J Periodontol, 1976. **47**(1): p. 1-18.

34. Listgarten, M.A. and L. Hellden, *Relative distribution of bacteria at clinically healthy and periodontally diseased sites in humans.* J Clin Periodontol, 1978. **5**(2): p. 115-32.

35. Loesche, W.J. and S.A. Syed, *Bacteriology of human experimental gingivitis: effect of plaque and gingivitis score.* Infect Immun, 1978. **21**(3): p. 830-9.

36. Syed, S.A. and W.J. Loesche, *Bacteriology of human experimental gingivitis: effect of plaque age.* Infect Immun, 1978. **21**(3): p. 821-9.

37. Tanner, A., et al., *The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria.* Periodontol 2000, 1994. **5**: p. 26-51.

38. Downes, J., et al., *Characterisation of Eubacterium-like strains isolated from oral infections.* J Med Microbiol, 2001. **50**(11): p. 947-51.

39. Staley, J.T. and A. Konopka, *Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats.* Annu Rev Microbiol, 1985. **39**: p. 321-46.

40. Socransky, S.S., et al., *The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms.* Arch Oral Biol, 1963. **8**: p. 275-80.

41. Socransky, S.S. and S.D. Manganiello, *The oral microbiota of man from birth to senility.* J Periodontol, 1971. **42**(8): p. 485-96.

42. Slots, J., *Subgingival microflora and periodontal disease.* J Clin Periodontol, 1979. **6**(5): p. 351-82.

43. Wolff, L.F., et al., *Fluorescence immunoassay for detecting periodontal bacterial pathogens in plaque.* J Clin Microbiol, 1991. **29**(8): p. 1645-51.

44. Wolff, L.F., et al., *Natural distribution of 5 bacteria associated with periodontal disease.* J Clin Periodontol, 1993. **20**(10): p. 699-706.

45. Loesche, W.J., et al., *Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard?* J Clin Microbiol, 1992. **30**(2): p. 418-26.

46. Gmur, R. and B. Guggenheim, *Monoclonal antibodies for the detection of 'periodontopathic' bacteria.* Arch Oral Biol, 1990. **35 Suppl**: p. 145S-151S.

47. Di Murro, C., et al., *Occurrence of Porphyromonas gingivalis, Bacteroides forsythus, and Treponema denticola in periodontally healthy and diseased subjects as determined by an ELISA technique.* J Periodontol, 1997. **68**(1): p. 18-23.

48. Ximenez-Fyvie, L.A., A.D. Haffajee, and S.S. Socransky, *Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis.* J Clin Periodontol, 2000. **27**(9): p. 648-57.

49. Socransky, S.S., C. Smith, and A.D. Haffajee, *Subgingival microbial profiles in refractory periodontal disease.* J Clin Periodontol, 2002. **29**(3): p. 260-8.

50. Socransky, S.S., et al., *Microbial complexes in subgingival plaque.* J Clin Periodontol, 1998. **25**(2): p. 134-44.

51. Haffajee, A.D., et al., *Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects.* J Clin Periodontol, 1998. **25**(5): p. 346-53.

52. Haffajee, A.D., et al., *Clinical and microbiological features of subjects with adult periodontitis who responded poorly to scaling and root planing.* J Clin Periodontol, 1997. **24**(10): p. 767-76.

53. Feres, M., et al., *Systemic doxycycline administration in the treatment of periodontal infections (I). Effect on the subgingival microbiota.* J Clin Periodontol, 1999. **26**(12): p. 775-83.

54. Feres, M., et al., *Change in subgingival microbial profiles in adult periodontitis subjects receiving either systemically-administered amoxicillin or metronidazole.* J Clin Periodontol, 2001. **28**(7): p. 597-609.

55. Dibart, S., et al., *Identification of bacterial species on or in crevicular epithelial cells from healthy and periodontally diseased patients using DNA-DNA hybridization.* Oral Microbiol Immunol, 1998. **13**(1): p. 30-5.

56. Cugini, M.A., et al., *The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results.* J Clin Periodontol, 2000. **27**(1): p. 30-6.

57. Colombo, A.P., et al., *Clinical and microbiological features of refractory periodontitis subjects.* J Clin Periodontol, 1998. **25**(2): p. 169-80.

58. Ali, R.W., et al., *Comparison of the subgingival microbiota of periodontally healthy and diseased adults in northern Cameroon.* J Clin Periodontol, 1997. **24**(11): p. 830-5.

59. Wecke, J., et al., *A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets.* FEMS Microbiol Lett, 2000. **191**(1): p. 95-101.

60. Leys, E.J., et al., *Association of Bacteroides forsythus and a novel Bacteroides phylotype with periodontitis.* J Clin Microbiol, 2002. **40**(3): p. 821-5.

61. Griffen, A.L., et al., *Prevalence of Porphyromonas gingivalis and periodontal health status.* J Clin Microbiol, 1998. **36**(11): p. 3239-42.

62. Woese, C.R., *Interpreting the universal phylogenetic tree.* Proc Natl Acad Sci U S A, 2000. **97**(15): p. 8392-6.

63. Pace, N.R., *A molecular view of microbial diversity and the biosphere.* Science, 1997. **276**(5313): p. 734-40.

64. Hugenholtz, P., et al., *Novel division level bacterial diversity in a Yellowstone hot spring.* J Bacteriol, 1998. **180**(2): p. 366-76.

65. Frank, D.N., et al., *Culture-independent molecular analysis of microbial constituents of the healthy human outer ear.* J Clin Microbiol, 2003. **41**(1): p. 295-303.

66. de la Torre, J.R., et al., *Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica.* Appl Environ Microbiol, 2003. **69**(7): p. 3858-67.

67. Blank, C.E., S.L. Cady, and N.R. Pace, *Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park.* Appl Environ Microbiol, 2002. **68**(10): p. 5123-35.

68. Angenent, L.T., et al., *Molecular identification of potential pathogens in water and air of a hospital therapy pool.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4860-5.

69. Paster, B.J., et al., *Prevalent bacterial species and novel phylotypes in advanced noma lesions.* J Clin Microbiol, 2002. **40**(6): p. 2187-91.

70. Paster, B.J., et al., *Bacterial diversity in human subgingival plaque.* J Bacteriol, 2001. **183**(12): p. 3770-83.

71. Kazor, C.E., et al., *Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients.* J Clin Microbiol, 2003. **41**(2): p. 558-63.

72. Becker, M.R., et al., *Molecular analysis of bacterial species associated with childhood caries.* J Clin Microbiol, 2002. **40**(3): p. 1001-9.

73. McClellan, D.L., A.L. Griffen, and E.J. Leys, *Age and prevalence of Porphyromonas gingivalis in children.* J Clin Microbiol, 1996. **34**(8): p. 2017-9.

74. de Lillo, A., et al., *Culture-independent identification of periodontitis-associated Porphyromonas and Tannerella populations by targeted molecular analysis.* J Clin Microbiol, 2004. **42**(12): p. 5523-7.

75. Spratt, D.A., A.J. Weightman, and W.G. Wade, *Diversity of oral asaccharolytic Eubacterium species in periodontitis-- identification of novel phylotypes representing uncultivated taxa.* Oral Microbiol Immunol, 1999. **14**(1): p. 56-9.

76. Kulik, E.M., et al., *Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis.* FEMS Microbiol Lett, 2001. **196**(2): p. 129-33.

77. Kroes, I., P.W. Lepp, and D.A. Relman, *Bacterial diversity within the human subgingival crevice.* Proc Natl Acad Sci U S A, 1999. **96**(25): p. 14547-52.

78. Tanner, A., et al., *Microbiota of health, gingivitis, and initial periodontitis.* J Clin Periodontol, 1998. **25**(2): p. 85-98.

79. Haffajee, A.D., et al., *Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects.* J Clin Periodontol, 1998. **25**(5): p. 346-353.

80. Munson, M.A., et al., *Molecular analysis of the microflora associated with dental caries.* J Clin Microbiol, 2004. **42**(7): p. 3023-9.

81. Paster, B.J., et al., *Bacterial diversity in human subgingival plaque.* J Bacteriol, 2001. **183**(12): p. 3770-83.

82. Wang, G.C. and Y. Wang, *The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species.* Microbiology, 1996. **142 (Pt 5)**: p. 1107-14.

83. Hill, K.E., et al., *Heterogeneity within the gram-positive anaerobic cocci demonstrated by analysis of 16S-23S intergenic ribosomal RNA polymorphisms.* J Med Microbiol, 2002. **51**(11): p. 949-57.

84. Murdoch, D.A., *Gram-positive anaerobic cocci.* Clin Microbiol Rev, 1998. **11**(1): p. 81-120.

85. Kuriyama, T., et al., *Bacteriology and antimicrobial susceptibility of gram-positive cocci isolated from pus specimens of orofacial odontogenic infections.* Oral Microbiol Immunol, 2002. **17**(2): p. 132-5.

86. Dymock, D., et al., *Molecular analysis of microflora associated with dentoalveolar abscesses.* J Clin Microbiol, 1996. **34**(3): p. 537-42.

87. van Winkelhoff, A.J., et al., *Smoking affects the subgingival microflora in periodontitis.* J Periodontol, 2001. **72**(5): p. 666-71.

88. Grant, D.A., M.J. Flynn, and J. Slots, *Periodontal microbiota of mobile and non-mobile teeth.* J Periodontol, 1995. **66**(5): p. 386-90.

89. Dzink, J.L., et al., *The predominant cultivable microbiota of crevicular epithelial cells.* Oral Microbiol Immunol, 1989. **4**(1): p. 1-5.

90. Nonnenmacher, C., R. Mutters, and L.F. de Jacoby, *Microbiological characteristics of subgingival microbiota in adult periodontitis, localized juvenile periodontitis and rapidly progressive periodontitis subjects.* Clin Microbiol Infect, 2001. **7**(4): p. 213-7.

91. Kumar, P.S., et al., *New bacterial species associated with chronic periodontitis.* J Dent Res, 2003. **82**(5): p. 338-344.

92. Stephens, P., et al., *Anaerobic cocci populating the deep tissues of chronic wounds impair cellular wound healing responses in vitro.* Br J Dermatol, 2003. **148**(3): p. 456-66.

93. Kremer, B.H. and T.J. van Steenbergen, *Peptostreptococcus micros coaggregates with Fusobacterium nucleatum and non-encapsulated Porphyromonas gingivalis.* FEMS Microbiol Lett, 2000. **182**(1): p. 57-62.

94. Kremer, B.H., et al., *Adherence of Peptostreptococcus micros morphotypes to epithelial cells in vitro.* Oral Microbiol Immunol, 1999. **14**(1): p. 49-55.

95. Siqueira, J.F., Jr. and I.N. Rocas, *Detection of Filifactor alocis in endodontic infections associated with different forms of periradicular diseases.* Oral Microbiol Immunol, 2003. **18**(4): p. 263-5.

96. Zhou, X., et al., *Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods.* Microbiology, 2004. **150**(Pt 8): p. 2565-73.

97. Brancaccio, M. and G.G. Legendre, *Megasphaera elsdenii endocarditis.* J Clin Microbiol, 1979. **10**(1): p. 72-4.

98. Gibson, G.R., G.T. Macfarlane, and J.H. Cummings, *Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut.* J Appl Bacteriol, 1988. **65**(2): p. 103-11.

99. Contreras, A., et al., *Importance of Dialister pneumosintes in human periodontitis.* Oral Microbiol Immunol, 2000. **15**(4): p. 269-72.

100. Nonnenmacher, C., et al., *Quantitative detection of periodontopathogens by real-time PCR.* J Microbiol Methods, 2004. **59**(1): p. 117-25.

101. Siqueira, J.F., Jr. and I.N. Rocas, *Dialister pneumosintes can be a suspected endodontic pathogen.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2002. **94**(4): p. 494-8.

102. Marchandin, H., et al., *Intra-chromosomal heterogeneity between the four 16S rRNA gene copies in the genus Veillonella: implications for phylogeny and taxonomy.* Microbiology, 2003. **149**(Pt 6): p. 1493-501.

103. Mikx, F.H. and J.S. Van der Hoeven, *Symbiosis of Streptococcus mutans and Veillonella alcalescens in mixed continuous cultures.* Arch Oral Biol, 1975. **20**(7): p. 407-10.

104. McBride, B.C. and J.S. Van der Hoeven, *Role of interbacterial adherence in colonization of the oral cavities of gnotobiotic rats infected with Streptococcus mutans and Veillonella alcalescens.* Infect Immun, 1981. **33**(2): p. 467-72.

105. Papapanou, P.N., et al., *"Checkerboard" versus culture: a comparison between two methods for identification of subgingival microbiota.* Eur J Oral Sci, 1997. **105**(5 Pt 1): p. 389-96.

106. Macuch, P.J. and A.C. Tanner, *Campylobacter species in health, gingivitis, and periodontitis.* J Dent Res, 2000. **79**(2): p. 785-92.

107. Socransky, S.S. and A.D. Haffajee, *Periodontal microbial ecology.* Periodontol 2000, 2005. **38**: p. 135-87.

108. Cebra, J.J., *Influences of microbiota on intestinal immune system development.* Am J Clin Nutr, 1999. **69**(5): p. 1046S-1051S.

109. van der Waaij, D., *The ecology of the human intestine and its consequences for overgrowth by pathogens such as Clostridium difficile.* Annu Rev Microbiol, 1989. **43**: p. 69-87.

110. Madden, J.A., et al., *Effect of probiotics on preventing disruption of the intestinal microflora following antibiotic therapy: a double-blind, placebo-controlled pilot study.* Int Immunopharmacol, 2005. **5**(6): p. 1091-7.

111. MacFarlane, T.W., et al., *Longitudinal study of untreated periodontitis (II). Microbiological findings.* J Clin Periodontol, 1988. **15**(5): p. 331-7.

112. Machtei, E.E., et al., *Longitudinal study of predictive factors for periodontal disease and tooth loss.* J Clin Periodontol, 1999. **26**(6): p. 374-80.

113. Papapanou, P.N., et al., *Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression.* J Periodontol, 1997. **68**(7): p. 651-66.

114. Cullinan, M.P., et al., *Acquisition and loss of Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Prevotella intermedia over a 5-year*

period: effect of a triclosan/copolymer dentifrice. J Clin Periodontol, 2003. **30**(6): p. 532-41.

115. van Steenberghe, D., et al., *A 15-month evaluation of the effects of repeated subgingival minocycline in chronic adult periodontitis.* J Periodontol, 1999. **70**(6): p. 657-67.

116. Wilson, K.H. and R.B. Blitchington, *Human colonic biota studied by ribosomal DNA sequence analysis.* Appl Environ Microbiol, 1996. **62**(7): p. 2273-8.

117. Kumar, P.S., Griffen, A.L., Moeschberger, M.L., Leys, E.J., *Identification of candidate periodontal pathogens and benficial species using quantitative 16S clonal analysis.* J Clin Microbiology, 2005. **43**(8).

118. Kumar, P.S., et al., *New bacterial species associated with chronic periodontitis.* J Dent Res, 2003. **82**(5): p. 338-44.

119. Hutter, G., et al., *Molecular analysis of bacteria in periodontitis: evaluation of clone libraries, novel phylotypes and putative pathogens.* Microbiology, 2003. **149**(Pt 1): p. 67-75.

120. Siqueira Junior, J.F. and I.N. Rocas, *Simultaneous detection of Dialister pneumosintes and Filifactor alocis in endodontic infections by 16S rDNA-directed multiplex PCR.* J Endod, 2004. **30**(12): p. 851-4.

121. Tanner, A., H.D. Bouldin, and M.F. Maiden, *Newly delineated periodontal pathogens with special reference to selenomonas species.* Infection, 1989. **17**(3): p. 182-7.

122. Riviere, G.R., et al., *Subgingival distribution of Treponema denticola, Treponema socranskii, and pathogen-related oral spirochetes: prevalence and relationship to periodontal status of sampled sites.* J Periodontol, 1995. **66**(10): p. 829-37.

123. Simonson, L.G., et al., *Bacterial synergy of Treponema denticola and Porphyromonas gingivalis in a multinational population.* Oral Microbiol Immunol, 1992. **7**(2): p. 111-2.

124. Dewhirst, F.E., et al., *The diversity of periodontal spirochetes by 16S rRNA analysis.* Oral Microbiol Immunol, 2000. **15**(3): p. 196-202.

125. Moter, A., et al., *Molecular epidemiology of oral treponemes associated with periodontal disease.* J Clin Microbiol, 1998. **36**(5): p. 1399-403.

126. Chen, C., *Distribution of a newly described species, Kingella oralis, in the human oral cavity.* Oral Microbiol Immunol, 1996. **11**(6): p. 425-7.

127. Carlier, J.P., et al., *Oribacterium sinus gen. nov., sp. nov., within the family 'Lachnospiraceae' (phylum Firmicutes).* Int J Syst Evol Microbiol, 2004. **54**(Pt 5): p. 1611-5.

128. Kroes, I., P.W. Lepp, and D.A. Relman, *Bacterial diversity within the human subgingival crevice.* Proc Natl Acad Sci U S A, 1999. **96**(25): p. 14547-14552.

129. Sakamoto, M., et al., *Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries.* Microbiol Immunol, 2000. **44**(8): p. 643-52.

130. Griffen, A.L., et al., *Prevalence of Porphyromonas gingivalis and periodontal health status.* J Clin Microbiol, 1998. **36**(11): p. 3239-3242.

131. McClellan, D.L., A.L. Griffen, and E.J. Leys, *Age and prevalence of Porphyromonas gingivalis in children.* J Clin Microbiol, 1996. **34**(8): p. 2017-2019.

132. Rumpf, R.W., et al., *Sequencing of the ribosomal intergenic spacer region for strain identification of Porphyromonas gingivalis.* J Clin Microbiol, 1999. **37**(8): p. 2723-5.

133. Fleiss, J.L., *Statistical Methods for Rates and Proportions*. 1981: Wiley.

134. Leys, E.J., et al., *Association of Bacteroides forsythus and a novel Bacteroides phylotype with periodontitis.* Journal of Clinical Microbiology, 2001: p. submitted for publication.

135. Haapasalo, M., *Black-pigmented gram-negative anaerobes in endodontic infections.* FEMS Immunol Med Microbiol, 1993. **6**(2-3): p. 213-7.

136. Petit, M.D., et al., *Porphyromonas endodontalis: prevalence and distribution of restriction enzyme patterns in families.* Oral Microbiology & Immunology, 1993. **8**(4): p. 219-24.

137. Uematsu, H., et al., *Eubacterium saphenum sp. nov., isolated from human periodontal pockets [corrected].* Int J Syst Bacteriol, 1993. **43**(2): p. 302-4.

138. Hashimura, T., M. Sato, and E. Hoshino, *Detection of Slackia exigua, Mogibacterium timidum and Eubacterium saphenum from pulpal and periradicular samples using the Polymerase Chain Reaction (PCR) method.* Int Endod J, 2001. **34**(6): p. 463-70.

139. Fosse, T., et al., *Prevalence of beta-lactamase-producing strains among 149 anaerobic gram-negative rods isolated from periodontal pockets.* Oral Microbiol Immunol, 1999. **14**(6): p. 352-7.

140. Teanpaisan, R., C.W. Douglas, and T.F. Walsh, *Characterisation of black-pigmented anaerobes isolated from diseased and healthy periodontal sites.* Journal of Periodontal Research, 1995. **30**(4): p. 245-251.

141. Willis, S.G., et al., *Identification of seven Treponema species in health- and disease-associated dental plaque by nested PCR.* J Clin Microbiol, 1999. **37**(3): p. 867-9.

142. Asai, Y., et al., *Detection and quantification of oral treponemes in subgingival plaque by real-time PCR.* J Clin Microbiol, 2002. **40**(9): p. 3334-40.

143. Nakazawa, F., et al., *Cryptobacterium curtum gen. nov., sp. nov., a new genus of gram-positive anaerobic rod isolated from human oral cavities.* Int J Syst Bacteriol, 1999. **49 Pt 3**: p. 1193-200.

144. Takeuchi, Y., et al., *Treponema socranskii, Treponema denticola, and Porphyromonas gingivalis are associated with severity of periodontal tissue destruction.* J Periodontol, 2001. **72**(10): p. 1354-63.

145. Riviere, G.R., et al., *Association of oral spirochetes from sites of periodontal health with development of periodontitis.* J Periodontol, 1997. **68**(12): p. 1210-4.

146. Ximenez-Fyvie, L.A., A.D. Haffajee, and S.S. Socransky, *Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis.* J Clin Periodontol, 2000. **27**(10): p. 722-32.

147. Ximenez-Fyvie, L.A., A.D. Haffajee, and S.S. Socransky, *Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis.* J Clin Periodontol, 2000. **27**(9): p. 648-57.

148. Wyss, C., et al., *Treponema lecithinolyticum sp. nov., a small saccharolytic spirochaete with phospholipase A and C activities associated with periodontal diseases.* Int J Syst Bacteriol, 1999. **49 Pt 4**: p. 1329-39.

149. Peltroche-Llacsahuanga, H., et al., *Investigation of infectious organisms causing pericoronitis of the mandibular third molar.* J Oral Maxillofac Surg, 2000. **58**(6): p. 611-6.

150. Suda, R., et al., *Eikenella corrodens in subgingival plaque: relationship to age and periodontal condition.* J Periodontol, 2002. **73**(8): p. 886-91.

151. Muller, H.P., et al., *Microbial ecology of Actinobacillus actinomycetemcomitans, Eikenella corrodens and Capnocytophaga spp. in adult periodontitis.* Journal of Periodontal Research, 1997. **32**(6): p. 530-42.

152. Papapanou, P.N., et al., *"Checkerboard" assessments of periodontal microbiota and serum antibody responses: a case-control study.* J Periodontol, 2000. **71**(6): p. 885-97.

153. Choi, B.K., et al., *Detection of major putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach.* J Periodontol, 2000. **71**(9): p. 1387-94.

154. Van Winkelhoff, A.J., et al., *Porphyromonas gingivalis, Bacteroides forsythus and other putative periodontal pathogens in subjects with and without periodontal destruction.* J Clin Periodontol, 2002. **29**(11): p. 1023-8.

155. Colombo, A.P., et al., *Subgingival microbiota of Brazilian subjects with untreated chronic periodontitis.* J Periodontol, 2002. **73**(4): p. 360-9.

156. Diaz, P.I., P.S. Zilm, and A.H. Rogers, *Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments.* Microbiology, 2002. **148**(Pt 2): p. 467-72.

157. Mombelli, A., et al., *Persistence patterns of Porphyromonas gingivalis, Prevotella intermedia/nigrescens, and Actinobacillus actinomyetemcomitans after mechanical therapy of periodontal disease.* J Periodontol, 2000. **71**(1): p. 14-21.

158. Willems, A. and M.D. Collins, *Evidence for the placement of the gram-negative Catonella morbi (Moore and Moore) and Johnsonella ignava (Moore and Moore) within the Clostridium subphylum of the gram-positive bacteria on the basis of 16S rRNA sequences.* Int J Syst Bacteriol, 1995. **45**(4): p. 855-7.