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Prevalence and diversity of *Synergistetes* taxa in periodontal health and disease

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Background and Objective: Members of the phylum *Synergistetes* have previously been identified within periodontitis subgingival plaque and are considered putative periodontopathogens. This study compared the diversity of subginigval *Synergistetes* in a cohort of subjects with periodontitis ($n = 10$) vs. periodontitis-free controls ($n = 10$).

Material and Methods: Pooled subgingival plaque samples from all deep periodontal pockets or all sulci were collected from the periodontitis and periodontitis-free subjects, respectively. Bacterial 16S rRNA genes were PCR-amplified from purified subgingival plaque DNA using a *Synergistetes* 'selective' primer set. PCR products were cloned and sequenced to analyze the prevalence and diversity of *Synergistetes* operational taxonomic units (OTUs) present in plaque samples of both subject groups.

Results: A total of 1030 non-chimeric 16S rRNA clones were obtained, of which 162 corresponded to members of the phylum *Synergistetes*. A significantly larger number of *Synergistetes* clones were obtained from periodontitis subgingival plaque than from periodontitis-free controls (25.4% vs. 5.9%, $p < 0.001$). All *Synergistetes* clones corresponded to cluster A oral *Synergistetes*, and fell into 31 OTUs (99% sequence identity cut-off). Twenty-nine *Synergistetes* OTUs were detected in the periodontitis group while eight were detected in the periodontitis-free group ($p < 0.001$). Five *Synergistetes* OTUs; including one OTU corresponding to the recently-characterized species *Fretibacterium fastidiosum*, were more prevalent in the periodontitis subjects ($p < 0.05$).

Conclusion: OTUs belonging to oral *Synergistetes* cluster A were more readily detectable and were more diverse in subgingival plaque from periodontitis subjects compared with periodontitis-free controls. Specific *Synergistetes* OTUs appear to be associated with periodontitis.

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Periodontitis is a chronic polymicrobial infectious inflammatory disease affecting the periodontium; which causes the progressive destruction of the soft and hard tooth supporting tissues (1,2). It is generally considered to result from microbial dysbiosis, i.e. a 'pathogenic shift' in the composition of the subgingival microbia that promotes and potentiates pathogenic processes (3,4). Of the 13 bacterial phyla known to inhabit the oral cavity (5), nine have been detected in the subgingival plaque from both periodontally healthy (6) and diseased subjects (7). The predominant phyla found in the periodontal niches are *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*; which have been estimated to account for more than 90% of the total bacteria present (6). In

surveys of the oral microbiome that have utilized 'universal' 16S rRNA molecular detection protocols, *Synergistetes* was defined as a 'rare' phylum, as it had a relatively low detection frequency of ca. 0.1–2% (6,8–10). However, other estimations based on microscopic fluorescence *in situ* hybridization (FISH) studies have indicated that *Synergistetes* taxa may account for as much as 11% of the microbiota within deep pockets (11).

Members of the *Synergistetes* inhabit diverse ecological environments (12,13). They are fastidious, gram-negative anaerobic straight or curved bacilli, some of which are motile (14,15). The *Synergistetes* phylum was only recently described, having nine component genera (16). A significant number of taxa, most of which were previously placed in the phyla *Deferribacteres* (7,10,17–19) and *Firmicutes* (20,21), were incorporated into this new phylum. In previous culture-independent surveys of oral bacterial diversity, 16S rRNA gene sequences corresponding to members of the phylum *Synergistetes* have been detected in a variety of oral niches; especially within diseased periodontal pockets, endodontic infections and carious dentine (6–9,22,23). The oral *Synergistetes* have been divided into two principal clusters, based on their phylogenetic origins (11,14). Cluster A is the larger group comprising mainly uncultivated phylotypes, with one member *Fretibacterium fastidiosum* (also referred to as SGP1) isolated and characterized recently (24,25). Cluster B is considerably smaller, containing two cultivated species: *Pyramidobacter piscolens* (26) and *Jonquetella anthropi* (27).

The *Synergistetes* population appears to be more diverse in periodontitis niches than in dental caries or endodontic infections (14). The presence or (clonal) abundance of certain *Synergistetes* taxa have previously been shown to be associated with periodontal disease, and consequently certain members of this phylum may considered putative periodontopathogens (7,11,28). In a recent UK-based study, Vartoukian *et al.* (11) compared the diversity and detection frequency of *Synergistetes* OTUs within several oral sites in a cohort of subjects with periodontitis ($n = 5$) vs. subjects with good periodontal health ($n = 5$). The authors found that the richness of *Synergistetes* OTUs, and the prevalence and abundance of one taxon in particular (OTU 4.2), were associated with periodontitis. Notwithstanding these results however, we still have a very limited understanding of the genetic diversity, distributions and disease-associations of oral *Synergistetes* taxa in global populations.

To directly address these issues, we employed a 16S rRNA clone library-based approach to investigate the diversity and prevalence of members of the *Synergistetes* phylum in the subgingival plaque of Chinese subjects with periodontitis, vs. periodontitis-free controls. We found that *Synergistetes* clones were significantly more prevalent and more diverse in periodontitis subgingival plaque compared with periodontitis-free controls. The presence of certain *Synergistetes* OTUs (phylotypes) was associated with periodontitis. Our results add to the growing body of evidence supporting the putative role of members of this phylum in the pathogenesis of periodontal disease.

Material and methods

Subject recruitment, clinical examination and sampling methods

Ten periodontitis patients and 10 periodontitis-free controls were recruited for this study with informed consent. Ethical approval was granted by the Institutional Review Board of the University of Hong Kong/Hospital Authority, Hong Kong West Cluster (UW 11-154). All subjects were non-smokers or former smokers who had quit smoking for more than 6 mo. All subjects were ethnically Chinese, who lived in Hong Kong. Exclusion criteria included a history of periodontal or antimicrobial therapy within the past 6 mo, any underlying systemic medical conditions; or < 20 standing teeth. The inclusion criteria for the periodontitis group were four or more posterior teeth with periodontal pockets of $\geq$ 6 mm and clinical attachment level (CAL) of $\geq$ 4 mm, plus radiographic evidence of alveolar bone loss. The periodontitis-free subjects had no probing depths of $\geq$ 3 mm, no detectable clinical attachment loss or radiographic bone loss. Full mouth bleeding on probing (BOP) scores and probing pocket depths (PPD) were recorded to characterize the periodontal status. After scrupulous removal of supragingival plaque, subgingival plaque samples were collected from as many periodontal pockets (sites with PPD $\geq$ 5 mm in periodontitis subjects) or sulci (periodontitis-free subjects) using sterile Gracey or universal curettes, and were pooled to generate a single subgingival plaque sample for each subject ($n = 20$). The collected plaque samples were suspended in 1.0 mL phosphate buffered saline (PBS, pH 8.0), placed on ice, and processed in the laboratory within 30 min after collection.

DNA extraction, amplification, cloning and sequencing

Subgingival plaque samples were washed twice (PBS, pH 7.4, 1 mL), and DNA was extracted (Wizard Genomic DNA Purification Kit, Promega, Madison, WI, USA) following the manufacturer's protocol. DNA pellets were dissolved in 100 μL of rehydration buffer. The universal forward primer TPU1 (5′-AGAGTTTGATCMTGGCTCAG-3′) (29) and the C90 reverse primer (5′-GTTACGACTTCACCCTCCT-3′) (5,19) were used to PCR-amplify ca. 1500 bp 16S rRNA gene fragments, using the purified DNA from subgingival plaque samples as templates. PCR mixtures (50 μL) contained: (5 ×) PCR buffer (10 μL), template DNA (2 μL, ca. 50 ng), $MgCl_2$ (2.5 mM), dNTPs (0.2 mM each), TPU1 and C90 primers (0.4 μM each), LongAmp DNA polymerase (1.5 U, New England Biolabs, Ipswich, MA, USA). PCR reactions were performed using a 'touchdown' protocol on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA): initial denaturation (94°C, 480 s); then six cycles of: denaturation (94°C, 30 s), annealing (52°C, 30 s, decreasing

0.5°C every cycle), extension (65°C, 150 s); followed 23 cycles of denaturation (94°C, 20 s), annealing (49°C, 20 s), extension (65°C, 120 s); final extension (65°C, 600 s). PCR products of ca. 1500 bp were gel purified (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). Purified PCR amplicons from each subject were individually TOPO-cloned into pCR2.1 vectors (TOPO TA Cloning Kit, Invitogen, Carlsbad, CA, USA). Ligation mixtures ($n = 20$) were electroporated into *Escherichia coli* DH10B, plated onto Luria-Bertani (LB) 1% agar plates supplemented with kanamycin (50 μg/mL) and X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside, 20 μg/mL), then incubated overnight at 37°C. Plasmid DNA was purified from 45 to 60 (white) colonies from each transformant plate (QIAprep Spin Miniprep Kits, Qiagen). About 45–60 plasmids from each subject were subsequently sequenced in both directions using M13 forward and reverse primers [Beijing Genome Institute (BGI-Hong Kong Co. Ltd), Tai Po, Hong Kong].

Primer and 16S rRNA gene sequence analysis

The selectivity and coverage of the C90 reverse primer (5′-GTTACGACTT-CACCCTCCT-3′) for amplifying 16S rRNA gene sequences from members of the *Synergistetes* phylum was estimated *in silico*, using the Probe Match programme of the Ribosomal Database Project (RDP) 10 online resource (http://rdp.cme.msu.edu/) (30). The 16S rRNA gene sequence data were assembled and trimmed using CODONCODE ALIGNER 3.7.1 (Codon Code Corporation, Dedham, MA, USA). Sequences were aligned using the 16S ribosomal SILVA bacteria dataset (31) as a template, and were manually corrected before further analysis. Chimeric sequences were identified using Chimera Slayer and 24 suspicious sequences were removed. RDP Classifier was used to taxonomically-classify valid sequences to at least the level of phylum (32).

Taxonomic assignment estimates of microbial diversity

A distance matrix was generated and sequences were assigned to Operational Taxonomic Units (OTUs) at various sequence identity cut-off levels (99%, 97%, 95%) using the furthest neighbor algorithm in the Mothur software package (32). This algorithm requires that the distance between every sequence within an OTU lie within the specified identity threshold (33). Representative sequences for each *Synergistetes* OTU found in this study were deposited in the NCBI GenBank (accession numbers JX010869 to JX010946, Table S2). Rarefaction curves for OTU were calculated using Mothur.

Community comparisons

Synergistetes OTU populations in each subject were compared and visualized as a dendrogram according to the Yue and Clayton theta structural diversity measure (34). The structure of *Synergistetes* communities present in the periodontitis and periodontitis-free groups were compared using ∫-libshuff (35,36), unweighted and weighted UniFrac analysis (37,38) and Parsimony *p*-tests (39). The *p*-value threshold was set to 0.05.

Phylogenetic relationships

An alignment comprising representative 16S rRNA sequences from each identified non-*Synergistetes* OTU were used as an input to examine phylogenetic relationships. NJ trees were constructed following evolutionary distance analysis of aligned sequences, corrected by the Jukes-Cantor substitution model using MEGA 5 (40), and were supported by 100 bootstrap replicates. The absolute clonal abundance for each OTU was visualized as a heat-map using ggplot2 from the R statistical computing package (http://www.r-project.org/) (41).

Statistical analysis

Standard descriptive statistical analysis was conducted to synthesize the clinical data. Assumption tests for normality and equality of variance were performed prior to all statistical comparative analyses of datasets obtained from the two cohorts. Non-parametric Mann–Whitney *U* tests were used for all statistical tests between the two cohorts. The *p*-value threshold was set to 0.05.

Results

Clinical evaluation of periodontal health status in the two subject groups

Subjects with periodontitis who sought treatment at the Prince Philip Dental Hospital and subjects with periodontitis-free attending regular check-up were recruited for this study, with informed consent and ethical approval. There was no difference in gender distribution between periodontitis and periodontitis-free control groups; but the average age of the periodontitis-free group was slightly higher than that of the periodontitis group (Table 1). The periodontal health status of all subjects was as summarized in Table 1. The mean percentage of BOP sites for the periodontitis group was significantly higher than that for the periodontitis-free group. None of the periodontitis-free group had probing pocket depths ≥ 4 mm, radiographic evidence of bone loss or furcation involvement in multi-rooted teeth (data not shown), confirming that they had good periodontal status. All subgingival sites in the periodontitis-free group were sampled to maximize the total amount of plaque collected (Table 1). Whilst it was possible to collect subgingival plaque from every diseased periodontal pocket sampled in the periodontitis group, it was not possible to obtain detectable levels of plaque from every sulcal site in the periodontitis-free group. As may be seen in Table 1, the sampled sites from the periodontitis subjects had a significantly higher average probing depth and percentage of BOP.

Table 1. Demography and clinical profile of study subjects

Characteristics	Categories	Periodontal status: Periodontitis (n = 10)	Periodontal status: Periodontitis-free (n = 10)	Statistics[a]	p-Value
Age (Mean ± SD, year)		40.7 ± 10.6	54.0 ± 10.6	U	0.014
Gender	Male	3	3	χ^2	NS
	Female	7	7		
Full mouth periodontal profile					
Standing teeth		28.0 ± 2.2	27.5 ± 1.1	U	NS
% BOP		88.6 ± 10.3	30.4 ± 12.7	U	<0.001
% PPD = 4–5 mm		22.0 ± 8.8	0	NA	
% PPD ≥ 6 mm		15.8 ± 15.0	0	NA	
Profile of sample sites					
No. of sites sampled	Mean	42.7 ± 31.2	165.0 ± 6.5	U	<0.001
	Range	8–81	156–180		
% BOP	Mean	99.8 ± 0.5	30.4 ± 12.7	U	<0.001
	Range	98.3–100.0	11.0–48.2		
Mean PPD (mm)	Mean	6.2 ± 0.5	1.9 ± 0.6	U	<0.001
	Range	5.21–6.63	1.3–2.4		

Results are number unless otherwise indicated; BOP, bleeding on probing; NS, not significant; NA, not applicable; PPD, probing pocket depth.
[a]U: Mann–Whitney U test; χ^2: chi square test.

In silico evaluation of PCR primer amplification specificity

In combination with a 'universal' forward PCR primer [e.g. TPU1 (29)], the C90 reverse primer (5,19) has previously been shown to predominantly amplify near-full length 16S rRNA (*rrs*) gene fragments (corresponding to positions 8-1503 of the *Escherichia coli* 16S rRNA gene) from members of the *Spirochaetes* (7,19,42,43) and *Synergistetes* phyla (previously referred to as *Synergistes*/*Flexistipes*/*Deferribacteres* taxa) (19). To systematically evaluate the hybridization specificity of the C90 primer, we used the Probe match software tool to analyze its sequence homology with 16S rRNA sequences from representative *Synergistetes* OTUs. Results indicated that the C90 primer should anneal with cluster B oral *Synergistetes* seque-nces without mismatches (Table S1). The C90 primer exactly matched some cluster A oral *Synergistetes* sequences, whilst there was a single G/A nucleotide mismatch with other sequences at position 1485 (*E. coli* numbering).

Construction and analysis of 16S rRNA clone libraries

Using the TPU1 plus C90 primer set, ca. 1500 bp 16S rRNA gene fragments were successfully amplified from DNA template purified from the pooled subgingival plaque samples from each of the 20 subjects. Plasmid libraries of 'TOPO-cloned' 16S rRNA genes were separately constructed for each subject, and the PCR amplicons within ca. 45–60 plasmid clones from each subject were sequenced. After poor-quality reads and chimeras were filtered out, a total of 1030 high-quality 16S rRNA sequences were obtained. There was no significant difference in the mean number of plasmid clones obtained from the periodontitis (52 ± 8.0) and periodontitis-free control (51 ± 8.4) groups. 16S rRNA sequences were assigned a taxonomy using RDP classifier (32), with results summarized in Table 2. In terms of clonal abundance (i.e. number of plasmid clones obtained), the predominant phyla detected were *Spirochaetes* (59.7%), *Synergistetes* (15.7%), *Actinobacteria* (16.2%), *Fusobacteria* (3.6%), *Proteobacteria* and *Firmicutes* (2.4%) and *Proteobacteria* (2.3%). This indicated that whilst the TPU1 plus C90 primer set predominantly amplified 16S rRNA genes from *Spirochaetes*, they exhibited a reasonably-good selectively for *Synergistetes*. The composition of the *Spirochaetes* clones obtained will be discussed elsewhere (You *et al.*, manuscript in preparation).

There were significantly higher numbers of *Synergistetes* clones amplified from periodontitis subjects compared with periodontitis-free controls (Table 2). Of the 162 *Synergistetes* clones detected, 132 (>80%) clones were obtained from the periodontitis subject group. The mean number of *Synergistetes* clones obtained per subject was 13.2 (range: 8–21) and 3.0 (range: 0–7) from the periodontitis and periodontitis-free groups, respectively.

Compared with the periodontitis group, a relatively large proportion of clones corresponding to members of the *Actinobacteria*, and a sizable number of clones belonging to *Firmicutes*, *Fusubacteria* and *Proteobacteria* phyla were detected in the periodontitis-free group (See Table 2). However, as the TPU1 and C90 primers do not have good coverage for members of these phyla (data not shown), these findings are probably not representative of the actual populations of these bacteria present; and as such, correlations with clinical health status was not performed. As has been previously proposed (7,11,19), this may possibly reflect the low abundance of stringently-matched taxa (e.g. *Spirochaetes*, *Synergistetes*), resulting in the amplification of more abundant and less-stringently matched 16S rRNA sequences (especially *Actinobacteria* spp.).

Prevalence of *Synergistetes* taxa within the periodontitis and periodontitis-free subject groups

The *Synergistetes* clones were divided into distinct taxonomic groups, i.e. OTUs, based on the levels of shared 16S rRNA gene sequence identity. The prevalence of OTUs in both subject groups were analyzed using three different sequence identity cut-off values: 99, 97 and 95% (Table 3). Rarefaction curves were plotted using this data to estimate the OTU richness within the two subject groups at each of these levels (see Figure S1). Graphs were plotted based on four different

Table 2. Absolute and relative clonal abundance at the phylum level

Phylum	Periodontitis (%)	Periodontitis-free (%)	Total (%)	*p*-Value[a]
Spirochaetes	365 (70.2)	250 (49.0)	615 (59.7)	<0.05
Synergistetes	132 (25.4)	30 (5.9)	162 (15.7)	<0.001
Actinobacteria	20 (3.8)	147 (28.8)	167 (16.2)	<0.05
Firmicutes	NA	25 (4.9)	25 (2.4)	<0.05
Fusobacteria	3 (0.6)	34 (6.7)	37 (3.6)	NS
Proteobacteria	NA	24 (4.7)	24 (2.3)	<0.05
Total	520 (100)	510 (100)	1030 (100)	NS

NA, not applicable; NS, not significant.
[a]Mann–Whitney *U* test.

parameters: the numbers of subjects sampled (Panel A); as well as the total number of clones sampled from both groups (Panel B), the periodontitis-free group only (Panel C) or the periodontitis group only (Panel D). In all four panels it may be seen that all the rarefaction curves plotted using OTU composition based on a 97% or 95% sequence identity cut-off level plateaued-out at low OTU richness values, whilst curves plotted used a 99% sequence identity cut-off did not plateau over the range shown. When rarefaction curves were respectively plotted for the number of OTUs observed vs. number of clones sequenced for the two cohorts using a 99% cut-off value, only the curve for the periodontitis-free group appeared to reach an asymptote. This result was consistent with the Chao 1 diversity estimates described below.

Using a 99% 16S rRNA sequence identity cut-off, a total of 31 *Synergistetes* OTUs were present in the overall dataset (Table 3). These OTUs were given alphanumeric names reflecting their origin (1P-10P for the 10 periodontitis subjects, 1H-10H for the periodontitis-free subjects). It should be noted that some 'P' OTUs were detectable in both periodontitis and periodontitis-free subjects; with the name indicating the subject group from which the representative clones were first obtained. Substantially more OTUs were detected in the periodontitis group ($n = 29$), compared with the periodontitis-free controls ($n = 8$). Twenty three OTUs were detected exclusively in the periodontitis group, six OTUs were common to both groups (5H23, 1P28, 7P1, 4P26, 8H6, 4P12), whilst only two OTUs (3H20, 5H10) was found exclusively in the periodontitis-free group (also see Fig. 1). The mean number of *Synergistetes* OTUs detected per subject was 6.7 (SD ± 3.0; range: 3–13) in the periodontitis group and 1.7 (SD ± 1.4; range: 0–4) in the periodontitis-free group ($p < 0.001$, Mann–Whitney *U* test). The total estimated number of *Synergistetes* OTUs (as calculated using a Chao 1 index) was 53.8; with a projected 47.5 and 8.5 OTUs present in the periodontitis and periodontitis-free groups, respectively.

The prevalence of the 31 *Synergistetes* OTUs within the subjects, and their clonal abundance (i.e. absolute numbers of plasmids encoding 16S rRNA genes that correspond to a specific OTU) were represented using a grey-scale heatmap (Fig. 1). The three most widely distributed OTUs were 4P26 (detected in 10 subjects), 7P1 (nine subjects) and 2P9 (seven subjects). Sixteen OTUs were present in two or more subjects, whilst 15 OTUs were only found in one subject. However, it should be noted that individual OTUs defined using a 99% sequence identity cut-off may comprise several closely-related *Synergistetes* 16S rRNA sequences. Non-parametric Mann–Whitney *U* tests revealed that five *Synergistetes* OTUs were detected with a significantly higher frequency in the periodontitis group, compared with the periodontitis-free group. These were OTUs 2P9 ($p < 0.01$; analogous to *Synergistetes* OTU 2 clone 7.1 (25)); 4P12 ($p < 0.01$; analogous to *F. fastidiosim* SGIP^T (1435/1440 nt, 99.7%; previously known as *Synergistetes* SGP1) (24,25) and the '*Deferribacteres*' oral clone W090 clone (1434/1440 nt, 99.6% identity) (17)); 7P1 ($p < 0.01$, analogous to '*Deferribacteres*' oral clone BH007 (1433/1434 nt, 99.9% identity) (7)); 7P22 ($p < 0.05$) and 6P18 ($p < 0.05$); also discussed below.

Table 3. The number of observed *Synergistetes* OTUs

Level	Periodontitis	Periodontitis-free	Total
Unique sequences	119	24	143
OTU (99%)	29	8	31
OTU (97%)	8	3	8
OTU (95%)	5	2	5

Phylogenetic relationships between *Synergistetes* OTUs

Phylogenetic relationships between the 31 *Synergistetes* OTUs identified in the clinical samples were analyzed using a Neighbor-joining (NJ) approach. The overall topology of the NJ phylogenetic tree generated was generally well-supported with high bootstrap values (Fig. 1). It may be seen that the OTUs belonged to various well-separated genetic lineages that diverged at an early point in their evolutionary history. Several OTUs appeared to be quite genetically distinct (e.g. 6P18, 2P9, 3P46), whilst others were part of 'branches' containing two or three OTUs that shared reasonably high levels (ca. 98.5–99.5%) of 16S rRNA gene sequence similarity (e.g. 4P36, 4P12, 3P2).

Figure 2 shows the phylogenetic relationships between the 31 OTUs detected in the subjects analyzed here and the three named *Synergistetes* species previously isolated from the oral cavity, as well as the corresponding *Synergistetes* Human Oral Taxa (HOT) defined in the Human Oral Microbiome Database (HOMD) (5) 23 of the 31 OTUs identified here could be assigned to an existing *Synergistetes* HOT (using a 98.5% sequence identity cut-off). Eight OTUs (6P48, 4P10, 5P11, 8P65, 4P40, 3H20, 4P23, 7P51) did not correspond to an existing *Synergistetes* HOT. We did not detect any

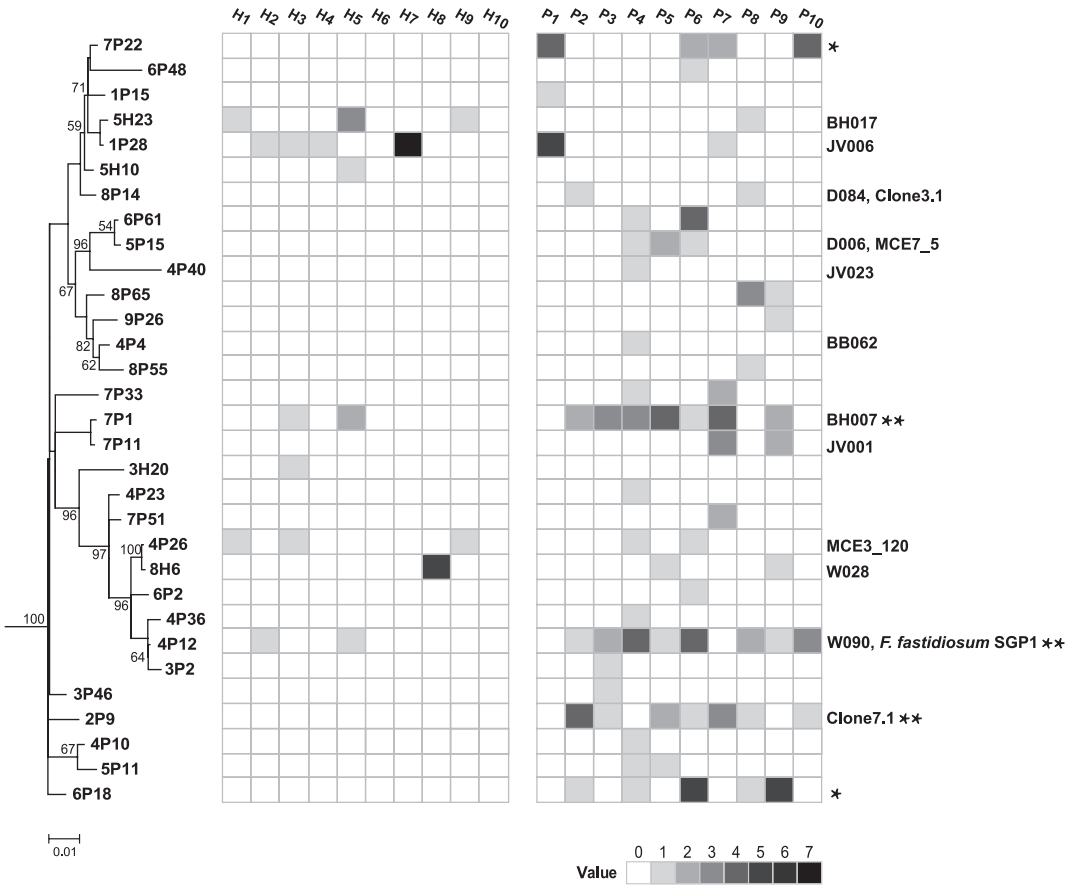

Fig. 1. Phylogenetic relationships and clonal abundance of *Synergistetes* OTUs identified in this study. Left hand side: NJ phylogenetic tree composed of the *Synergistetes* OTUs identified in this study. Bootstrap values $\geq$ 50% are shown beside branch points. Right hand side: The absolute clonal abundance of the *Synergistetes* OTUs detected in each subject is displayed using a heat map with grey-scale boxes; with corresponding values indicated in the key shown near the base of the figure. The names of previously reported oral *Synergistetes* clones or isolates that correspond to the OTUs defined here are indicated. The statistical significance of the differences in clonal abundances of specific OTUs between the periodontitis (P) and periodontitis-free (H) subject groups are indicated (*$p < 0.05$, **$p < 0.01$, Mann–Whitney *U* test).

clones or isolates corresponding to oral cluster B *Synergistetes* within either of our subject groups.

Community-based analysis of *Synergistetes* OTUs detected in subjects

A hierarchical OTU-based comparison analysis was performed to determine whether the two subject groups contained similar or disparate communities of *Synergistetes* OTUs (99% cut-off). As may be seen in the dendrogram shown in Fig. 3, based on their resident populations of identified OTUs, the subjects formed two quite distinct clusters that correlated closely to their periodontal health status. The only notable outlier was periodontitis subject P1, whose resident *Synergistetes* clones were similar to those present in healthy subjects H7 and H4. ∫-libshuff, unweighted and weighted UniFrac and Parsimony *p*-tests were all used to systematically compare the differences in communities of *Synergistetes* OTUs present in the periodontitis and periodontitis-free subject sets. All these hypothesis tests (that employ different algorithms) indicated that the populations within the two groups were significantly different, with overall *p*-values of < 0.001 obtained.

Discussion

In this study, we used a 16S rRNA gene-based approach to systematically analyze and compare members of the *Synergistetes* phylum that were present in pooled subgingival plaque sampled from periodontal pockets of 10 periodontitis patients, vs. pooled subgingival plaque collected from the sulci of an equal number of periodontitis-free controls. Of the 162 *Synergistetes* clones identified, significantly more clones were obtained from the periodontitis group ($n = 132$, 81.5%) than the periodontitis-free group ($n = 30$, 18.5%; $p < 0.001$), consistent with the hypothesis that certain members are 'periodontopathogens'.

Subgingival niches in subjects with periodontal or endodontic infections are populated with anaerobic microbial biofilm communities that are able to produce peptides and free amino

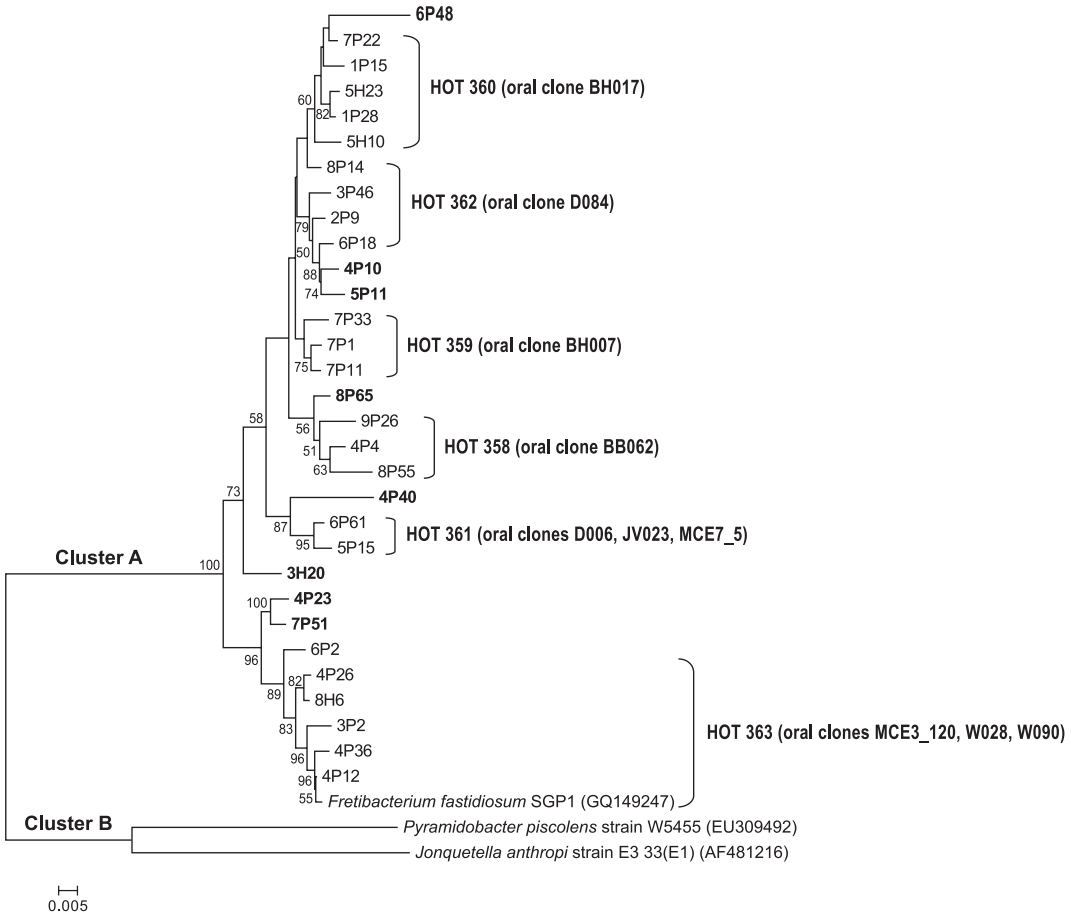

Fig. 2. Phylogenetic relationships between *Synergistetes* OTUs detected here and previously identified species of oral *Synergistetes* species and Human Oral Taxon (HOT) numbers listed in the Human Oral Microbiome Database (HOMD). The phylogenetic tree was constructed from corresponding 16S rRNA gene sequences using a Neighbour-joining method (Jukes-Cantor model), and was rooted against *Treponema socranskii* subsp. *socranskii* ATCC 35536. Bootstrap values > 50% are shown beside the branch points. *Synergistetes* OTUs were assigned to HOTs using a 98.5% sequence identity cut-off value. The eight OTUs that do not correspond to existing *Synergistetes* HOTs are indicated in bold text.

acids through the proteolytic degradation of host tissues (44). Such niches appear to be ideal environments for members of the *Synergistetes* taxa, which are proposed to acquire nutrients predominantly via amino acid metabolism (14). Correspondingly, a significant number of *Synergistetes* taxa have been detected or isolated from diseased periodontal sites (7,10,17,20,45,46) and endodontic infections (19,21,47–51); as well as dental caries lesions (52). Using a similar 16S rRNA clone-based approach, it has previously been shown that the *Synergistetes* levels detected in subgingival plaque was associated with periodontal health status; constituting ca. 1.5% of all clones obtained from periodontal pockets, but only 0.1% of clones from periodontally-healthy sites (10). Although *Synergistetes* have been frequently identified in periodontitis subjects, several studies targeting periodontal healthy subjects have failed to detected taxa belonging to the *Synergistetes* phylum (53,54). In our cohort, *Synergistetes* could be detected, albeit at low frequencies, in 8/10 periodontitis-free subjects, and in all periodontitis subjects. In agreement with previous reports (6), our results support the hypothesis that *Synergistetes* are typically present in 'healthy' human oral microbiota at low levels.

Taxa within the *Synergistetes* phylum share < 75% 16S rRNA sequence identity with other phyla. *Synergistetes* taxa that have previously been (co)-detected within specific niches; such as in animal hosts, soil samples, or human subgingival plaque generally share only ca. 80% 16S rRNA sequence similarity (12,16). *Synergistetes* thus far identified within the oral cavity form two major clusters: A and B, with the majority of taxa belonging to cluster A (14,25). To date, only one species of cluster A oral *Synergistetes* has been isolated and characterized: *Fretibacterium fastidiosum* (24), which was previously referred to as the SGP1 clone (25). The smaller cluster B includes two cultivated species: *Pyramidobacter piscolens*, with several strains isolated from odontogenic abscesses, inflamed gingival crevices or periodontal pockets; and *Jonquetella anthropi,* with

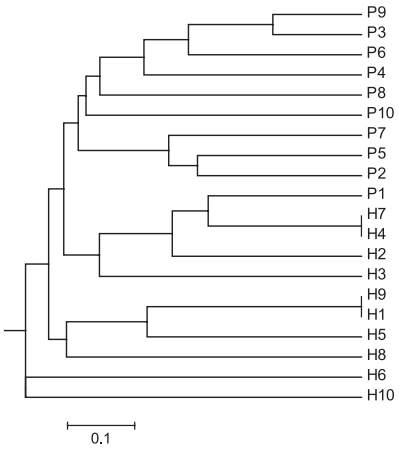

Fig. 3. Similarities in the composition of *Synergistetes* OTUs detected in the 20 subjects (H1-H10, P1-P10) included in the study. The dendrogram indicates the similarity of *Synergistetes* communities detected in each subject according to the structure-based Yue and Clayton theta coefficient, using a 99% 16S rRNA sequence identity cut-off for defining OTUs. P: Periodontitis, H: Periodontitis-free (control) subject groups.

strain E3_33 isolated from an endodontic infection (14,26,27).

Synergistetes populations within periodontitis subgingival plaque were previously reported to be more diverse than those in endodontic infections or dental caries, and certain taxa were identified exclusively in periodontal sites (14). Using a '*Synergistetes* specific' primer set that amplified a ca. 800 bp fragment of the 16S rRNA gene, Vartoukian *et al.* (11) detected 12 *Synergistetes* OTUs (using a 99% cut-off value) in plaque sampled from the periodontal environment in five healthy and five periodontitis subjects. Here, we used the '*Synergistetes* selective' TPU1 + C90 primers to PCR-amplify near full-length 16S rRNA fragments (ca. 1500 bp), enabling us to identify 31 *Synergistetes* OTUs. Twelve of the *Synergistetes* OTUs in our dataset corresponded to known cluster A members, and 19 corresponded to previously undescribed OTUs. Analogous to Vartoukian *et al.* (11), we defined the *Synergistetes* OTUs using a 99% sequence identity cut-off. Whilst this OTU definition gives higher levels of taxonomic resolution, it may over-estimate the numbers of 'species-level' phylotypes present. We detected 16S rRNA sequences belonging to OTUs that corresponded to all 15 of the previously-identified oral *Synergistetes* cluster A taxa listed in Table S1, even though many of these putatively encode a G/A single nucleotide mismatch with the C90 reverse primer. This indicated that we were able to sample a broad range of oral *Synergistetes* taxa using our approach. It should be noted however, that the recently-reported M98 *Spirochaetes* and *Synergistetes* selective reverse primer (5) contains a degenerate Y (C/T) residue in place of a T residue which is present in the C90 primer, and should theoretically have a better range of coverage.

Consistent with previous reports (11), we did not detect any oral cluster B taxa in our subject groups. Other molecular analyses of oral (especially periodontitis) microbiota have similarly noted that the vast majority of *Synergistetes* taxa (previously referred to as *Deferibacterres*/*Flexistipes*/*Synergistes*) corresponded to cluster A, with relatively few cluster B taxa detected (7,10,17,45). Sequence analysis indicated that the C90 reverse primer can perfectly complement all previously-identified cluster B OTUs, indicating that they should be detected if present at levels comparable to those of the cluster A taxa (see Table S1). Consequently, it is not possible to state whether oral cluster B *Synergistetes* are absent, or are present at very low levels within our subject set.

The communities of *Synergistetes* were significantly more OTU-rich in the periodontitis subjects (mean: 6.7 OTUs, range: 3–13) compared with the periodontitis-free subjects (mean: 1.7, range: 0–4). This is in good agreement with the results of Vartoukian *et al.* (11), who noted that periodontitis cases (mean: 7.8 OTUs, range: 7–10) also had significantly higher OTU-richness than controls (mean, 3.4, range 0–6). These authors also noted that certain individuals could be 'dominated' with a specific *Synergistetes* OTU (up to 64% relative clonal abundance), whilst others contained a greater OTU diversity with a fairly even prevalence. Similarly, in our data, control subject H7 contained 7 clones corresponding to a single OTU (1P28), whilst periodontitis subject P6 harbored 13 different OTUs, which were each present on 1–6 plasmid clones.

In their landmark (US-based) study published in 2001, Paster and co-workers identified *Synergistetes* clones BH017, D084, W028 and W090 as putative periodontal pathogens on the basis of their prevalence in diseased subjects, and absence in healthy subjects (7). Using primer sets specific for clones BH017/D084 and clone W090, Kumar and co-workers subsequently found that the BH017 and D084 clones, but not W090, were associated with chronic periodontitis in a US cohort (17). Based on results from a 16S rRNA clone-based investigation, Kumar *et al.* (10) later found that the occurrence and prevalence of *Synergistetes* clones BH007 and W090, but not BH017, were strongly associated with periodontal disease. In a UK-based study, Vartoukian *et al.* (11) subsequently found that a previously undescribed *Synergistetes* taxon (OTU 4.2) was significantly associated with periodontitis; whilst other phylotypes including: BH007, W090 and W028 had no significant correlation with disease. Notably, we did not detect a sequence corresponding to OTU 4.2 within our cohort. Therefore, despite consistent results linking members of the *Synergistetes* phylum with (chronic) periodontitis, the association between specific *Synergistetes* clones and periodontal disease remains somewhat inconclusive.

Here, we found that the increased prevalence of five *Synergistetes* OTUs were associated with periodontitis: 7P1, 2P9 and 4P12 ($p < 0.01$); and 7P22 and 6P18 ($p < 0.05$). Whilst OTUs 7P22 and 6P18 are novel; OTU 7P1 corresponds to clone BH007 (10); 2P9 corresponds to clone 7.1 (25). OTU 4P12 shares > 99.6% sequence identity with clone W090 (7,10) and *F. fastidiuosum* SGIP^T (24,25), which may both be designated as Human Oral Taxon 363 (*Synergistetes* [G-3] sp.) according to the Human Oral

Microbiome Database (HOMD) classification scheme (5). Although our understanding of taxonomy in the *Synergistetes* phylum is rudimentary, it seems probable that both the 4P12 and W090 clones correspond to *F. fastidiosum* strains. Our results suggest that certain *Synergistetes* taxa belonging to cluster A may be widely distributed in global populations, as they have now been detected in the oral cavities of subjects residing in the UK, US and China. Whilst our finding that 4P12 and 7P1 OTUs are strongly associated with periodontitis is consistent with two previous studies performed in the US (7,10), they are not consistent with results from two other studies performed in the UK (11) and US (17). This highlights the fact that the phylogeny, global distributions and disease-associations of oral *Synergistetes* taxa require further detailed investigation.

In brief conclusion, there was a greater diversity and clonal abundance of OTUs belonging to cluster A oral *Synergistetes* detected in subgingival plaque from periodontitis patients, compared to periodontitis-free controls. Five *Synergistetes* OTUs were found predominantly or exclusively in the periodontitis group. Our results are consistent with the hypothesis that members of the phylum *Synergistetes* play a role in the occurrence or pathogenesis of periodontal disease.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rarefaction curves for *Synergistetes* OTUs defined using 99% 97% and 95% 16S rRNA sequence similarity cut-off values.

Table S1 Primer match results for C90 reverse primer to representative oral *Synergistetes taxa*.

Table S2 *Synergistetes* OTU taxonomy and related sequences in the NCBI GenBank.

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