Microbial complexes in subgingival plaque

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Abstract. It has been recognized for some time that bacterial species exist in complexes in subgingival plaque. The purpose of the present investigation was to attempt to define such communities using data from large numbers of plaque samples and different clustering and ordination techniques. Subgingival plaque samples were taken from the mesial aspect of each tooth in 185 subjects (mean age 51 ± 16 years) with (n=160) or without (n=25) periodontitis. The presence and levels of 40 subgingival taxa were determined in 13,261 plaque samples using whole genomic DNA probes and checkerboard DNA-DNA hybridization. Clinical assessments were made at 6 sites per tooth at each visit. Similarities between pairs of species were computed using phi coefficients and species clustered using an averaged unweighted linkage sort. Community ordination was performed using principal components analysis and correspondence analysis. 5 major complexes were consistently observed using any of the analytical methods. One complex consisted of the tightly related group: *Bacteroides forsythus, Porphyromonas gingivalis* and *Treponema denticola*. The 2nd complex consisted of a tightly related core group including members of the *Fusobacterium nucleatum/periodontium* subspecies, *Prevotella intermedia, Prevotella nigrescens* and *Peptostreptococcus micros*. Species associated with this group included: *Eubacterium nodatum, Campylobacter rectus, Campylobacter showae, Streptococcus constellatus* and *Campylobacter gracilis*. The 3rd complex consisted of *Streptococcus sanguis, S. oralis, S. mitis, S. gordonii* and *S. intermedius*. The 4th complex was comprised of 3 *Capnocytophaga* species, *Campylobacter concisus, Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* serotype a. The 5th complex consisted of *Veillonella parvula* and *Actinomyces odontolyticus*. *A. actinomycetemcomitans* serotype b, *Selenomonas noxia* and *Actinomyces naeslundii* genospecies 2 (*A. viscosus*) were outliers with little relation to each other and the 5 major complexes. The 1st complex related strikingly to clinical measures of periodontal disease particularly pocket depth and bleeding on probing.

The complexity of the subgingival microbiota has been recognized since the 1st microscopic examination of this ecosystem by Van Leeuwenhoek in 1683 (Tal, 1980). Since that time, numerous studies have evaluated the composition of plaque using light and electron microscopy, cultural techniques and more recently immunologic or DNA probe techniques. All techniques reinforce Van Leeuwenhoek's initial observation that subgingival plaques are comprised of a large complex mixture of bacterial species. Indeed it has been estimated that 400 or more species re-

side in this area. Cursory examination of a series of plaque samples suggests that there is little order in the microbiota that colonizes gingival sulci or periodontal pockets. However, examination of sections of plaque by light and electron microscopy indicates a surprising degree of order in colonization patterns. For example, early supragingival plaque demonstrated columnar arrangement of morphologically distinct bacterial species from the tooth surface to the outer surface of the plaque (Listgarten et al. 1975). Subgingival plaque was frequently characterized by

a zone of gram negative and/or motile species located adjacent to the epithelial lining of the pocket while gram positive rods and cocci appeared to be forming a tightly adherent band of organisms on the enamel or root surface (Listgarten 1976, Listgarten 1994).

Cultural, immunologic or DNA probe assessments of plaque have demonstrated that certain species frequently occur together in subgingival plaque samples. For example, *Porphyromonas gingivalis* is almost always observed in samples that are harboring *Bacteroides forsythus*. It has been speculated that *B.*

forsythus in some fashion precedes colonization by *P. gingivalis* since *B. forsythus* is detected more frequently by itself (Gmur et al. 1989). Other complexes that have been observed include *P. gingivalis* and *Treponema denticola* (Simonson et al. 1992b) and *Fusobacterium nucleatum* and *Prevotella intermedia* (Ali et al. 1994).

Understanding the relationship among bacterial species is useful in understanding the biology of subgingival plaque and in planning strategies for its control. Knowledge of the ecological relationships among bacterial species can direct and focus investigations on critical bacterial interactions.

Technological developments permit the evaluation of large numbers of bacterial species in large numbers of plaque samples from a wide range of subjects. Such procedures provide a data base in which associations among bacterial species can be precisely examined. Associations between a pair of species is often performed using contingency tables, sometimes 2×2 and sometimes with more levels of each species. Cluster analysis has been useful in describing closely related species when more than a few pairs of species are examined. Community ordination is a procedure that attempts to indicate closely related species within a community and then demonstrate the relatedness among different communities of species within the ecosystem of interest. The purpose of the present investigation was to use cluster analysis and community ordination techniques to examine relationships among bacterial species in subgingival plaque samples, and relate the complexes to clinical parameters of periodontal disease.

Material and Methods

Subject population

185 subjects ranging in age from 20–87 years who were considered to be periodontally healthy (n=25) or with evidence of prior attachment loss (n=160) were selected for study. All subjects had been 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 pre-medication for monitoring or therapy. No subject with localized juvenile periodontitis, rapidly progressive peri-

odontitis or acute necrotizing ulcerative gingivitis was included in the study.

Clinical monitoring

Subjects were screened for suitability and if accepted, were asked to sign informed consent forms. All subjects were clinically monitored at baseline and subjects with periodontitis were monitored at 3 month intervals post therapy. 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 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars at each visit. The baseline clinical features of the 185 subjects are presented in Table 1.

Microbiological assessment

Subgingival plaque samples were taken from the mesio-buccal aspect of each tooth in each subject at each monitoring visit. Counts of 40 subgingival species were determined in each plaque sample using the checkerboard DNA-DNA hybridization technique (Socransky et al. 1994). After the removal of supragingival plaque, subgingival plaque samples were taken with individual sterile Gracey curettes from the mesial aspect of each tooth. The samples were placed in separate Eppendorf tubes containing 0.15 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). 0.15 ml of 0.5 M NaOH was added to each sample and the sample boiled in a water bath for 5 min. The samples were neutralized using 0.8 ml 5 M ammonium

acetate. The released DNA was placed into the extended slots of a Minislot (Immunetics, Cambridge MA) and then concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed to the membrane by exposure to ultraviolet light followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of 28 different plaque samples in individual "lanes" on a single 15×15 cm nylon membrane as well as 2 control lanes containing 10^5 or 10^6 cells of each test species. The membrane with fixed DNA was placed in a Miniblotter 45 (Immunetics, Cambridge MA), with the "lanes" of DNA at 90° to the channels of the device. A 30×45 "checkerboard" pattern was produced with 5 of the probe lanes remaining empty to permit accurate localization. Each channel was used as a hybridization chamber for separate DNA probes. Signals were detected by chemiluminescence.

DNA–DNA hybridization

The membranes were prehybridized at 42°C for 1 hr in 50% formamide, 5×SSC (1×SSC=150 mM NaCl, 15 mM Na citrate, pH 7.0), 1% casein (Sigma, St. Louis MO), 5 X Denhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer Mannheim). Digoxigenin-labeled, whole chromosomal DNA probes were prepared using a random primer technique (Feinberg & Vogelstein 1983). The probes and hybridization buffer were placed in individual lanes of the Miniblotter and the whole apparatus placed in a sealed plastic bag. Membranes were hybridized overnight at 42°C in a hybridizing solution containing 45% formamide,

Table 1. Baseline clinical characteristics of subject group (n=185)

|	Mean (±SD)	Range
age (years)	51±16	20–87
no. missing teeth	3.1±3.1	0–8
% males	43	
mean pocket depth (mm)	3.1±0.7	1.7–6.6
mean attachment level (mm)	3.1±1.2	0.8–6.8
% sites with:		
plaque	69±30	0–100
red	72±30	0–100
BOP	49±37	0–100
suppuration	1.7±5.4	0–49
pocket depth <4 mm	74±20	11–100
pocket depth 4–6 mm	23±17	0–76
pocket depth >6 mm	3±6	0–37
attachment level <4 mm	72±24	7–100
attachment level 4–6 mm	23±18	0–67
attachment level >6 mm	5±9	0–61
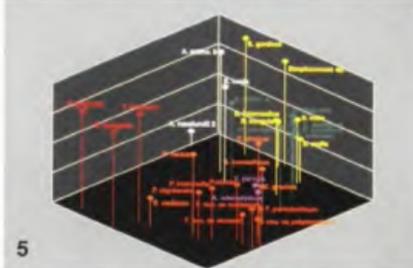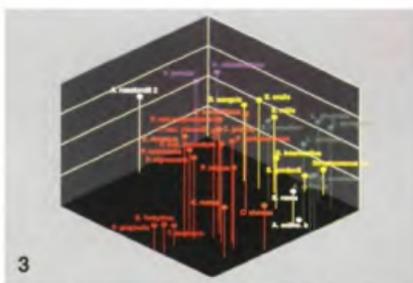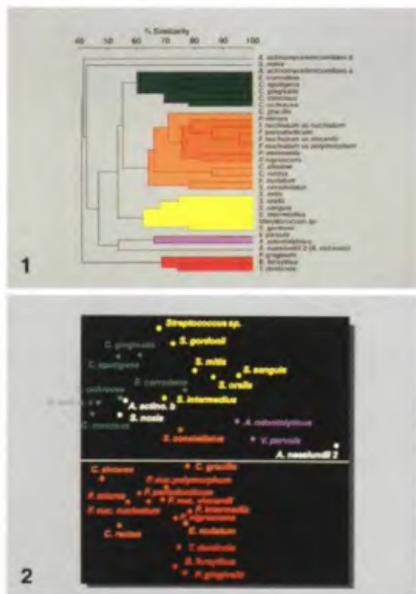
Fig. 1. Dendrogram of a cluster analysis of 32 subgingival taxa. The similarity between pairs of species was computed using a phi coefficient, the coefficients were scaled and then sorted using an average unweighted linkage sort. Clusters were formed with a threshold level of 60%. The color coding used to delineate the groups in this figure were employed in all figures.

Fig. 2. Community ordination of 32 subgingival taxa using correspondence analysis. The relationships among species were evaluated using the levels of the species at each of the sampled sites. Correspondence analysis was performed as described by Ludwig & Reynolds (1988) and the species were plotted along the first (x-axis) and second (y-axis) axes. The colors indicate taxa within the same group. A. naeslundii genospecies 2 (*A. viscosus*), *A. actinomycetemcomitans* serotype b and *S. noxia* were not part of any complex.

Fig. 3. Community ordination of 32 subgingival taxa using correspondence analysis. The analysis was performed as described in Fig. 2; however, the z-axis has been added. This presentation demonstrates that species that appeared to be closely associated in Fig 2 such as *E. nodatum* and *T. denticola* were widely separated in the 3-dimensional presentation.

Fig. 4. Community ordination of 32 subgingival taxa using principal components analysis. The relationships among species were evaluated using the levels of the species at each of the sampled sites. Principal components analysis was performed as described by Ludwig & Reynolds (1988) and the species were plotted along the first (x-axis) and second (y-axis) principal components.

Fig. 5. Community ordination of 32 subgingival taxa using principal components analysis. The analysis was performed as described in Fig. 4; however, the third principal component (z-axis) has been added. This presentation demonstrated that species that appeared to be closely associated in Fig. 4 such as *E. nodatum* and *T. denticola* were widely separated in the 3 dimensional presentation.

5×SSC, 1×Denhardt's reagent, 20 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dextran sulfate and 1% casein. Membranes were washed at low stringency to remove loosely bound probe and then at high stringency (68°C, 0.1×SSC, 0.1%

SDS, 20 min, twice) in a Disk Wisk apparatus (Schleicher and Schuell, Keene NH).

Detection and enumeration

To detect hybrids, membranes were blocked and then incubated with a

1:25,000 dilution of anti-digoxigenin antibody conjugated with alkaline phosphatase using the modification described by Engler-Blum et al. (1993). After washing, the membranes were incubated in Lumiphos 530 (Lumigen, Southfield, MI) for 45 min at 37°C,
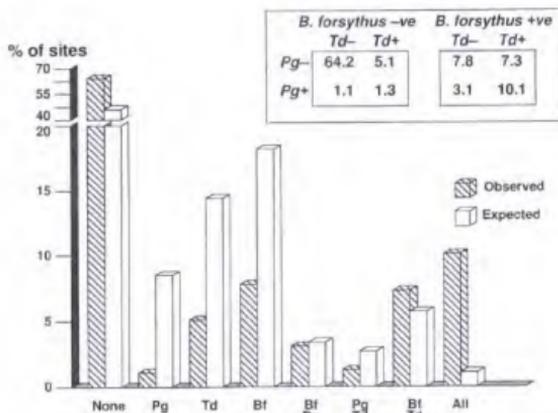
	B. forsythus −ve		B. forsythus +ve	
	Td−	Td+	Td−	Td+
Pg−	64.2	5.1	7.8	7.3
Pg+	1.1	1.3	3.1	10.1

Fig. 6. Bar chart of the observed and expected frequencies of members of the red complex (*B. forsythus* (*Bf*), *T. denticola* (*Td*) and *P. gingivalis* (*Pg*)) individually and in different combinations. The observed frequencies of the 3 species were summarized in a 2×2×2 contingency table and configural frequency analysis (von Eye 1990) was performed to compute the expected frequencies. The inset presents the % of sites in each of the 8 cells.
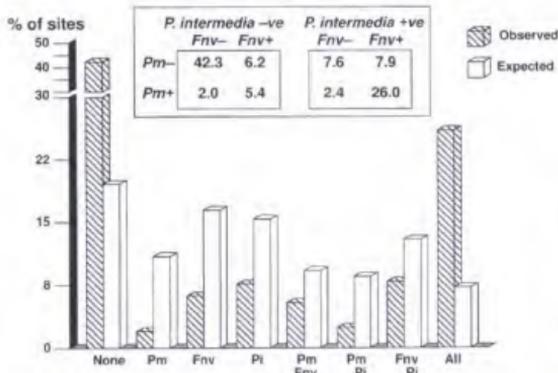

	P. intermedia −ve		P. intermedia +ve	
	Fnv−	Fnv+	Fnv−	Fnv+
Pm−	42.3	6.2	7.6	7.9
Pm+	2.0	5.4	2.4	26.0

Fig. 7. Bar chart of the observed and expected frequencies of representative members of the orange complex (*P. micros* (*Pm*), *F. nucleatum* ss *vincentii* (*Fnv*) and *P. intermedia* (*Pi*)) individually and in different combinations. Observed and expected frequencies were computed as described for Fig. 6.

placed in a film cassette with Reflection NEF film (Dupont, Boston MA) for 1 h at 37°C and then developed. Two lanes in each run contained standards at different concentrations. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given spe-

cies by adjusting the concentration of each DNA probe. This procedure was carried out in order to provide the same sensitivity of detection for each species. Failure to detect a signal was recorded as zero, although conceivably, counts in the 1 to 1000 range could have been

present. Signals were evaluated visually by comparison with the standards for the test species. They were recorded as: 0, not detected; 1,$<10^5$ cells; 2, $\sim10^5$; 3, 10^5 to 10^6; 4, $\sim10^6$; 5 $>10^6$ cells.

Statistical analysis

Microbiological data available for each subject included the level of each of 40 test species from up to 28 plaque samples (mean 25.2 samples) at each visit. The prevalence (the number of sites at which the species was detected) of each species was also computed for each subject at each visit. A total of 13,261 plaque samples were evaluated. Data for 32 of the species examined were used in the following analyses. The species were chosen on the basis of frequency of detection; species detected in< 5% of sites were omitted. Two by two tables were set up for each of the 496 pairs of species in which the presence or absence of each species was summarized. A phi coefficient was computed to assess association between each pair of species and scaled to range between 0 and 100%. The resulting similarities were clustered using an average unweighted linkage sort (Sneath & Sokal, 1973). For comparative purposes, other similarity coefficients were employed including the Bray Curtis, Mahalanobis d^2 and the correlation coefficients.

Community ordination was performed using principal components analysis and correspondence analysis (Ludwig & Reynolds 1988). The data employed were the ranks representing different counts of the test species at the 13,261 sampled sites. The data were plotted as described by Ludwig & Reynolds (1988).

In order to examine the robustness of the observed relationships, subsets of the data were analyzed using the cluster and community ordination procedures. These subsets included use of first visit data only and samples from sites within specified pocket depth ranges including 0–3 mm, 4–6 mm and >6 mm as well as pre- and post-therapy data.

Associations among species within a complex were examined using configural frequency analysis. Significance of differences between observed and expected values within cells were determined as described by von Eye (1990).

The relationship between the clinical parameters and individual species and/ or complexes was examined after aver-

aging data for a chosen parameter within the subject and then averaging across subjects. For example, the levels of a species or complex were averaged for all non bleeding on probing sites in a subject as well for all bleeding on probing sites in that subject prior to averaging within BOP categories across subjects. Significance of differences for clinical parameters were sought using the Kruskal-Wallis test.

Results
Associations among bacterial species as determined by cluster analysis

Fig. 1 presents a dendrogram of the results of the cluster analysis of all 13,261 samples using ϕ coefficients and an average unweighted linkage sort. 5 clusters were formed with >60% similarity and included 29 of the 32 taxa evaluated. The red cluster consisted of *P. gingivalis*, *B. forsythus* and *T. denticola*. The orange cluster consisted of *F. nucleatum* subspecies, *P. intermedia* and *P. nigrescens*, *Peptostreptococcus micros* and *Campylobacter rectus*, *Campylobacter showae*, *Campylobacter gracilis*, *E. nodanum* and *S. constellatus*. The 3 *Capnocytophaga* species, *Campylobacter concisus*, *Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* serotype a formed the green cluster, while a group of streptococci made up the yellow cluster. *Streptococcus mitis*, *Streptococcus sanguis* and *Streptococcus oralis* were most closely related within this group. *Actinomyces odontolyticus* and *Veillonella parvula* formed the purple cluster. *Actinomyces naeslundii* genospecies 2 (*Actinomyces viscosus*), *Selenomonas noxia* and *A. actinomycetemcomitans* serotype b did not cluster with other species.

Associations among bacterial species as determined by community ordination

The results of community ordination using correspondence analysis are presented in Fig. 2. This analysis reinforced the relationships demonstrated in Fig. 1 and showed the relationships among the different microbial complexes. For example, the red complex consisting of *P. gingivalis*, *B. forsythus* and *T. denticola* was closely associated with the orange complex that included *F. nucleatum* subspecies, *P. intermedia* and *P. nigrescens*, *P. micros*, *E. nodatum* and *S. constellatus* and 3 of the *Campylobacter* species. Fig. 2 also dem-
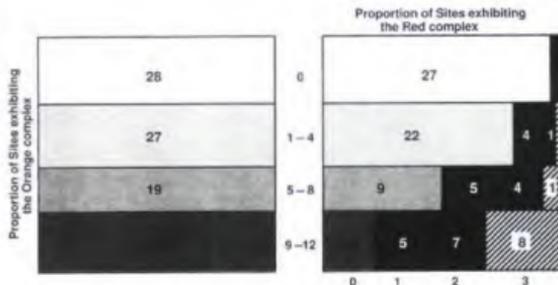

Fig. 8. Area plots depicting the percentage of sites colonized by members of the orange and red complexes. The data are based on examination of 13,261 subgingival plaque samples. The left hand panel indicates the percentage of sites colonized by 0, 1-4, 5-8 and 9-12 members of the orange complex. The right hand panel presents the same data, but overlaid with the percentage of sites in each of those categories that exhibited 1, 2 or 3 members of the red complex.
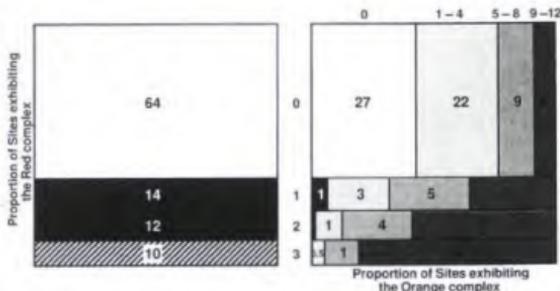

Fig. 9. Area plots depicting the % of sites colonized by members of the red and orange complexes. The left hand panel indicates the % of sites colonized by 0, 1, 2 or 3 members of the red complex. The right hand panel presents the same data, but overlaid with the percentage of sites in each of those categories that exhibited 0, 1-4, 5-8 and 9-12 members of the orange complex.

onstrated the relationships among the *Capnocytophaga* species, most of the *Streptococcus* species, *E. corrodens* and *C. concisus*. These species were closely related to each other and somewhat related to the orange complex. *A. actinomycetemcomitans* serotype b appeared to be closely related to *C. ochracea*, but examination of the 3 dimensional plot of the correspondence analysis (Fig. 3) indicated that these 2 species were distant from one another. The relationship between *A. odontolyticus* and *V. parvula* suggested by cluster analysis was confirmed by correspondence analysis. Further, the outlier status of *S. noxia*, *A. actinomycetemcomitans* serotype b

and *A. naeslundii* genospecies 2 (*A. viscosus*) was evident (Figs. 2, 3).

Figs. 4, 5 present principal components analysis of the same data and indicate similar groupings of species. This analysis reinforces the distinction of the red (*P. gingivalis*, *B. forsythus* and *T. denticola*) and orange clusters as well as the separation of these 2 groups from the green and yellow complexes.

Associations among species within complexes

The associations between species in the red complex are summarized in Fig. 6. 64% of sites harbored none of the spe-
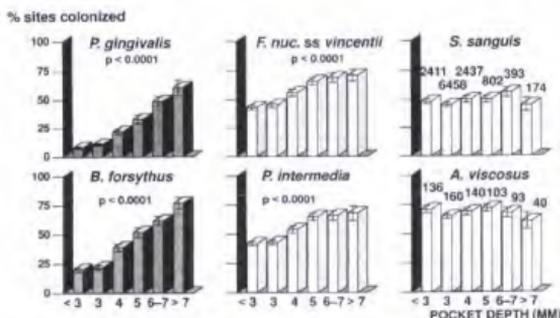
% sites colonized

Fig. 10. Bar charts of the mean (± SEM) of the % of sites colonized by 6 subgingival species at selected pocket depths. The percentage of sites colonized by each species at each pocket depth category was computed for each subject and then averaged across subjects. Significance of differences among pocket depth categories was tested using the Kruskal-Wallis test. The total number of subjects was 185. The number of subjects exhibiting the different pocket depths is shown above the bars in the *A. naeslundii* genospecies 2 (*A. viscosus*) panel, while the number of sites in each pocket depth category is shown above the bars for *S. sanguis*.

% sites colonized
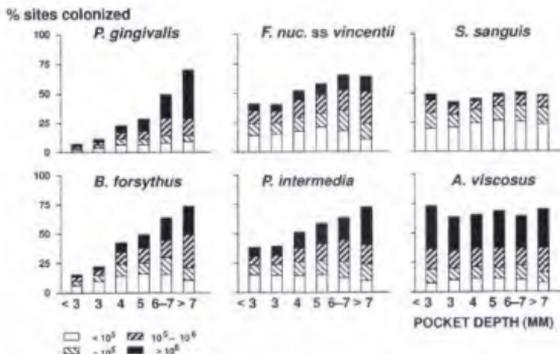

□ = <10⁴   ▨ = 10⁴ - 10⁶   ▧ = >10⁶

Fig. 11. Stacked bar charts of the % of sites colonized by different levels of 6 subgingival species at selected pocket depths. The number of subjects and sites providing the data were presented in Fig. 10.

cies in this complex, while 10% of sites harbored all 3 species. These percentages were strikingly elevated as determined by configural frequency analysis. In contrast, the individual species were detected less frequently than expected. The same analysis was repeated using representative members of the orange complex (Fig. 7). In a fashion similar to the red complex, the % of sites harboring none or all of the species were significantly higher than expected, while the individual species and pairs of spe-

cies were detected less frequently than expected. Similar results were obtained for the yellow and green complexes (data not shown).

Relationship among complexes

The relationship between the red and orange complexes is illustrated in Figs. 8, 9. The left hand panel of Fig. 8 depicts the % of sites colonized by 0, 1–4, 5–8 and 9–12 members of the orange complex. The right hand panel presents

the same data together with the percentage of sites in each category that also harbored 1, 2 or 3 members of the red complex. It is readily apparent that members of the red complex are rarely found in the absence of members of the orange complex. With increasing colonization by the orange complex, more sites were colonized by increasing numbers of the red complex. Fig. 9 presents the same data but with the left hand panel depicting the % of sites colonized by members of the red complex. Only 36% of sites harbored one or more of the red complex species compared with 72% of sites which harbored members of the orange complex (Fig. 8). The right hand panel indicates that a large proportion of sites in which the red complex was not detected was colonized by members of the orange complex. Taken together the data suggest that species in the orange complex precede colonization by species of the red complex.

Relationship between bacterial species and clinical parameters

The relationship of the species in the different complexes to pocket depth was examined. Species in the red complex exhibited a very strong relationship with pocket depth (Figs. 10, 11). For example. *B. forsythus* and *P. gingivalis* (and *T. denticola*, data not shown) increased in prevalence and numbers with increasing pocket depth. Similarly, all species in the orange complex showed a significant association with increasing pocket depth as exemplified by *P. intermedia* and *F. nucleatum ss vincentii*. *A. naeslundii* genospecies 2 (*A. viscosus*) and *S. sanguis* provide examples of the remaining species which showed no statistically significant relationship with pocket depth.

Since species in the red complex were so strongly related to pocket depth, the relationship between individual and combinations of species in this complex and pocket depth was examined further (Fig. 12). Sites with none of the species exhibited the shallowest mean pocket depth, while sites harboring all 3 showed the deepest. It is interesting to note that sites harboring *P. gingivalis* alone or in combination with the other 2 species exhibited the deepest mean pocket depths. The red complex and the individual species in that group were also strongly associated with bleeding on probing (Fig. 13).
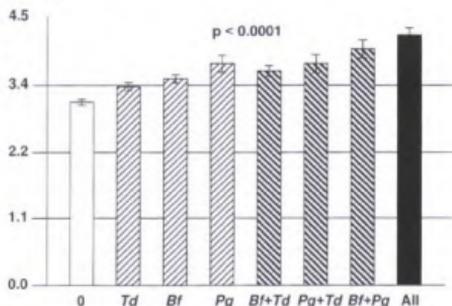
Mean PD (mm)

Fig. 12. Bar chart of mean (±SEM) pocket depth at sites harboring none, all or different combinations of the species in the red complex (*B. forsythus* (*Bf*), *T. denticola* (*Td*) and *P. gingivalis* (*Pg*)). The average pocket depth for each of the microbial combinations was computed within a subject and then averaged across subjects.
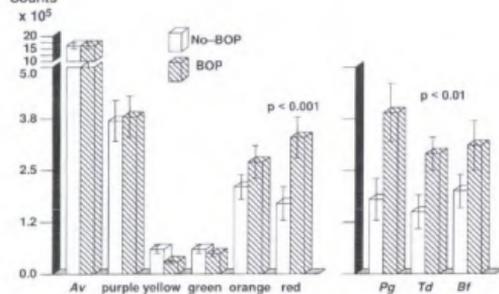

Fig. 13. Bar chart of the mean (±SEM) % of species in each complex at sites that bled or did not bleed on probing. The % of species in a complex present at each site was computed. The values were averaged for each BOP category within each subject and then averaged across subjects. The only significant difference between bleeding and non bleeding sites was observed for the red complex. The individual members of that complex differed significantly as depicted in the right panel.

Fig. 14. Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes. This diagram was based on the results of multiple cluster and community ordination analyses using the entire data base as well as subsets of data.

Discussion

The goal of the present investigation was to attempt to understand the nature of the microbial complexes that exist in subgingival plaque. Any representation of these complexes whether by cluster analysis or community ordination techniques suffers the limitation that one is attempting to represent multi-dimensional relationships in two or three dimensions. Thus, different presentations of relationships are bound to suggest somewhat different associations among species. In spite of these reservations, the associations observed were quite robust, in that different similarity coefficients, different methods of community ordination or subsets of the data, such as first visit data only or data from sites with different baseline pocket depths, provided essentially identical groupings. The only exceptions were the occasional movement of *E. nodatum* into the red complex, *A. actinomycetemcomitans* serotype a joining *A. actinomycetemcomitans* serotype b and *A. naeslundii* genospecies 2 (*A. viscosus*) joining the purple complex. Fig. 14 is an attempt to summarize the complexes and the relationships among complexes observed in multiple analyses of the full data base and subsets of the base. The potential for human error in interpretation of the multi-dimensional data is high, however, Fig. 14 may serve as a point of departure for further evaluation of subgingival microbial relationships.

One concern in interpreting the data would be the sensitivity and specificity of the DNA probes employed. The sensitivity of the assay was set to 10^4 cells of a species by adjusting the concentration of each probe in the hybridization buffer. The specificity of the probes was examined using a recently acquired Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA) and Artophos (Amersham Life Science, Arlington Heights, Illinois, USA) instead of Lumiphos 530 (Lumigen, Southfield, Michigan, USA) in the detection step. Over 92% of all probe:heterologous species reactions did not exhibit cross-reactions under the conditions of the assay. Some probes such as those to *B. forsythus*, *P. gingivalis* and *T. denticola* did not exhibit cross-reactions with any heterologous species tested. When cross-reactions were observed they were always within genera and were quite limited. For example, of the 6 *Campylobacter*

species evaluated, only *C. showae* and *C. rectus* cross-reacted but at a homologous:heterologous ratio >100:1. There were no cross-reactions among the 3 *Capnocytophaga* species. Cross-reactions within the *Streptococci* were minimal always exceeding 100:1 for homologous:heterologous species, although *S. oralis*, *S. mitis* and *S. sanguis* showed no cross-reactions with *S. intermedius* and *S. constellatus*. Thus, the majority of the observed relationships were unlikely to be due to cross-reactions of the DNA probes although some influence cannot be entirely ruled out. A second concern might have been the fact that pre- and post-therapy samples were included for periodontitis subjects, but only first visit data were used for periodontally healthy subjects. It must be emphasized that the relationships depicted in this manuscript were observed when only 1st visit data were employed, i.e., each subject contributed approximately equal numbers of samples. The authors chose to present the entire data base since it included pre- and post-therapy samples adding a degree of robustness to the observed relationships.

The relationship of *P. gingivalis*, *T. denticola* and *B. forsythus* (red complex) appears in one guise or another in the Figures provided in this manuscript as well as when subsets of data were examined from pre or post therapy visits or for sites subset in different pocket depth ranges (data not shown). This complex have been described in the literature (Gmur et al. 1989, Simonson et al. 1992ab). Gmur et al. (1989) described a strong relationship between *B. forsythus* and *P. gingivalis* in subgingival plaque samples from pockets of different depths in adult subjects. Both species were detected more frequently and in higher numbers in deeper periodontal pockets. In addition, *P. gingivalis* was never detected in the absence of *B. forsythus*. Simonson et al. (1992a) found a strong association between *P. gingivalis* and *T. denticola* in plaque samples taken from subgingival sites in 74 Fijians, 24 Colombians and 25 Americans stationed in the Sinai desert. Peterson et al. (1994) demonstrated a similar relationship in a continental US population. Members of the red complex were found together in high numbers in lesions of adult periodontitis (Hosaka et al. 1994, Umeda et al. 1996), in particular, in sites with deeper pockets or more advanced lesions (Si-

monson et al. 1992b, Kojima et al. 1993, Wolff et al. 1993, Ali et al. 1994, Kamma et al. 1995, Kigure et al. 1995). In particular, Kigure et al. (1995) using immunohistochemical techniques, provided graphic demonstration of the relationship between *T. denticola* and *P. gingivalis* in biopsies of subgingival plaque, epithelial and connective tissues from different pocket depths in human periodontitis subjects. They demonstrated that both species were predominant in pockets >4 mm. In 4–6 mm pockets, *T. denticola* was detected at the surface layer of the plaque, while *P. gingivalis* cells were detected in the layer beneath. In deeper pockets, the species co-existed in large numbers. Other studies demonstrated a reduction in the species of this complex after scaling and root planing (Simonson et al. 1992b, Haflajee et al. 1997). Possible mechanisms of pathogenicity of species in the red complex have been reviewed (Socransky & Haffajee 1991, Haffajee & Socransky 1994); however, it is of interest that this trio has been shown to produce proteolytic enzymes such as those sought in the "BANA" test (Loesche et al. 1992) and in the proposed SK013 peptidase test (Seida et al. 1992). The biological basis of the association among *P. gingivalis*, *T. denticola* and *B. forsythus* is not known. However, it has been shown that members of this complex coaggregate strongly in vitro (Grenier 1992a, Onagawa et al. 1994, Yao et al. 1996) and one species of the complex may produce growth factors required by another in that complex (Grenier 1992b, Nilius et al. 1993).

A second complex that was observed in multiple analyses was the orange complex consisting of the *F. nucleatum* subspecies, *F. periodonticum*, *P. micros*, *P. intermedia*, *P. nigrescens*, *Streptococcus constellatus*, *E. nodatum*, *C. showae*, *C. gracilis* and *C. rectus*. The species in this group were closely associated with one another and this complex appeared closely related to the red complex. Other studies have shown an association between members of this complex. For example, Ali et al. (1994) found that *P. intermedia* was always detected in the presence of *F. nucleatum* in subgingival plaque samples from deep pockets in a group of adult periodontitis subjects. *P. micros* and *C. rectus* were occasionally elevated in samples from mobile teeth compared with non-mobile teeth (Grant et al. 1995). Von Troil-Linden et al. (1995)

found that *P. intermedia*, *C. rectus* and *P. micros* were significantly elevated in saliva samples from subjects with advanced periodontitis compared with samples from subjects with initial or no periodontitis. Further, treatment that included systemically administered metronidazole decreased levels of these species and improved periodontal status. Members of the orange complex have been associated with infections in non-periodontal sites. Sundqvist (1992) sampled 65 infected root canals. The most frequently detected organisms included *F. nucleatum*, *P. intermedia*, *P. micros*, *P. anaerobius*, *Eubacterium* sp. and *C. rectus*. Strong associations were found between *F. nucleatum* and *P. micros*, *F. nucleatum* and *C. rectus* and *P. intermedia* and *P. micros*. Summanen et al. (1995) found that the most commonly detected anaerobes in subcutaneous abscesses of intravenous drug users were *F. nucleatum*, *P. micros*, *P. intermedia*, *P. nigrescens*, *A. odontolyticus* and *V. parvula* while *F. nucleatum*, *P. intermedia*, *P. nigrescens*, *P. micros* and *Eubacterium* species were most commonly detected in 46 cases of anaerobic empyema (Civen et al. 1995). *P. intermedia* has been shown to stimulate the growth of *S. constellatus* in vitro and the combination of these species often produced fatal pulmonary infections in a mouse model system (Shinzato & Saito 1994). One possible mechanism for co-aggregation between *E. nodatum* and strains of *F. nucleatum* was described by George and Falkler (1992). The coaggregation was thought to involve a protein receptor on *F. nucleatum* and a heat-stable protein or polysaccharide component on *E. nodatum*.

While species within complexes were closely associated, the complexes themselves seem to have specific relationships with one another (Fig. 12). Species such as *A. naeslundii* genospecies 2 (*A. viscosus*) and members of the yellow and green complexes were less commonly associated with members of the red and orange complexes than with each other. The 2 species in the purple complex were strongly related to each other and to a lesser extent to members of the orange, green and yellow complexes. The reasons for these relationships among complexes is unclear but it might be speculated that antagonistic relationships (Grenier 1996) may exist or that environments selective for one group may be less hospitable to a second group of organisms.

On observing Fig. 14, it is tempting to speculate that the relationships observed mimic to some extent the microbial succession patterns that may exist in developing plaque. It seems likely that *A. naeslundii* genospecies 2 (*A. viscosus*) and the streptococcal species are early colonizers followed by *Capnocytophaga* sp., *C. concisus*, and *E. corrodens*. *A. odontolyticus* and *V. parvula* may be bridging species leading to the orange complex and ultimately, at some sites, to the red complex. This pattern of colonization is part speculation and in part based on data about the composition of plaques in various clinical conditions (Moore & Moore 1994). It is interesting to note that a treatment such as SRP has a profound effect on the species of the red complex and virtually no effect on the majority of other species except for an increase in *A. naeslundii* genospecies 2 (*A. viscosus*) and some of the streptococcal species (Haffajee et al. 1997).

The red complex showed the strongest relationship with the clinical parameters considered most meaningful in periodontal diagnosis. For example, the individual species in the complex as well as the complex itself related very strongly with pocket depth and bleeding on probing. As described earlier in the Discussion, other investigators have noted the relationship between pocket depth and members of the red complex (Gmur et al. 1989, Kigure et al. 1995, Simonson et al. 1992b). The orange complex also related to pocket depth, although this relationship and the relationship to other clinical parameters was less striking.

Knowledge of the associations between subgingival species maybe used to interpret and guide periodontal therapy. The recognition that an inter-related complex of species such as the red complex exists suggests that therapies that affect 1 of these species may influence the colonization of the other species in this group. In addition, it might be speculated that altering the orange complex might prevent subsequent colonization of species in the red complex providing the therapist with a second route to control this group of organisms. *A. actinomycetemcomitans* serotype b related poorly to members of the red and orange complexes suggesting that therapies effective against one set of pathogens may not necessarily be effective against others. This indeed appears to be the case for SRP

Further, other studies have suggested that different therapies affect different segments of the microbiota and the use an incorrect therapy may be ineffective in lowering the target species and might adversely affect other members of the microbial community (Haffajee et al. 1996).

The data of the present investigation suggest the nature of some of the microbial complexes in subgingival plaque. Although the number of plaque samples was quite large and the number of species evaluated was reasonably extensive, the investigation represents an initial attempt at evaluating inter-relationships among subgingival species. While some of the associations depicted may not be completely accurate, certain associations were repeatedly using different analytical techniques and were in accord with data in the literature. Further, certain complexes, and members within the complexes, related strongly to clinical parameters of inflammation and periodontal destruction. The data provide a framework for understanding the complex ecology observed in plaque and could be used to guide approaches to diagnosis and therapy of periodontal diseases.

Zusammenfassung

Mikrobielle Komplexe in der subgingivalen Plaque

Es wurde seit einiger Zeit erkannt, daß bakterielle Spezies in der subgingivalen Plaque in Komplexen existieren. Der Zweck der vorliegenden Untersuchung war es, die Definition solcher Gemeinschaften unter Verwendung der Daten einer großen Anzahl von Plaqueproben sowie verschiedener Clusterund Ordnungstechniken zu versuchen. Bei 185 Personen (Altersdurchschnitt 51 ± 16 Jahre) wurden von der Mesialfläche eines jeden Zahnes mit (n=160) oder ohne Parodontitis (n=25) subgingivale Plaqueproben genommen. Unter Verwendung von Ganz-Chromosomen-DNA-Sonden und der Schachbrett-DNA-DNA-Hybridisierung wurden die Vorkommen und der Anteil von 40 subgingivalen Taxa bei 13261 Plaqueproben bestimmt. Zu jedem Termin wurden an 6 Stellen pro Zahn klinische Messungen durchgeführt. Ähnlichkeite zwischen Speziespaaren wurde unter Verwendung von Phi-Koeffizienten er-

rechnet und die Speziescluslerung erfolgte unter Verwendung einer imgewichteten Durchschnitt-orientierten Sortierung. Mit alsanalytischen Methoden wurden 5 Hauptkomplexe bestandig beobachtet. Ein Komplex bestand aus der eng verbundenen Gruppe: *Bacteroides forsythus*, *Porphyromonas gingivalis* und *Treponema denticola*. Der zweite Komplex bestand aus einer eng verbundenen Zentralgruppe mit den Subspezies von *Fusobacterium nucleatum/periodonticum*, *Prevotella intermedia*, *Prevotella nigrescens* und *Peptostreptococcus micros*. Spezies, die mit dieser Gruppe assoziiert waren, sind folgende: *Eubacterium nodatum*, *Campylobacter rectus*, *Campylobacter showae*, *Streptococcus constellatus* und *Campylobacter gracilis*. Der dritte Komplex bestand aus *Streptococcus sanguis*, *S. oralis*, *S. mitis*, *S. gordonii* und *S. intermedius*. Der vierte Komplex umfaßte die 3 *Capnocytophaga* Spezies, *Campylobacter concisus*, *Eikenella corrodens* und *Actinobacillus actinomycetemcomitans* Serotyp a. Der fünfte Komplex bestand aus *Veillonella parvula* und *Actinomyces odontolyticus*. *A. actinomycetemcomitans* Serotyp b, *Selenomonas noxia* und *Actinomyces naeslundii* Genospezies 2 (*A. viscosus*) lagen außerhalb der Clustergebilde. Es wurde eine auffallende Verbindung zu den klinischen Parametern der Parodontalerkrankung, insbesondere Taschentiefe und Sondierungsblutung.

Résumé

Complexes microbiens dans la plaque sousgingivale

Il est reconnu depuis quelques temps que les espèces bactériennes existent sous forme de complexes dans la plaque sous-gingivale. Le but de la présente étude était de tenter de définir ce genre de communautés en utilisant un nombre important sur un grand nombre d'échantillons de plaque et différentes méthodes de groupement et de classement. Des échantillons de plaque sous-gingivale ont été prélevés dans la plaque sous-gingivale de 185 sujets (âge moyen 51 ± 16 ans) avec (*n*=160) ou sans (*n*=25) parodontite. La présence et les niveaux de 40 taxons sous-gingivaux ont été déterminés dans 13261 échantillons de plaque, en utilisant des sondes d'ADN génomique entier et un hybridation ADN-ADN en damier. Les mensurations cliniques ont été faites dans 6 sites par dent à chacune des visites. Les corrélalts entre les paires d'espèces ont été calculées en utilisant les coefficients phi et les espèces ont été groupées à l'aide d'un tri moyen des liaisons sans pondération (average unweighted linkage sort). Le classement des communautés a été fait à l'aide de l'analyse des composantes principales et de l'analyse des correspondances. 5 complexes principaux ont été régulièrement observés avec toutes les méthodes d'analyse. L'un des complexes était constitué par le groupe étroitement apparenté: *Bacteroides forsythus*, *Porphyromonas gingivalis* et *Trepo-*

nema denticola. La second complexe était constitué par un noyau étroitement apparenté; le comprenant des membres des sous-espèces *Fusobacterium nucleatum/periodonticum*, *Prevotella intermedia*, *Prevotella nigrescens* et *Peptostreptococcus micros*. Les espèces associées avec ce groupe comprenaient: *Eubacterium nodatum*, *Campylobacter rectus*, *Campylobacter showae*, *Streptococcus constellatus* et *Campylobacter gracilis*. Le troisième complexe était composé de *Streptococcus sanguis*, *S. oralis*, *S. mitis*, *S. gordonii* et *S. intermedius*. Le quatrième complexe était constitué par 3 espèces de *Capnocytophaga*, *Campylobacter concisus*, *Eikenella corrodens* et *Actinobacillus actinomycetemcomitans* sérotype a. Le cinquième complexe était composé par *Veillonella parvula* et *Actinomyces odontolyticus*. *A. la* périphérie. *A. actinomycetemcomitans* sérotype b, *Selenomonas noxia* et *Actinomyces naeslundii* génospecies 2 (*A. viscosus*), avaient très peu de relation entre eux et avec les 5 complexes principaux. Le premier complexe avait une relation frappante avec les mesures cliniques de la maladie parodontale, en particulier la profondeur des poches et le saignement au sondage.

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