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Microbial complexes in supragingival plaque

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Background/aims: To examine microbial communities in supragingival biofilm samples.
Methods: Supragingival plaque samples were taken from 187 subjects at baseline (n = 4745). Fifty-five subjects provided supragingival plaque samples at 1–7 days after professional tooth cleaning (n = 1456); 93 subjects provided 8044 samples between 3 and 24 months post-therapy. All samples were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. Microbial associations among species were sought using cluster analysis and community ordination techniques for the three groups separately.
Results: Six complexes were formed for the baseline samples. Similar complexes were formed for the samples taken 3–24 months post-therapy. However, distinct changes were observed in microbial communities in samples taken during the 7 days of plaque redevelopment. The complexes related to clinical parameters of periodontal disease.
Conclusion: There were specific microbial complexes in supragingival plaque that were similar to those found in subgingival plaque samples with a few minor differences. The relation of previously unclustered taxa to the complexes was also described.

Key words: bacteria; ecology; microbiology; periodontal; supragingival plaque

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In 1998, we published a paper describing microbial associations among bacterial species in subgingival biofilms (7). Examination of over 13,000 subgingival plaque samples, taken from both periodontally healthy subjects and patients with chronic periodontitis, using cluster analysis and community ordination techniques revealed specific associations among bacterial species in the subgingival biofilm as well as relationships among the different microbial groups or complexes detected. The paper was well received because it 'simplified' description of the subgingival microbiota from multiple individual species to complexes of bacteria that had specific relationships to periodontal health or disease. For example, the 'red complex', comprising *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, has become recognized as a 'disease-related' complex by many.

Similar analyses were not performed on supragingival plaque samples in 1998, largely because we had too few samples to provide meaningful and robust data. However, since that time, supragingival plaque samples have been collected from a large number of subjects participating in different clinical studies at The Forsyth Institute. The purpose of the present investigation was to examine relationships among bacterial species in supragingival plaque samples using cluster analysis and community ordination techniques.

Materials and methods

Subject population

A total of 187 subjects, ranging in age from 22 to 74 years, who were considered to be periodontally healthy (n = 38) or who had evidence of prior attachment loss (n = 149) were selected for study. Fifty-five of the subjects (38 periodontally healthy and 17 with chronic periodontitis) were part of a study examining short-term plaque redevelopment, while 132 patients with chronic periodontitis were part of a study to examine the effects of different periodontal therapies. Of these subjects, 93 provided post-therapy microbiological data. All subjects had at least 20 teeth. Exclusion criteria included pregnancy, periodontal therapy or antibiotics in the previous 3 months, any systemic condition which might have affected the progression or treatment of periodontitis, and the need for premedication for monitoring or therapy. No subject with localized juvenile periodontitis, rapidly progressive periodontitis, or acute necrotizing ulcerative gingivitis was included in the study. The studies in which the subjects were participating had been approved by The Forsyth Institute Institutional Review Board.

Table 1. Clinical characteristics of the 187 subjects at baseline

	Mean ± SD	Range
Age	46.8 ± 11.9	22–74
% male subjects	47	
% current smokers	17	
% of sites with:		
Plaque	73 ± 24	4–100
Gingival redness	68 ± 30	0–100
Bleeding on probing	34 ± 24	0–96
Suppuration	1 ± 3	0–30
Mean pocket depth (mm)	3.32 ± 0.84	1.68–7.35
Mean attachment level (mm)	3.20 ± 1.20	0.86–7.04

Clinical monitoring

Subjects were screened for suitability and, if accepted, they provided signed informed consent. All subjects were clinically monitored at baseline. Plaque accumulation (0/1), overt gingivitis (0/1), bleeding on probing (0/1), suppuration (0/1), probing pocket depth, and probing attachment level were measured at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) at all teeth excluding the third molars at each visit. The baseline clinical features of the 187 subjects are presented in Table 1.

Experimental design

All 187 subjects provided full-mouth supragingival plaque samples at baseline, before any professional oral hygiene procedures, giving a total of 4745 samples. These baseline samples represented a 'mature' supragingival biofilm of unknown age. Fifty-five subjects provided full-mouth supragingival plaque samples at 1, 2, 4, and 7 days of plaque redevelopment after a thorough professional tooth cleaning. The samples from these subjects were considered to be short-term biofilm redevelopment samples and were analyzed as a second group. Ninety-three periodontitis subjects had received periodontal therapy consisting of scaling and root planing alone or combined with weekly professional supragingival plaque removal for 3 months with or without a 2-week course of systemically administered metronidazole. The 3–24 months post-therapy samples from these subjects (a total of 325 subject visits) represented long-term plaque redevelopment and were collected before maintenance scaling and root planing.

Microbiological assessment

Supragingival biofilm samples were taken using individual sterile Gracey curettes from the mesial surface of each tooth (excluding third molars) and placed into separate Eppendorf tubes containing 0.15 ml Tris–EDTA buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid, pH 7.6). Then, 0.10 ml of 0.5 M NaOH was added immediately to each sample. Each sample was evaluated for its content of 40 bacterial species using checkerboard DNA–DNA hybridization (9, 11). In brief, the samples were lyzed and the DNA was placed in lanes on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 bacterial taxa were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using antibody to digoxigenin, conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL) and were read using a STORM FLUORIMAGER (Molecular Dynamics, Sunnyvale, CA), a computer-linked instrument that reads the intensity of the fluorescence signals resulting from the probe–target hybridization. Two lanes in each run contained standards at concentrations of 10^5 and 10^6 cells of each species. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. Signals were evaluated using the STORM FLUORIMAGER and converted to absolute counts by comparison with standards on the same membrane. Failure to detect a signal was recorded as zero.

Data analysis

Microbiological data available for each subject included the level of each of 40 test species from up to 28 plaque samples at each visit. A total of 4745 supragingival plaque samples (mean 25.4 per subject) were evaluated at baseline, 1456 supragingival samples (mean 26.5 per subject) for the short-term plaque redevelopment phase and 8044 supragingival samples (mean 24.8 per subject per visit) for the long-term plaque redevelopment phase. Two-by-two tables were set up for each of the 780 pairs of species. The data in these tables were used to generate similarity coefficients between each species pair using nine similarity coefficients (3) including: Bray Curtis, chord, Gower, Canberra, Sigleo, Euclidean distance, and Mahalanobis d^2 correlation coefficients as well as the phi coefficient based on the presence or absence of counts $>10^5$ in the samples. The resulting similarities were clustered using an average unweighted linkage sort (5), providing nine individual dendrograms of microbial associations.

Community ordination was performed using principal components analysis and correspondence analysis (3). These analyses reduced the dimensionality of the data set that consisted of the counts of 40 species in each of 4745 supragingival biofilm samples. Since one cannot envision data in 40 dimensional space, the dimensionality was reduced by the analyses to provide two- and three-dimensional plots in which species that were frequently encountered together were in close proximity in the plots. The data were plotted as described by Ludwig & Reynolds (3).

All of the above analyses were performed separately for the three data sets described above; i.e. the baseline data from 187 subjects representing 'mature' plaque, the short-term plaque redevelopment data from 55 subjects and the long-term plaque redevelopment data from 93 treated periodontitis subjects. The consensus microbial communities depicted in Figs 1 and 4 were based on a composite of the nine dendrograms and the two- and three-dimensional representations of the principal component and correspondence analyses.

The relationship between the clinical parameters and individual species within complexes was examined after averaging data for a chosen clinical parameter within the subject and then averaging across subjects. For example, the counts of each species were averaged for all non-bleeding-on-probing sites and separately for all bleeding-on-probing sites in a subject before averaging within bleeding-on-probing categories across subjects. In a similar fashion, mean species levels were

compared at pockets with depths <4, 4–6, and >6 mm and at sites in attachment level categories of <2, 2–4, and >4 mm. Sites were also subset into those that exhibited recession; i.e. attachment level measurement greater than the pocket depth measurement, and those that did not exhibit recession; i.e. pocket depth equal to or greater than attachment level. Significance of differences for species counts subset according to clinical parameters was sought using analysis of variance (ANOVA) and adjusted for 40 comparisons (8).

Results

Associations among bacterial species in 'mature' supragingival biofilm samples

Figure 1 presents a diagram of the relationships among 40 bacterial species detected in 4475 supragingival biofilm samples from 187 subjects at baseline. The figure is based on cluster analyses that employed nine similarity coefficients described in the Materials and methods section as well as two community ordination techniques: principal components analysis and correspondence analysis. Figure 2 is an example of one dendrogram based on the count data using the Bray

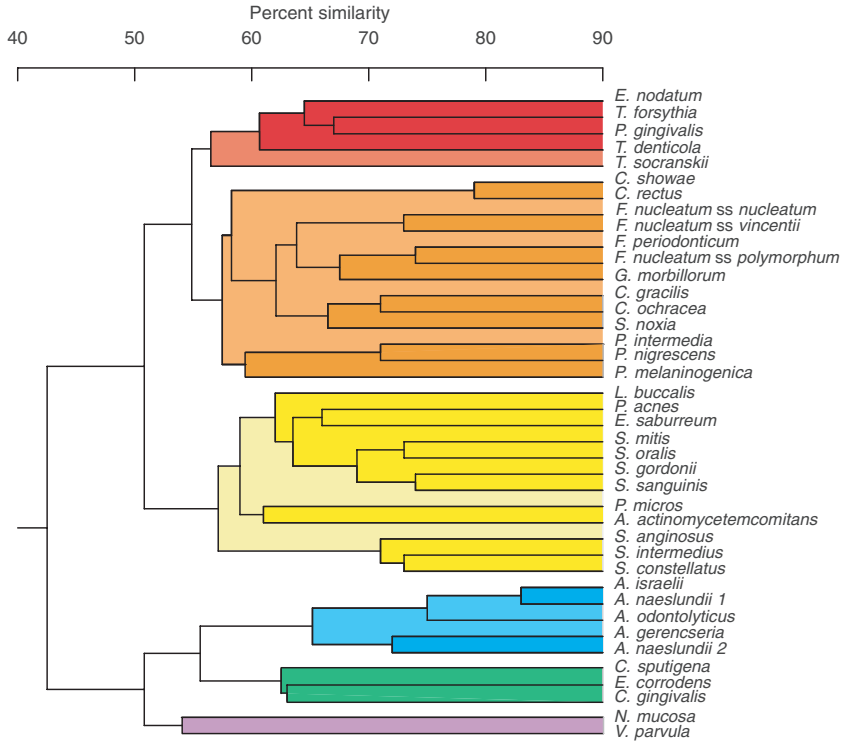

Fig. 2. Dendrogram of a cluster analysis of 40 subgingival taxa using the Bray Curtis coefficient and an average unweighted linkage sort. The data employed were the counts of the 40 taxa in supragingival plaque samples taken at baseline in 187 subjects. Cluster groups have been colored according to the clusters depicted in Fig. 1.

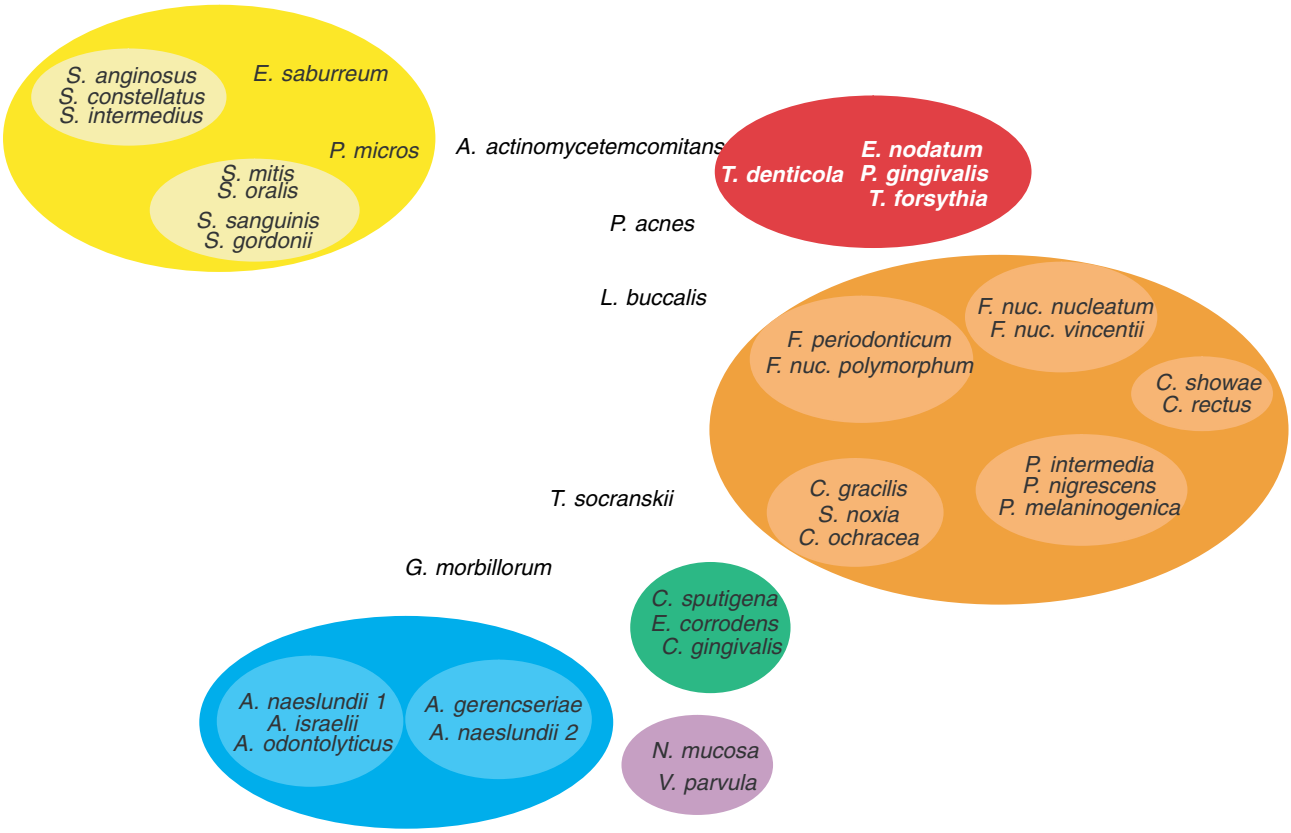

Fig. 1. Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes in supragingival biofilm samples. This diagram was based on the results of nine cluster and two community ordination analyses using the baseline data from 187 subjects.

Curtis similarity coefficient and an average unweighted linkage sort. The colors used in the dendrogram correspond to the final groupings in Fig. 1. As may be seen in Fig. 2, a red complex community was formed that contained the three species previously identified as the red complex in subgingival plaque, *T. forsythia*, *P. gingivalis*, and *T. denticola*. *Eubacterium nodatum* was also part of this complex and *Treponema socranskii* was loosely associated with these four species. A number of species previously identified in subgingival plaque as orange complex species were also detected as part of an orange complex in supragingival plaque. These included *Campylobacter showae*, *Campylobacter rectus*, *Fusobacterium nucleatum* subsp. *nucleatum*, *F. n.* subsp. *vincentii*, *Fusobacterium periodonticum*, *F. n.* subsp. *polymorphum*, *Campylobacter gracilis*, *Prevotella intermedia*, and *Prevotella nigrescens*. These taxa were joined by *Gemella morbillorum*, *Capnocytophaga ochracea*, *Selenomonas noxia*, and *Prevotella melaninogenica*. A yellow complex was formed primarily of the *Streptococcus* species *S. mitis*, *S. oralis*, *S. gordonii*, *S. sanguinis* and, somewhat separately, *S. anginosus*, *S. intermedius*, and *S. constellatus*. These species were joined by *Leptotrichia buccalis*, *Propionibacterium acnes*, *Eubacterium saburreum*, *Peptostreptococcus micros*, and *Aggregatibacter actinomycetemcomitans*. A tight cluster of *Actinomyces* species was formed including *A. israelii*, *A. naeslundii* 1, *A. odontolyticus*, *A. gerencseriae*, and *A. naeslundii* 2. A green complex consisting of *Capnocytophaga sputigena*, *Eikenella corrodens*, and *Capnocytophaga gingivalis* was formed as well as a loose purple complex consisting of *Neisseria mucosa* and *Veillonella parvula*. There were minor differences in the clusters formed when using the eight other similarity coefficients; however, the general features of the clusters were virtually identical.

Community ordination was used to not only describe relationships between pairs of species but to examine relationships among the different clusters. Figure 3 (left panel) presents a correspondence analysis of the relationships among the 40 species examined in the supragingival plaque samples. This panel presents the first and second axes and Fig. 3 (right panel) is a representation of the sample analyses providing the first, second, and third axes. The figure demonstrates that the *Actinomyces* complex and the yellow complex formed tight cluster groups that were quite separate from each other. The red complex species appeared to be somewhat associated with both the yellow and orange complexes, while the green complex was associated with the *Actinomyces* and with species in the orange complex, particularly *C. gracilis*, *S. noxia*, and *C. ochracea.* Some of the taxa in Fig. 1, such as *A. actinomycetemcomitans*, *P. acnes*, and *L. buccalis*, moved in and out of the yellow complex depending on the analysis employed and thus were not placed in the yellow cluster. *T. socranskii* and *G. morbillorum* also appeared to relate to different clusters in different analyses and thus were placed between clusters.

Associations among bacterial species in supragingival biofilm samples during long-term redevelopment

Ninety-three of the subjects were part of a longitudinal study to examine the clinical and microbial effects of different periodontal therapies. These subjects pro-

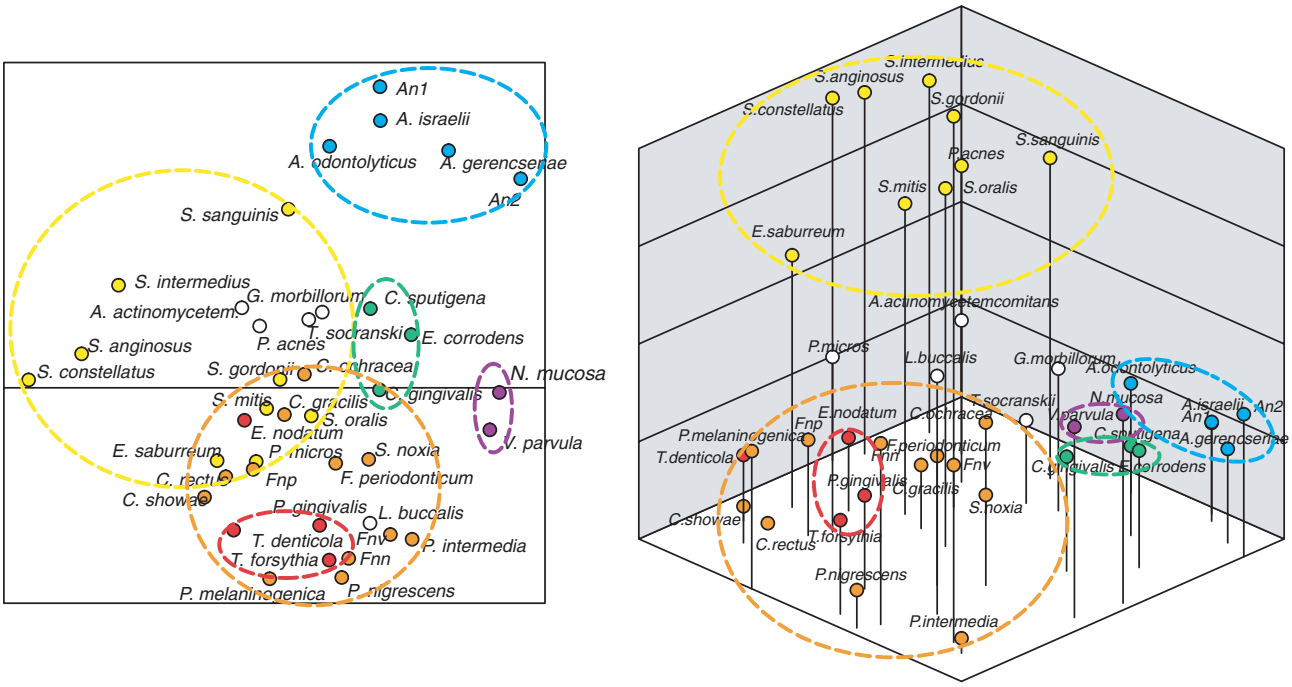

Fig. 3. Community ordination of 40 bacterial species in supragingival plaque samples from 187 subjects at baseline using correspondence analysis. The relationships among species were evaluated using the levels of the species at each of the sampled sites. This reduction in dimensionality was carried out using correspondence analysis (3). The left panel presents the two major components of the analysis as a two-dimensional plot with the species plotted along the first component (*x*-axis) and the second component (*y*-axis). Species that are in close proximity in the plot tend to frequently cohabit the plaque samples, while distant species are less commonly found together. The right panel presents a three-dimensional plot presenting species locations for the first, second, and third components, which are represented by the *x*, *y*, and *z* axes, respectively. The first, second, and third components accounted for 29.1, 8.1, and 7.8% of the variability, respectively. The color-coding is as shown in Fig. 1. The white circles represent species that were not part of any complex.

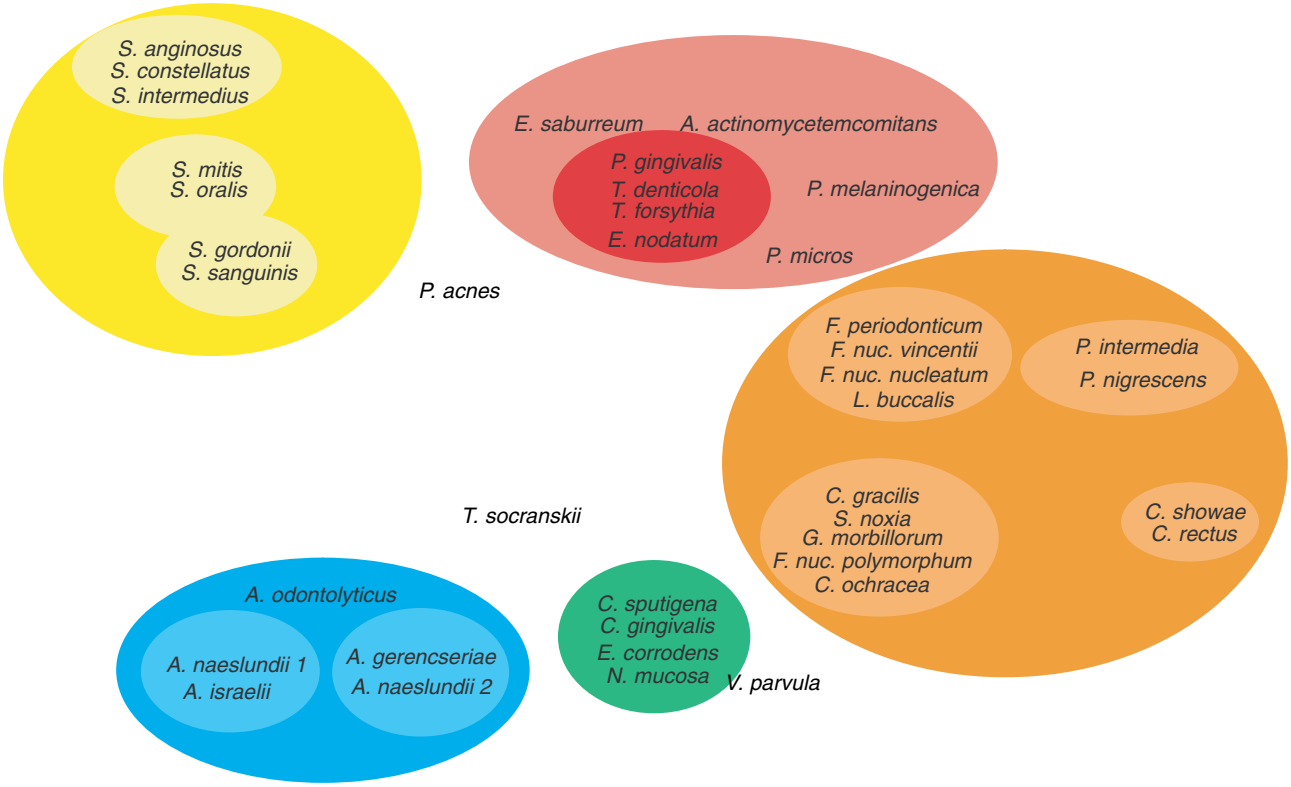

Fig. 4. Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes in supragingival biofilm samples. This diagram was based on the results of nine cluster and two community ordination analyses using the long-term plaque redevelopment data from 93 subjects with post-therapy microbiological data from 3–24 months.

vided supragingival plaque samples at monitoring visits from 3 to 24 months post-therapy. The analyses that were performed for the mature plaque samples were repeated for this set of 8044 samples from 325 subject visits. The relationships among the 40 bacterial species are depicted in Fig. 4 and are based on nine cluster analyses and two community ordination analyses. The complexes formed were similar to those described for 'mature' plaque in Fig. 1. The *Actinomyces* group was essentially identical. The green complex that consisted of *C. sputigena*, *C. gingivalis*, and *E. corrodens* was joined by *N. mucosa* and a loosely related *V. parvula*. The orange complex was quite similar to that in Fig. 1, having four subsets of closely related taxa. The major differences from Fig. 1 were the addition of *G. morbillorum* and *L. buccalis* and the movement of *P. melaninogenica* to a loose relationship with the red complex. The red complex was identical to that presented in Fig. 1 except that *E. saburreum* and *P. micros*, both previously in the yellow complex, as well as *A. actinomycetemcomitans*, and *P. melaninogenica* were loosely related to this complex. The purple complex was integrated into the green complex of Fig. 1. Figure 5 presents the two- and three-dimensional representations of the correspondence analysis performed on this data set. The relationships depicted in this figure are, in large part, consistent with the community structure depicted in Fig. 4. The colors in Fig. 5 are based on those presented in Fig. 4.

Associations among bacterial species in supragingival biofilm samples during short-term redevelopment

To determine the immediate effect of a 'disruptive' intervention (supragingival plaque removal) on the community structure of the biofilm, the relationships among bacterial species were examined using the same cluster analyses and community ordination techniques used for the mature and long-term redevelopment assessments. Fifty-five subjects received a thorough tooth cleaning. These subjects were asked not to perform home care procedures and were sampled at 1, 2, 4, and 7 days post-cleaning, providing a total of 1456 supragingival samples. A cluster analysis dendrogram using the Bray Curtis coefficient and an average unweighted linkage sort is presented in Fig. 6, while Fig. 7 presents the results of the correspondence analysis. The species are colored according to the communities depicted in Fig. 1. The data indicate that some of the relationships between and among species were similar to those seen in mature and long-term post-therapy biofilms. For example, the green complex and the associated purple complex remained relatively unchanged. In addition, certain pairs of species were still very closely associated, such as *S. mitis* and *S. oralis*, *A. israelii* and *A. gerencseriae*, and *A. odontolyticus* with *A. naeslundii* 1. However, the tight *Actinomyces* and yellow complexes depicted in Figs 1 and 4 were fragmented and the orange and red complexes were dispersed and intermingled with taxa of other complexes.

Relationship between bacterial species and clinical parameters

The relationship of the mean counts of the 40 test species with gingival redness or bleeding on probing is presented in Fig. 8. While the mean counts for all species were higher in samples from the sites that bled on probing or exhibited gingival redness, statistically significant differences were

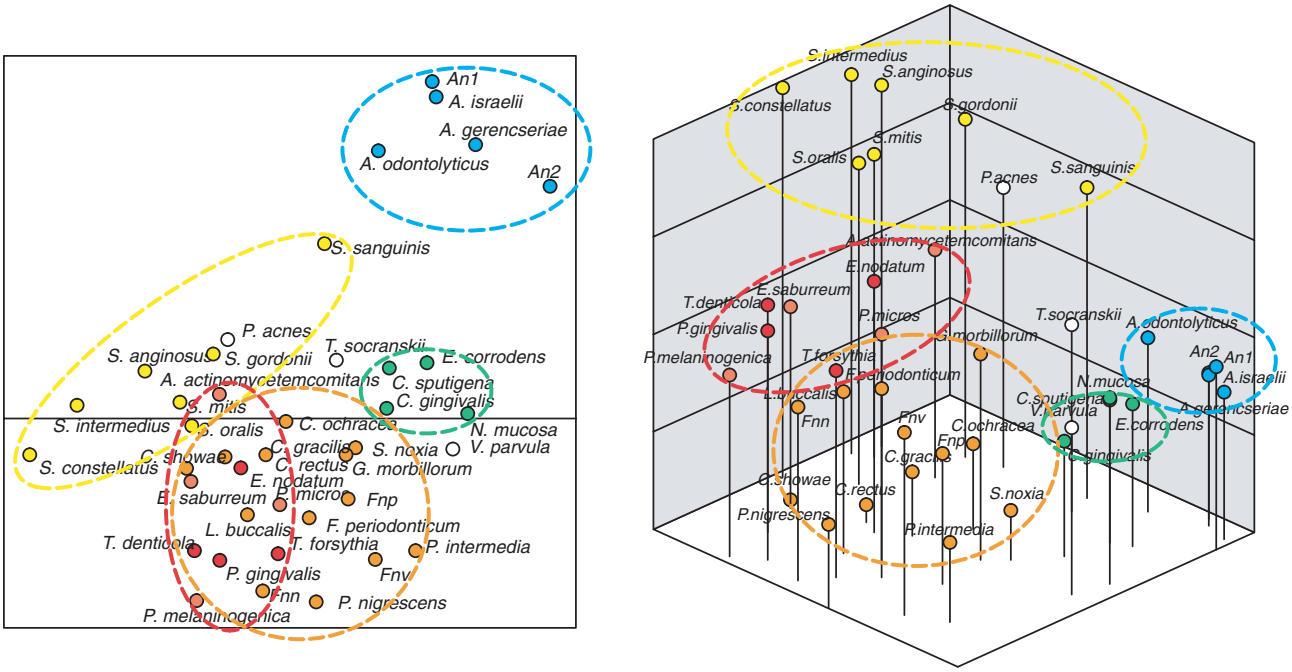

Fig. 5. Community ordination of 40 bacterial species in supragingival plaque samples from 93 subjects described in Fig. 4. The relationships among species were evaluated using the levels of the species at each of the sampled sites. Correspondence analysis and color-coding were as described in Fig. 3.

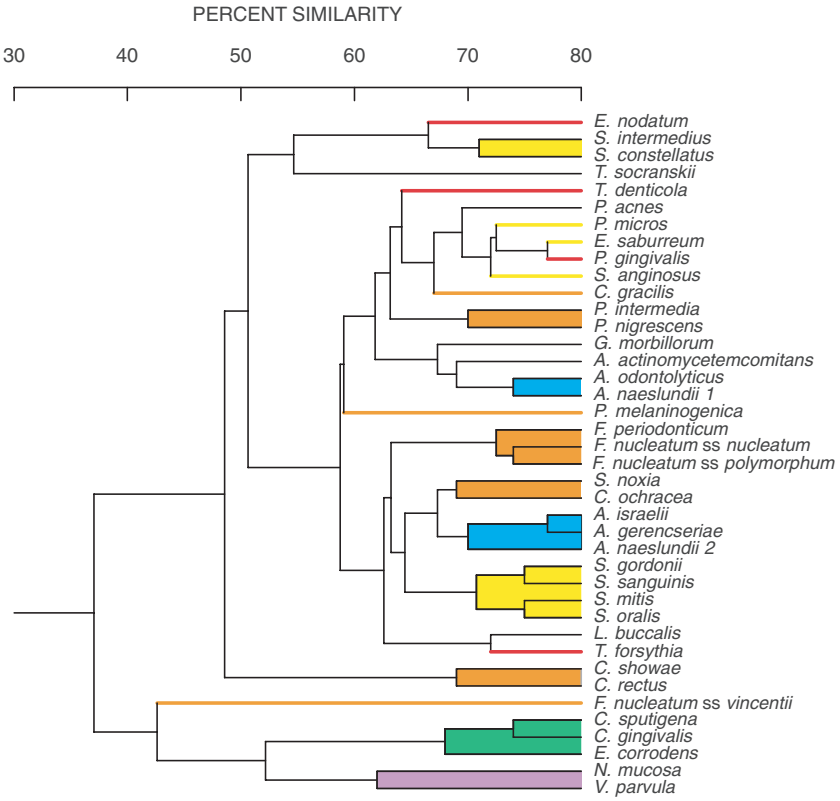

Fig. 6. Dendrogram of a cluster analysis of 40 subgingival taxa using the Bray Curtis coefficient and an average unweighted linkage sort. The data employed were the counts of the 40 taxa in supragingival plaque samples taken in the short-term (1–7 days) plaque redevelopment phase from 55 subjects. Cluster groups have been colored according to the clusters depicted in Fig. 1.

seen primarily for species in the orange and red complexes. *S. sanguinis* was in significantly higher mean proportions in sites that did not bleed on probing, while orange complex species, including, *S. noxia*, *C. gracilis*, *P. nigrescens*, *C. rectus*, and *C. showae*, were found in significantly higher mean proportions in sites that exhibited gingival redness (data not shown).

Figure 9 presents the relationship of the 40 test species to pocket depth, attachment level, and recession. The left panel presents the mean counts of the 40 test species in supragingival plaque samples taken from sites in pocket depth categories, <4, 4–6, and >6 mm. There were significantly higher mean counts of eight orange and two red complex species at sampled sites adjacent to deeper periodontal pockets. Since increased pocket depth was related to greater frequency of gingival redness, the analysis was repeated with the data subset according to the presence or absence of gingival redness. Similar relationships with pocket depth were seen in both subset categories as those observed for all sites. Seven species were at significantly higher mean counts at sites with gingival redness adjacent to deeper pockets (*A. naeslundii* 1, *C. gracilis*, *P. intermedia*, *P. nigrescens*, *C. rectus*, *C. showae*, *P. gingivalis*). Only *P. gingivalis* was significantly elevated in mean counts at non-reddened sites adjacent to deeper pockets (data not shown).

In a similar fashion, baseline attachment level measurements were subset into categories <2, 2–4, and >4 mm and the analyses were repeated (Fig. 9, middle panel). Species that were at increased mean counts at sites with the greatest

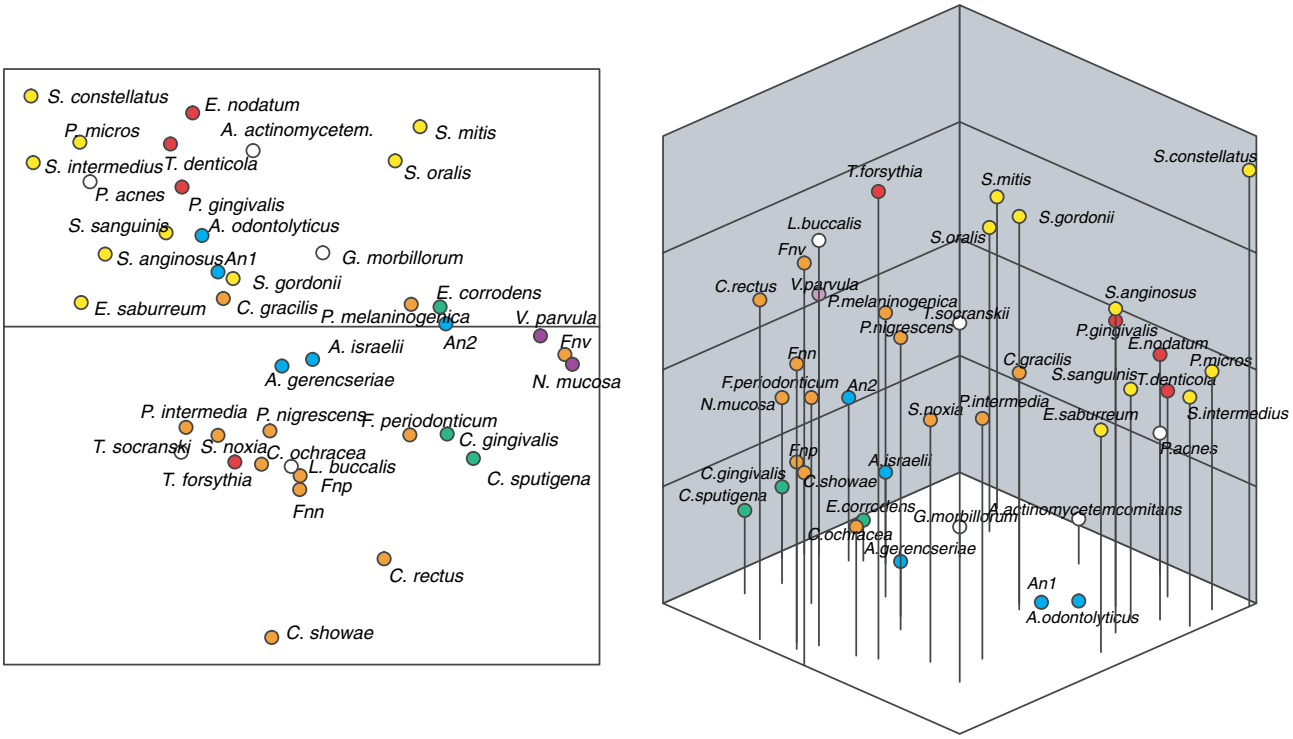

Fig. 7. Community ordination of 40 bacterial species in supragingival plaque samples from the 55 subjects described in Fig. 6. The relationships among species were evaluated using the levels of the species at each of the sampled sites. Correspondence analysis and color-coding were as described in Fig. 3.

attachment loss included *C. gingivalis*, *C. ochracea*, *S. noxia*, *C. gracilis*, *C. rectus*, *C. showae*, and *P. gingivalis*. Figure 9 (right panel) presents the mean counts of the 40 test species in supragingival plaque samples from sites that did or did not exhibit gingival recession. Mean counts of all species were higher in the sites with recession than in samples from sites with no gingival recession. Several statistically significant differences were observed for species in the *Actinomyces*, green, orange, and yellow complexes.

Discussion

The goal of the present investigation was to attempt to understand the nature of the microbial complexes that exist in supragingival plaque. As pointed out for the detection of subgingival complexes (7), any representation of these complexes, whether by cluster analysis or community ordination techniques, suffers the limitation that one is attempting to represent multidimensional relationships in two or three dimensions. Thus, different presentations of relationships are bound to suggest somewhat different associations among species. Cluster analyses are particularly effective in describing associations between pairs of species, while ordination techniques provide additional information relating communities of species to one another. The two- and three-dimensional representations both provide the relationship using the first two components of the ordination technique but the three-dimensional representation provides additional information, although at the expense of greater difficulty in visualization of relationships among species. In spite of these reservations, the associations observed for a given data set, such as the 'mature' biofilm samples, were quite robust, in that different similarity coefficients and different methods of community ordination provided essentially identical groupings. Figures 1 and 4 were attempts to summarize the complexes and the relationships among complexes observed in multiple analyses of the databases for the mature and long-term redevelopment samples, respectively. The potential for human error in interpretation of the multidimensional data is high and the figures should be considered to be a point of departure for further evaluation of supragingival microbial relationships.

The present manuscript differs from the 1998 paper in that all samples were of supragingival rather than subgingival biofilms. Forty species were included in the analyses, namely, the 32 described in the 1998 paper plus the eight species that have been designated as 'other' in many publications. Finally, the data represented three different phases of plaque development. The first was samples of mature plaque, of undetermined age, that were present in the subjects as they entered the study. The second was supragingival biofilm samples taken within 7 days after professional full-mouth tooth cleaning. The third set of data represented samples from subjects who had completed periodontal therapy and were in a post-therapy, monitoring phase. The conjecture was that microbial complexes/communities would be revealed in the mature biofilm, that these communities would be in some degree of disarray a short time after careful mechanical disruption, but that the basic community structure would re-establish after a longer period of post-therapy monitoring. This essentially was the case. The community structure of the mature and long-term redevelopment biofilms were quite similar with only a few species showing different community relationships. In contrast, the short-term redevelopment biofilm structure was a mixture of the communities observed in the more mature biofilms. The reason for these differences may be, in part, because the subject populations were not the same for each of the three phases.

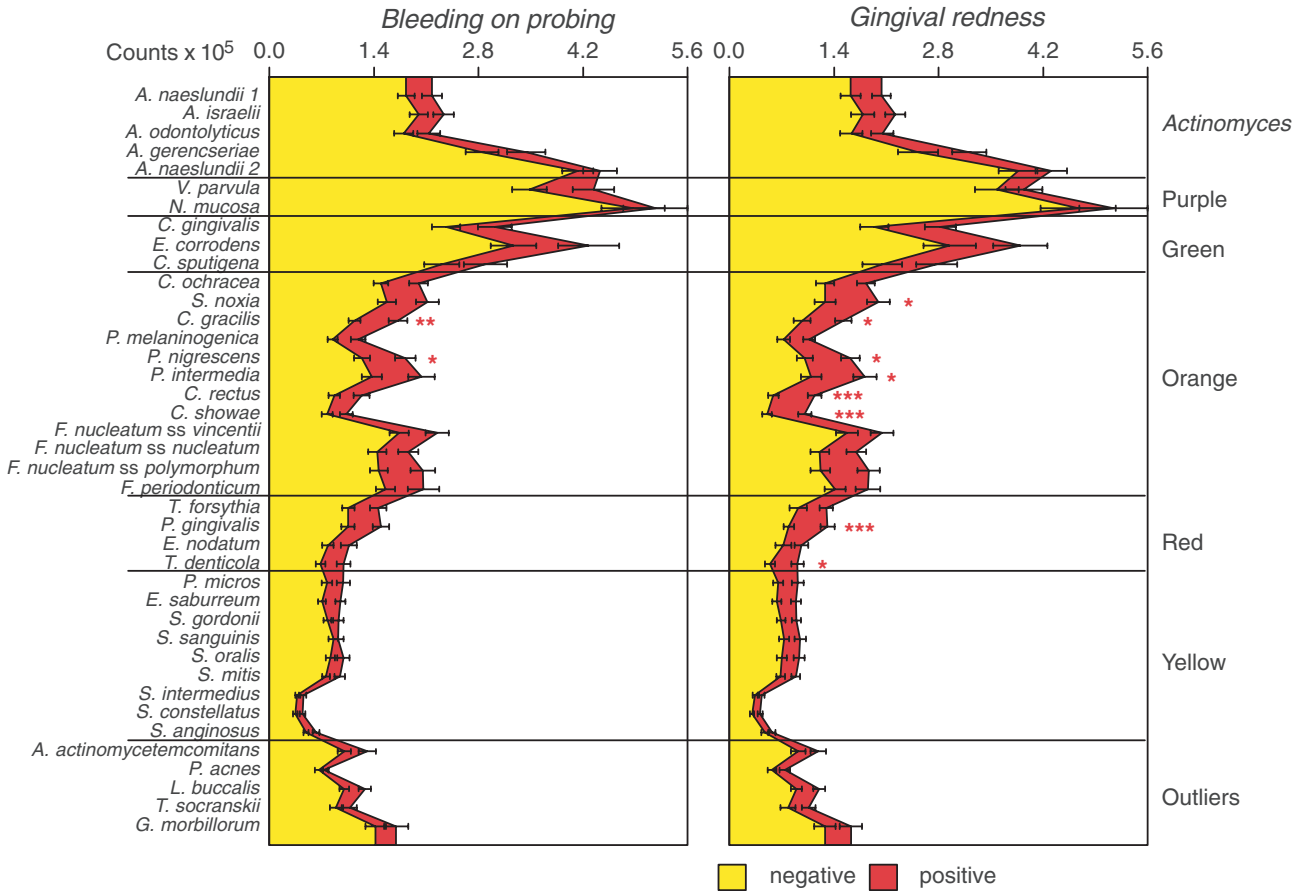

Fig. 8. Panel plots of the mean counts (× 10^5, ± SEM) of the 40 test species in supragingival plaque samples from 187 subjects at baseline at sites that did or did not exhibit bleeding on probing (left panel) or did or did not exhibit gingival redness (right panel). For both parameters, counts of each species were averaged within a subject at sites that were positive or negative for the parameter and then averaged across subjects for the positive and negative sites separately. Significance of differences between positive and negative sites was determined using ANOVA and adjusted for 40 comparisons (8). The species were ordered according to the complexes depicted in Fig. 1.

The mature plaque samples included samples from all 187 subjects, 38 of whom were periodontally healthy. Samples for the short-term phase were from 55 subjects including the 38 periodontally healthy subjects. For the long-term redevelopment phase, samples were collected from 93 patients with periodontitis at post-therapy monitoring visits ranging from 3 to 24 months. A second reason for the differences among the three phases may have been the differences in the within-plaque recolonization rate of different species. For example, members of the yellow complex, such as *S. mitis* and *S. oralis*, return rapidly after tooth cleaning (2, 6), while the return of *Actinomyces* species is much slower (4, 6, 10). Other species, including members of the red complex, are even further delayed in their return in supragingival biofilms (6). Thus, there is a reshuffling in biofilm development that takes time before 'maturation' may occur. Though this time must vary from tooth surface to tooth surface, the data presented in this paper indicate that 7 days is probably not sufficient for full development of the final climax community.

Perhaps the tightest knit communities were those of the yellow and *Actinomyces* complexes. These groups were even more related in supragingival than in subgingival biofilms. Part of the reason for this relationship was alluded to in the previous section; namely, that the streptococcal species dominate in early supragingival biofilm formation and are replaced over time by the *Actinomyces* species. This has been shown in cultural studies by Ritz (4), Socransky et al. (10) and Zee et al. (13). It was also demonstrated in studies using molecular techniques by Li et al. (2) and Socransky & Haffajee (6). One might surmise that a subset of the plaque samples from the mature biofilms might have recently been disrupted by toothbrushing and flossing, and these samples might be predominated by yellow complex species. Other samples might have been taken from plaque that had been undisturbed for an extended period of time (less effective home-care procedures) where *Actinomyces* would be more likely to predominate. Another factor that might contribute to the tightness of the yellow complex is that there appears to be more interspecies coaggregation among species of the genus *Streptococcus* than for other genera (1).

The green complex described for subgingival biofilm samples was somewhat modified for the supragingival samples. *C. gingivalis*, *C. sputigena*, and *E. corrodens* were still tightly grouped but *C. ochracea* appeared to relate somewhat more strongly to members of the orange complex. In some of the analyses, *V. parvula* and *N. mucosa* appeared to comprise a loose 'purple' complex in which *N. mucosa* had replaced the *A. odontolyticus* described in the subgingival microbial complexes. However, in the long-term

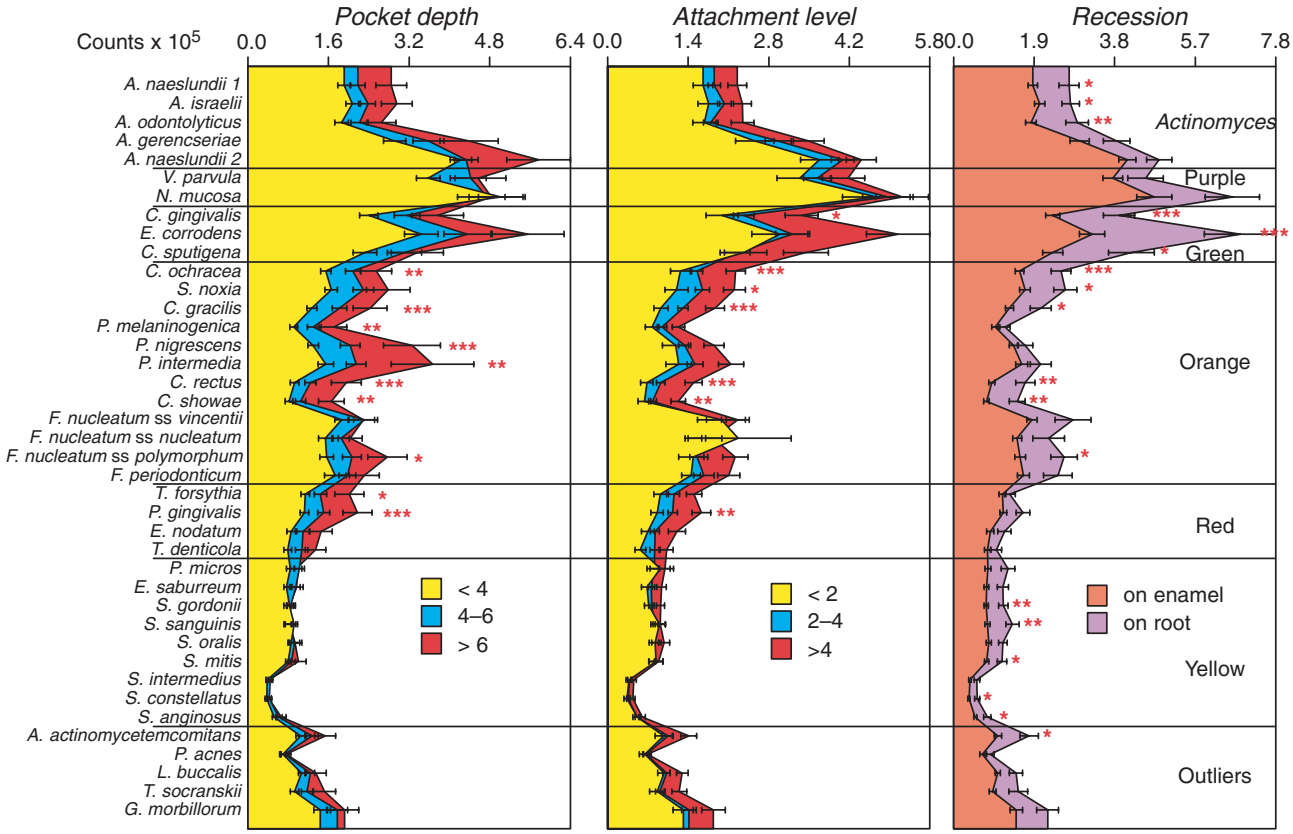

Fig. 9. Panel plots of the mean counts (× 10^5, ± SEM) of the 40 test species in supragingival plaque samples from 187 subjects at baseline. The left panel presents data at sample sites adjacent to pockets <4, 4–6, and >6 mm in depth; the middle panel presents data for sites in attachment level categories of <2, 2–4, and >4 mm; in the right panel the data have been subset according to whether the sites exhibited gingival recession or not. For all parameters, counts of each species were averaged within a subject at sites in the different categories and then averaged across subjects for the various site categories separately. Significance of differences among pocket depth and attachment level categories and between sites with and without recession was determined using ANOVA and adjusted for 40 comparisons (8). The species were ordered according to the complexes depicted in Fig. 1.

redevelopment analyses, *N. mucosa* and to a lesser extent *V. parvula* appeared to join the green complex. The orange complex was the largest complex observed in the supragingival samples in terms of number of species included. There appeared to be distinct subsets within this complex. For example, *C. gracilis*, *S. noxia*, and *C. ochracea* were a tight subset within the orange complex community. *P. intermedia* and *P. nigrescens*, as well as certain fusobacteria, also formed distinct subsets within this complex. The highly motile *Campylobacter* species, *C. rectus* and *C. showae*, were tightly related, presumably because of their similar nutrient requirements and perhaps a sheltered location for colonization. There seemed to be greater tightness of subsets within the orange complex of supragingival plaque compared with that observed in subgingival samples. Remarkably, the core red complex species, *P. gingivalis*, *T. forsythia*, and *T. denticola*, observed in subgingival plaque were also observed in supragingival plaque. This core group was joined by *E. nodatum* both in the mature and the long-term redevelopment biofilms. There was also a loose association of red complex species with *A. actinomycetemcomitans*, *E. saburreum*, *P. micros*, and *P. melaninogenica* in the long-term redevelopment biofilm samples. As might be expected from the species name, *T. socranskii* did not fit in well with any of the cluster groups.

The relationship between the microbial composition of supragingival plaque samples and clinical measures of inflammation was quite strong and many species were significantly elevated in mean counts at sites that exhibited gingival redness or bleeding on probing. The species that were most increased adjacent to the inflamed sites were members of the orange and red complexes. This relationship of orange and red complex species with inflammation was in accord with findings for subgingival biofilms (6). Since the samples of biofilm were taken from the supragingival area, it was expected that there would be less of a relationship between the composition of the supragingival biofilm with parameters of periodontal destruction, such as pocket depth, than was observed for subgingival plaque. This was not the case. There was a strong relationship of supragingival counts to measures of pocket depth and attachment level. Our initial thought was that this might have been because many periodontal pockets exhibited inflammation as determined by gingival redness or bleeding on probing. However, when counts of species in the different pocket depth categories were examined for sites that did or did not exhibit inflammation, the increased levels of orange and red complex species were still observed at the sites with deep pockets irrespective of the level of inflammation. There are several explanations for these observations. A deeper periodontal pocket not only harbors more plaque, including orange and red complex species, but is likely to produce more gingival crevicular fluid. Both of these factors could impact the supragingival biofilm, by providing a source of inocula for colonization and

essential nutrients. It is well known that the careful, repeated removal of supragingival biofilm beneficially affects the composition of the subgingival biofilm (12). The data in the present manuscript suggest that diminution of periodontal pockets and local inflammation would decrease counts of species, particularly those of the orange and red complexes, in the supragingival biofilm.

The mean counts of species at sites that exhibited recession were higher than at sites where the gingival margin was at or above the cemento-enamel junction. The higher mean counts at the sites with recession may have been the result, in part, of an increased surface area for sample taking or to a preferential adherence of certain species to cementum or dentin. This appeared to be most marked for *E. corrodens* and to a lesser extent, *C. ochracea* and *C. gingivalis*. It will be of interest to compare the composition of samples that adhere to enamel with those that adhere to the root surface on the same tooth in future studies.

The data in the present investigation support the notion that there are distinct microbial communities that exist in supragingival biofilms. These communities appear to exist, in part, because of selective adhesion of specific species to tooth or pellicle-coated surfaces and because of the distinct patterns of coaggregation that occur among bacterial species. However, these may not be the sole factors that influence community structure because the clinical status of the adjacent periodontal structures also influenced the composition of the supragingival biofilm, possibly by providing interspecies binding molecules and by contributing nutrients leading to the enrichment of certain communities. Therapy had an interesting effect on community structure. Short-term (1–7 days), the community structure was imperfectly developed, suggesting that greater time was needed to produce the 'typical' mature biofilm. Longer-term (3–24 months) community structure was essentially redeveloped, although there were lower numbers of species post-therapy than existed before the treatment procedures. The communities described in the present investigation should provide a basis for examining ecological relationships among bacterial species and between the species and the host as well as for studies of interactions among species in *in vitro* systems.

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