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Identification of Candidate Periodontal Pathogens and Beneficial Species by Quantitative 16S Clonal Analysis†

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Most studies of the bacterial etiology of periodontitis have used either culture-based or targeted DNA approaches, and so it is likely that pathogens remain undiscovered. The purpose of this study was to use culture-independent, quantitative analysis of biofilms associated with chronic periodontitis and periodontal health to identify pathogens and beneficial species. Samples from subjects with periodontitis and controls were analyzed using ribosomal 16S cloning and sequencing. Several genera, many of them uncultivated, were associated with periodontitis, the most numerous of which were gram positive, including *Peptostreptococcus* and *Filifactor*. The genera *Megasphaera* and *Desulfobulbus* were elevated in periodontitis, and the levels of several species or phylotypes of *Campylobacter*, *Selenomonas*, *Deferribacteres*, *Dialister*, *Catonella*, *Tannerella*, *Streptococcus*, *Atopobium*, *Eubacterium*, and *Treponema* were elevated in disease. *Streptococcus* and *Veillonella* spp. 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. The microbial profile of periodontal health also included the less-abundant genera *Campylobacter*, *Abiotrophia*, *Gemella*, *Capnocytophaga*, and *Neisseria*. These newly identified candidates outnumbered *Porphyromonas 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 between subjects with periodontitis and healthy subjects than between deep and shallow sites within the same subject. 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.

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 the 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* (24), 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 as-yet-uncultivable 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 specific 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 used 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 (10, 28). Investigations of oral bacteria using these tools have used enrichment primers for rare taxa, e.g., *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.

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The purpose of the present 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 by 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 after 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 3 months, oral prophylactic procedures within the last 3 months, fewer 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) after isolation and supragingival 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 nonadjacent 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 by using a previously described methodology (20). Briefly, bacteria was removed from the paper points by adding 750 μl of sampling buffer, followed by vortexing for 1 min. The paper points were then removed, the sample pelleted, and the supernatant discarded. The pellet was suspended in 1% sodium dodecyl sulfate in Tris-EDTA (TE), proteinase K was added, and the samples were incubated overnight. DNA was isolated on glass beads and eluted in TE.

Amplification of 16S rRNA. Bacterial 16S rRNA genes were amplified from the community DNA with universal eubacterial 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 1 μl of community DNA to a reaction mixture (50-μl final volume) containing 20 nmol of each primer, 40 nmol of deoxynucleotide triphosphates, and 1 U of *Taq* polymerase. The following cycling conditions were used: denaturation at 94°C for 1 min, annealing at 42°C for 2 min, and elongation at 72°C for 3 min. A final, 10-min elongation at 72°C followed 22 cycles of amplification. The PCR products were purified using the QiaQuik PCR purification kit (QIAGEN, Valencia, CA).

Cloning and sequencing. The 16S amplicons generated by PCR were cloned into *Escherichia coli* by 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-Bertani 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-β-D-galactopyranoside). The presence of inserts of the correct molecular size (≅1,500 bp) was confirmed by PCR amplification of the white colonies with the same primers used for initial amplification 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 to 800 bp were obtained from each amplicon. The sequences generated were compared to the GenBank database to identify the closest relatives by 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 by 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 was constructed from the alignments by the method of Jukes and Cantor. Phylogenetic trees were constructed by 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 >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 by using Kruskal-Wallis analysis of variance. Within-subject comparisons between deep and shallow sites for individual species were made by using the Wilcoxon signed-rank test. Chi-square 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 years (standard deviation [SD] = 9.1), and the mean age of the control group was 60.2 years (SD = 11.3). The difference, as determined by a Student *t* test, was not significant. The healthy group was 71% male, whereas the periodontitis group was 73% male. No significant difference was found by chi-square 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 4,500 clones. The identification of 100 clones per sample provided a 95% probability of detecting species present at ≥3% of total bacteria, and a 60% probability at ≥2%, calculated by using the binomial probability distribution. A total of 42 clones were <98% identical to current GenBank entries, and these clones were grouped into six novel phylotypes (GenBank accession numbers AY947495 to AY947500). A total of 274 species or phylotypes were identified. Table 1 lists these species in order of their ranking by overall prevalence and shows the mean prevalences in the three groups of samples. A table showing the same data sorted by phylogeny (Table S1A in the supplemental material) is available online.

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. Gram-positive but not gram-negative bacteria showed significant differences in relation to periodontal health status: gram-positive facultative bacteria accounted for a greater fraction of total bacteria in healthy subjects than in subjects with periodontitis, and gram-positive anaerobic species were more common in subjects with periodontitis than in healthy subjects.

Overall, 59.9% of the clone population was made up of as-yet-uncultivable 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*,

TABLE 1. Species and phylotypes from three sample groups showing the percentage of total clones and the mean prevalence in each group arranged in order of decreasing overall prevalence

Overall rank	Species/phylotype(s)	% 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	*Veillonella* 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. strain E2_20 E1 oral isolate	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* oral clone CK035	1.51	0.5 ± 0.8	0.8 ± 1.3	3.3 ± 4.7
16	*Megasphaera* oral clone BB166	1.42	0.2 ± 0.4	2.1 ± 3.7	1.9 ± 2.7
17	*Desulfobulbus* 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	*Neisseria 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	*Eubacterium* sp. oral clone BP1-82	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* (*Anaerosphaera*) *micronuciformis*	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. strain 1754-94	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. clone FX38-1	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

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TABLE 1—*Continued*

Overall rank	Species/phylotype(s)	% Clones	Mean prevalence ± SD		
			Healthy subjects	Shallow sites	Deep sites
69	*Selenomonas*-like 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* sp. oral clone OH1A	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. 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
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. oral clone AP060	0.18	0.2 ± 0.8	0.3 ± 0.6	0.0 ± 0.0
110	*Selenomonas* sp. oral clone OH4A	0.18	0.1 ± 0.4	0.3 ± 0.6	0.1 ± 0.4
111	*Campylobacter* sp. oral clone OH5A	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 CI002	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	*Selenomonas*-like sp. oral clone GAA14	0.13	0.1 ± 0.3	0.1 ± 0.5	0.2 ± 0.4
128	*Abiotrophia* sp. oral clone OH2A	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

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TABLE 1—*Continued*

Overall rank	Species/phylotype(s)	% Clones	Mean prevalence ± SD		
			Healthy subjects	Shallow sites	Deep sites
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. strain 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	*Neisseria 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. strain B	0.09	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.3
163	*Treponema* sp. strain Smibert-5	0.09	0.0 ± 0.0	0.1 ± 0.4	0.1 ± 0.4
164	*Treponema* sp. strain 6:H:D15A-4	0.09	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.4
165	*Treponema* sp. strain 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* sp. oral clone OH3A	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 goodfellowii*	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	*Lactobacillus* sp. MR-2	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. strain 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
185	*Neisseria* genomosp. P1 clone P4PC_20	0.07	0.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
186	*Neisseria* 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	*Streptococcus* sp. oral clone 3192A	0.04	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
199	*Streptococcus* sp. oral clone CH016	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	*Eubacterium* sp. equine clone 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

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TABLE 1—*Continued*

Overall rank	Species/phylotype(s)	% Clones	Mean prevalence ± SD		
			Healthy subjects	Shallow sites	Deep sites
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* sp. oral clone OH6A	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	*Actinobacillus actinomycetemcomitans*	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. oral clone BP2-13	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. strain 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. strain CLE-4	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
238	*Lactobacillus* sp. strain 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. strain ADV 04.01	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
244	*Mogibacterium* (*Eubacterium*) *timidum*	0.02	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
245	*Eubacterium* sp. strain WFeA1-59	0.02	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
246	*Eubacterium* sp. equine clone 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* (*Peptostreptococcus*) *lacrimalis*	0.02	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0
254	*Veillonella* sp. strain 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. strain 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. strain 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	*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

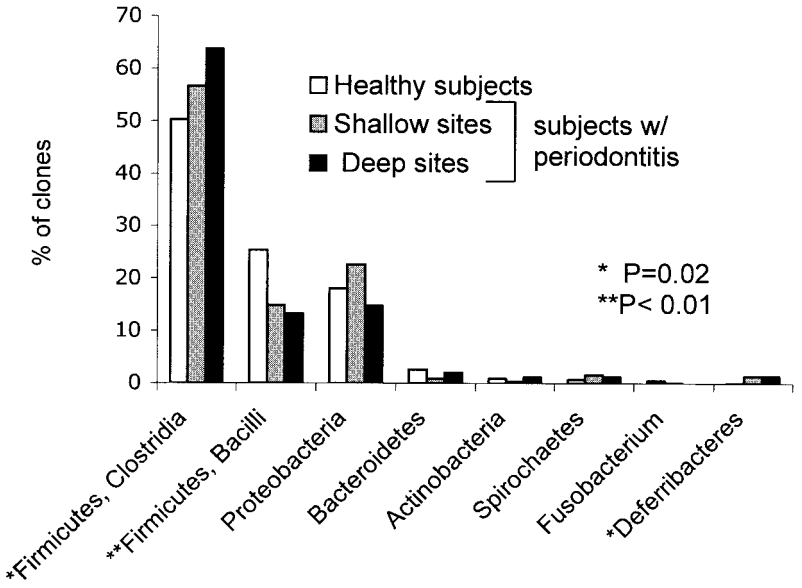

FIG. 1. Distribution of bacterial phyla in health and disease. Two classes of *Firmicutes* (*Bacilli* and *Clostridia*) are displayed individually due to their high prevalence.

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 used 16S PCR amplification with 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.

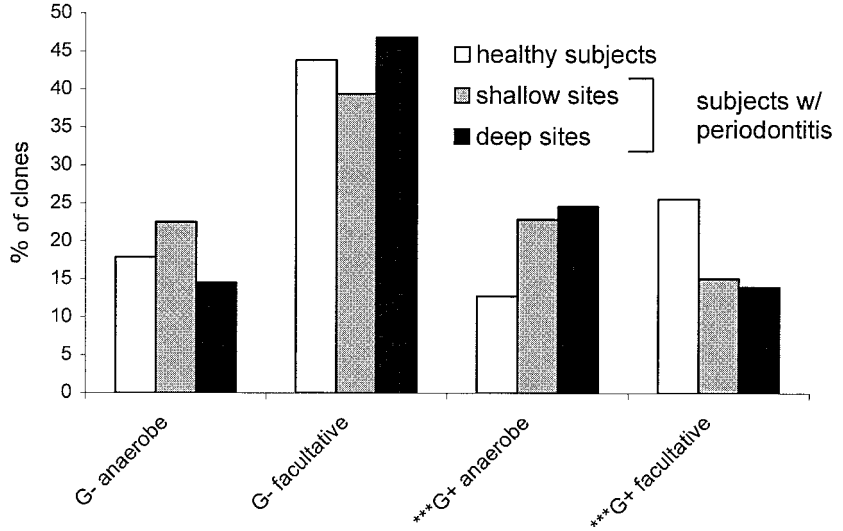

FIG. 2. Distribution of gram-positive (G+) and gram-negative (G−) anaerobes and facultative species in relation to disease status. The gram status of uncharacterized phylotypes was inferred from that of their closest neighbor. ***, $P < 0.005$.

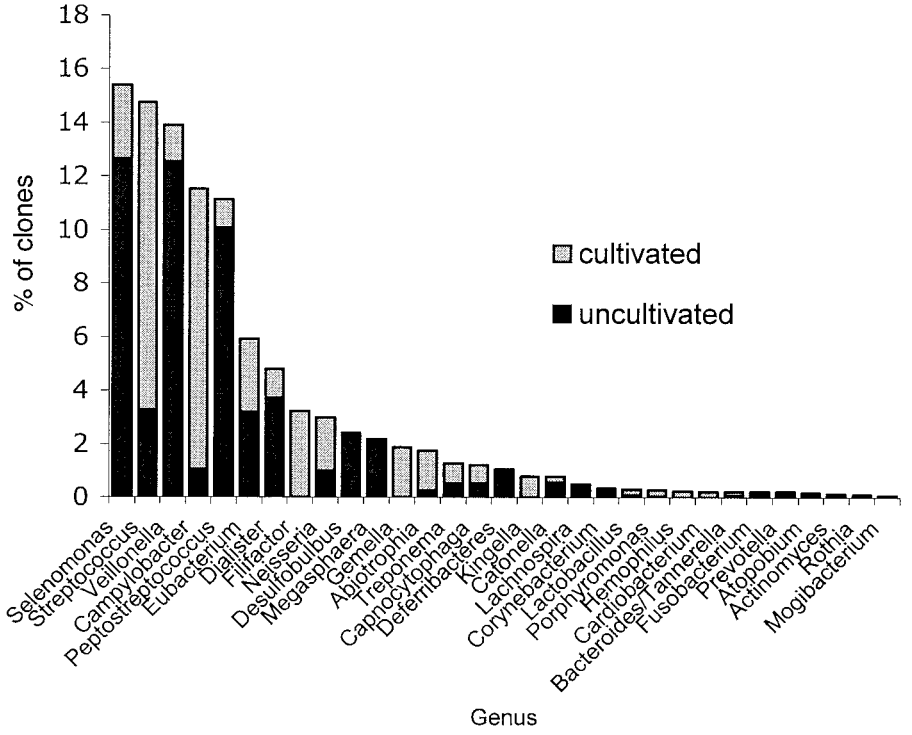

FIG. 3. Distribution of cultivated and uncultivated bacteria by genus for all samples.

Molecular approach. Subgingival bacterial populations have previously been explored by 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* (3, 28), *Eubacterium* (32), and even *Archaea* (14), or subtraction systems to eliminate major species, such as streptococci (13), and have

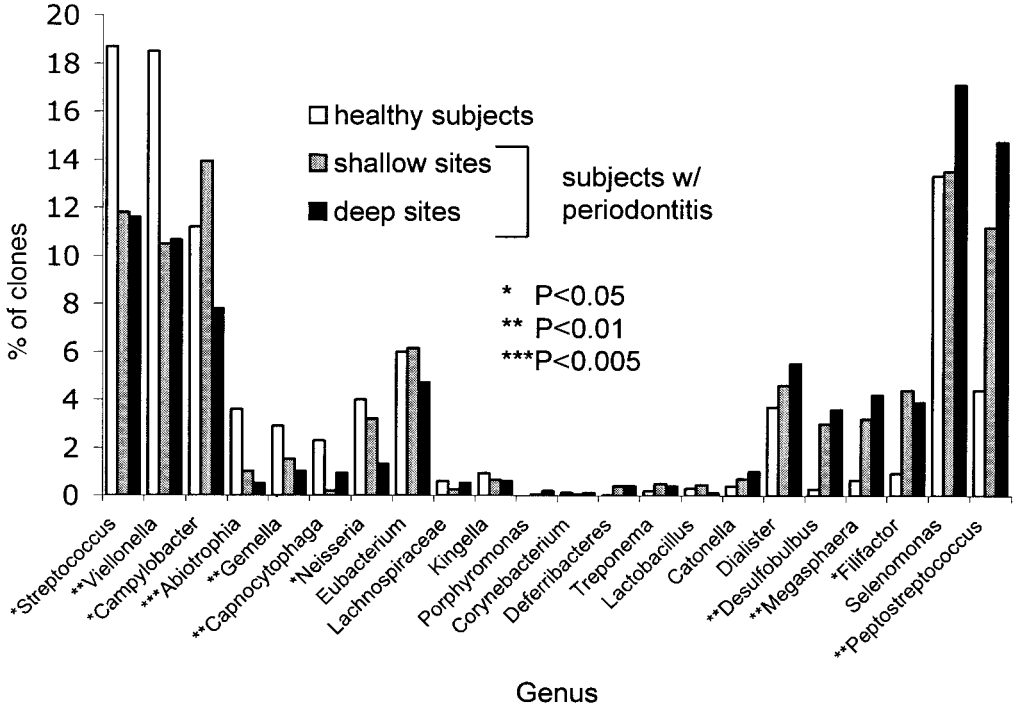

FIG. 4. Distribution by health status for genera accounting for >0.025% of total bacteria are shown. The genera are arranged in a gradient from those predominant in health shown on the left to those predominant in periodontitis shown on the right.

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

Clinical status and overall rank	Species and/or phylotype	*P*		
		Levels		Presence (between subjects[c])
		Between sites[a]	Between subjects[b]	
Disease				
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	*Streptococcus* 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. strain 6:H:D15A-4			0.04
172	*Streptococcus* sp. oral clone DP009		0.04	0.008
Health				
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 test.
[b] Comparison of levels between healthy and subjects with periodontitis by Kruskal-Wallis analysis of variance.
[c] Comparison of presence or absence of species by chi-square test.

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 used to avoid plateau effects, and a set of broad, universal eubacterial 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 four 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* (Fig. 4). These genera were all detected in a previous culture-based study of periodontal bacteria (34), although all but *Streptococcus* appeared to account for a relatively smaller fraction of total bacteria. Other major groups of bacteria detected in previous studies by using DNA hybridization included *Fusobacterium* and, by using cultivation and DNA hybridization, *Actinomyces* (8, 34). Both were rare in the present study. The greater sensitivity of cultivation compared to molecular analyses for the detection of actinobacteria has been previously reported (22). 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, a finding also consistent with earlier studies (31, 34, 35), and spirochetes were also found in low numbers. Centrifugation, freezing, and long storage times 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 the present study may have been slightly biased toward 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 the copy numbers of ribosomal genes. For the present 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 six novel phylotypes were detected (Table 1), and they belonged to six different phyla (Fig. 1). Consistent with earlier observations (13, 28), ca. 60% of these species were uncultivated. Several of the most numerous genera, including *Selenomonas*, *Veillonella*, and *Peptostreptococcus*, were composed primarily of uncultivated species (Fig. 3). Distributions of several uncultivated bacteria were found to differ between healthy subjects and subjects with 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 the present study. Studies using similar approaches have found 1 to 15% of clones to be chimeric sequences (28). For the current study, formation of chimeras was minimized by limiting the PCR cycle number (37). 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 were analyzed at the level of species (alpha = 0.10) to identify candidate species for subsequent investigation. Because of the non-normal distributions typically observed with bacterial counts, nonparametric statistics were used 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 class *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 (9, 23) such as *Anaerococcus*, *Peptococcus*, *Micromonas*, and *Peptonephilus* (9). In addition, several uncultivated peptostreptococci were detected in large numbers in the present 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* strains 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 (23). Most infections involving GPAC are polymicrobial and appear to involve synergistic interactions with other bacteria (23). Previous epidemiologic evidence has linked peptostreptococci with dental infections, although investigations have been limited to *Peptostreptococcus micros*, a rare species in the current study (and not associated with disease). *P. micros* has been associated with odontogenic infections (4, 16) and is significantly higher in smokers, a population that has more extensive and severe periodontitis than nonsmokers (36). It is also more common around mobile teeth (7) and has been found at higher levels in epithelium-associated plaque compared to unattached plaque in the gingival sulcus (5). Targeted DNA approaches have also found *P. micros* to be elevated in advanced chronic periodontitis (26) and more common in subjects with periodontitis (15). 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 (33). *P. micros* demonstrates both adhesion to epithelium and coaggregation with other species such as *P. gingivalis* and *F. nucleatum* mediated by extracellular polysaccharides (11, 12). 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, was also common in the samples (Table 2), and was significantly elevated in subjects with disease (Fig. 2). This organism has been previously associated with both chronic periodontitis (15) and endodontic lesions (29).

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 cases of periodontitis, and at the species level *Megasphaera* oral clones BB166, MCE3_141, and BS073 were associated with disease. *Megasphaera* clone BB166 has been previously associated with chronic periodontitis (15). *Megasphaera* spp. have been reported as normal inhabitants of the gut and vagina (38), and *M. elsdenii* has been implicated in bacterial endocarditis in immunocompromised patients (1).

The genus *Desulfobulbus* was also associated with disease, and at the species level both *Desulfobulbus* CH031 and R004 were significantly associated with deep sites. *Desulfobulbus* species have been previously detected in the gingival sulcus (15, 28) and the human gut (6). *Desulfobulbus* are sulfate-reducing bacteria and have been frequently detected in aquatic environmental samples.

Campylobacter sputorum subsp. *sputorum* and *Campylobacter* strain 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 (34). The less numerous and uncultivated *Selenomonas* phylotypes D0-042, EY047, and AH132 were associated with disease, and, in contrast, *Selenomonas* strain 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 (2, 25) and to endodontic infections (30). *Deferribacteres* phylotypes W090 and BH007 were associated with periodontitis, and W090 has been previously linked to disease (15). 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, *T. denticola*, and *T. forsythia* were rarely detected in the present study and, of these, only *T. forsythia* was associated with disease. Strong associations with 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 that 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* spp. 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* sp. oral clone X042. Both *Streptococcus* and *Veillonella* have been previously associated with periodontal health (8, 34). *Veillonella* oral clone X042 is very closely related to *V. parvula* and *V. dispar* by 16S phylogeny and may be part of an indistinguishable cluster (18). 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 (21), and it has been shown that veillonellae will not colonize tooth surfaces without streptococci (19).

The microbial profile of periodontal health also included the less-abundant genera *Campylobacter*, *Abiotrophia*, *Capnocytophaga*, *Gemella*, and *Neisseria*. This confirms earlier studies linking *Capnocytophaga* (8, 27, 34) and *Campylobacter gracilis* (17) to health.

Levels of the genera *Streptococcus* 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 shifts 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 whether explorations should be conducted at the level of genus, species, or even virulence genes. 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. *Peptostreptococcus* and *Filifactor* were elevated in subjects with periodontitis, and *Streptococcus*, *Abiotrophia*, and *Gemella* were elevated in healthy subjects. Differences were also observed among the gram-negative bacteria: *Veillonella*, *Campylobacter*, and *Capnocytophaga* levels were higher in the plaque of healthy subjects, and *Megasphaera* and *Desulfobulbus* levels were increased in cases of 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 whether 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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