

# Periodontal microbial ecology

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The authors have taken the liberty of presenting this manuscript in two parts. The first is a brief 'primer' on microbial ecology, because, although the importance of microbial ecology in periodontal diseases is widely recognized, most of us do not know precisely what is meant by the term. The second section is a rather extensive overview of current studies of oral microbial ecology based almost entirely on recent *in vivo* studies.

## Principles of microbial ecology

Microbial ecology is concerned with the interrelationships between microorganisms and their environments. Habitat ecologists, including periodontal microbiologists, examine the microorganisms of a particular habitat and attempt to analyze the effects of the microorganisms on their environment and the influence of the habitat on its residents. Many of the principles of microbial ecology were outlined in a superb monograph by Alexander (2) and will be briefly described below along with some periodontally relevant examples to highlight some of the points.

### The ecosystem

A key concept in microbial ecology is the ecosystem. An ecosystem is the complex of organisms in a specified environment and the nonmicrobial surroundings with which the organisms are associated. The ecosystem includes the assemblage of species and the organic and inorganic constituents characterizing that particular site. Each ecosystem has a collection of organisms and nonmicrobial components unique to it and it alone. The organisms inhabiting a given site constitute a community. The assemblage of organisms constituting a community contains populations of individual microbial species. This leads to a hierarchy from ecosystem to community to population to the single cell. One of the goals of this issue of

*Periodontology 2000* will be to define the assemblage of species in the periodontal habitat, to determine how changes in the habitat affect the community and how the community affects its habitat.

### Habitat and niches

The habitat is the site at which a population or community grows, reproduces or survives. The role of an organism in a habitat is its niche. Niche does not connote location but rather function. A species can have one niche in one habitat and a different niche in another habitat.

### Microbial succession

In a developing ecosystem, certain species termed pioneer organisms colonize first. These species are often replaced by other species after they have altered the habitat, making it suitable for colonization by other species. There are two kinds of microbial succession. In autogenic succession, the sequence of species is brought about because the resident populations alter their surroundings in such a manner that they are replaced by species better suited to the modified habitat. In allogenic succession, one type of community is replaced by another because the habitat is altered by nonmicrobial factors such as changes in the physical or chemical properties of the region or changes in the host. Factors contributing to succession are:

- provision by one community of a nutrient that confers an ecologic advantage to the species in the next stage of succession;
- the making available by one population of a constituent present in insufficient supply to allow for growth of a later population;
- alteration in concentration of an inorganic nutrient;
- modification of heterogeneous substrates such as animal tissue;
- an autointoxication effect;

- elimination of an organism by physical means;
- the appearance of barriers due to environmental feedback.

The development of gingivitis provides an example of microbial succession as well as species habitat interaction. Loe et al. (90) and Theilade et al. (170) demonstrated that dental plaque caused gingivitis. It was shown that withdrawal of toothbrushing for 28 days in periodontally healthy volunteers resulted in the rapid accumulation of plaque on the teeth. Gingivitis developed in all subjects in 10–21 days. Re-establishment of oral hygiene procedures removed the plaque and reversed the gingivitis. Stained smear preparations obtained during the 28-day time course revealed initial colonization by gram-positive cocci and rods, followed by gram-negative cocci and rods, then fusobacteria and filaments, and finally spirilla and spirochetes. Appearance of clinical gingivitis related to the appearance of the gram-negative forms. These data were in accord with other studies that demonstrated microbial succession in plaque development (140, 161, 188). The Danish studies associated certain bacterial morphotypes with a change in the clinical status of the site, i.e. the development of gingivitis. Data presented below demonstrate that species of the red and orange complexes (described later) are more prevalent and found in higher numbers in lesions of established gingivitis. The same taxa are even more prevalent and found in higher numbers in suppurating lesions. Thus, a change in plaque composition appears to affect the habitat, resulting in clinically apparent gingivitis. Other studies indicate

that a change in habitat such as the development of gingivitis also affects plaque development. Plaque accumulated much more rapidly after cleaning, at sites that exhibited gingivitis than at sites that were periodontally healthy (25, 132). Thus, it is possible to postulate a scheme of microbial succession followed by reciprocal host–bacterial interaction (Fig. 1). The members of each color-coded complex are described in a later section. Initial colonization appears to involve members of the yellow, green, and purple complexes along with *Actinomyces* species. This leads to autogenic succession in which members of the orange and then red complexes become more dominant. The presence of increased levels of the last two complexes is hypothesized to lead to a change in the habitat, manifested clinically as gingivitis. The gingivitis in turn favors further proliferation by members of not only the orange and red complexes, but probably members of the early colonizing species as well. This cycle could be broken in a number of ways. The first way would be to eliminate all plaque; this partially successful strategy is the one most commonly employed today. The second would be to eliminate members of the red and/or orange complexes. This would probably limit gingivitis and its feedback effect of greater plaque development. The third would be to decrease gingivitis by a non antimicrobial approach, leading to decreased plaque accumulation and possibly diminished red and orange complex development. It is worth noting that the effect of a prevention or treatment regimen would be an example of allogenic succession.

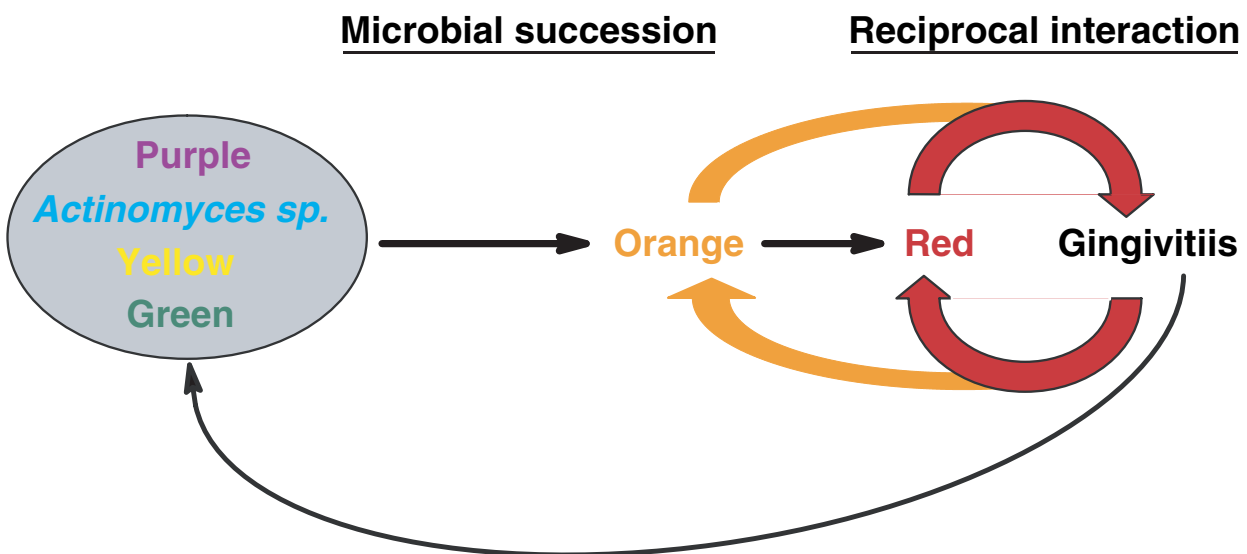


Fig. 1. Hypothesized relationship between the addition of species during microbial succession leading to the development of gingival inflammation. In turn, the increased

inflammation would result in increased growth of colonizing species.

## Factors limiting colonization

Certain factors limit colonization. One obvious limitation is the available physical space. Preemptive colonization is a second situation in which prior colonization by one species excludes another. Exclusion results from the occupation of a niche by one species that might have been taken by the other. The first species performs the activities, uses the nutrients and/or occupies the physical sites of the excluded species. Environmental resistance is the restriction in numbers of individuals or biomass imposed by physical, chemical or biological factors of the ecosystem. The condition holding the population in check is considered to be a barrier. Preemptive colonization is a major means of exclusion of late-comers, and it is, together with the physical and chemical barriers in the locale, one of the components of environmental resistance. Any periodontal investigator who has attempted to introduce a test species into periodontal plaque can attest to the effectiveness of these barriers.

## Dissemination of organisms

In order for organisms that are restricted in nature to live in association with a suitable host to continue to survive, their dispersal is essential. Dissemination can be via active or passive means. For example, growth or motility can actively move a species from one site to another within the oral cavity. Passive dissemination occurs both within the oral cavity and

from subject to subject. Microorganisms show centers of dispersal, regions from which species are spreading or have spread. At this site, conditions are favorable for an increase in density of the species and the site serves as a point from which the species can emanate. This site is referred to as a reservoir of infection. The greater the efficiency of the dispersal mechanism, the smaller the number of organisms needed for dispersion. Oral species might disseminate from subject to subject via droplet infection or infection via inanimate objects. The tools of molecular epidemiology have been used to demonstrate vertical transmission (parent to offspring) for *Actinobacillus actinomycetemcomitans* (124, 125, 128) and *Porphyromonas gingivalis* (125), as well as horizontal transmission from spouse to spouse for *P. gingivalis* (143, 175). Another example of transmission comes from early studies of acute necrotizing ulcerative gingivitis, also known as Trench mouth. This disease was transmitted among troops in World War I and later to the civilian population (154).

Riviere et al. (141) found that spirochetes and *P. gingivalis* were more prevalent in diseased sites of diseased subjects than in healthy sites of diseased subjects (Fig. 2). Of major significance was the additional finding that healthy sites of diseased subjects harbored these species more frequently than healthy sites of healthy subjects. This finding has been confirmed in other studies, as will be described below. The authors speculated that the deeper pockets of diseased subjects were acting as reservoirs for spread of infection to healthy sites. This is indeed

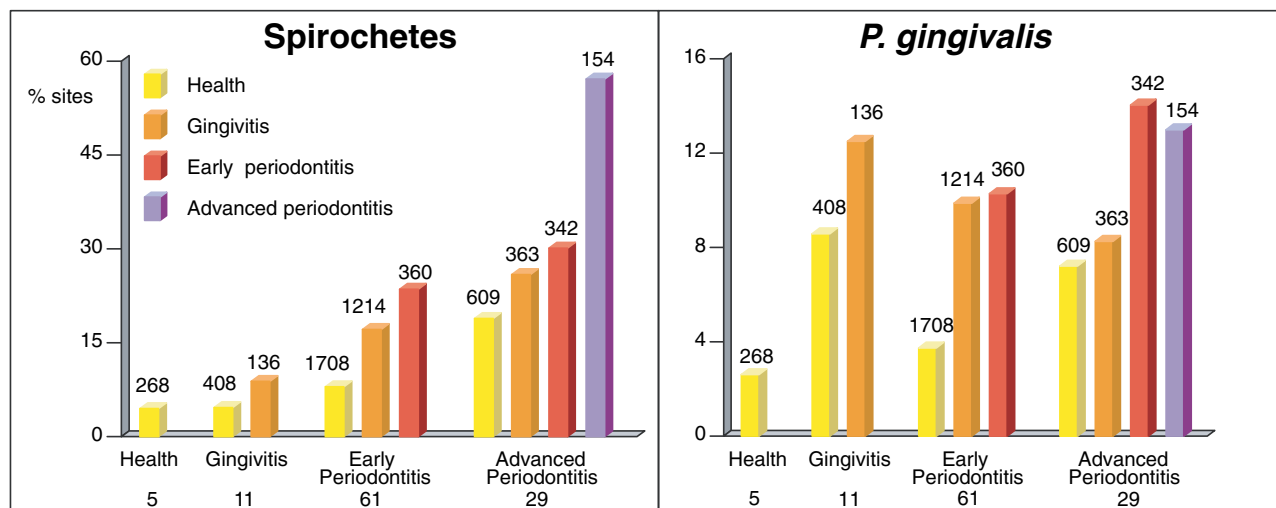


Fig. 2. Bar charts of the frequency of detection of spirochetes and *P. gingivalis* in sites and subjects with different clinical characteristics. Spirochetes were identified using phase contrast microscopy and *P. gingivalis* by immunocytochemistry. The numbers over the bars indicate the

number of samples examined. The bar colors indicate the characteristics of the sampled site. The labels under the bars indicate the classification of the subject, and the numbers under the bars indicate the number of subjects examined. Data adapted from (141).

a possibility, although alternative hypotheses are possible.

## Infectious disease

Infectious diseases represent a category of population–environment interactions involving a host plus a microorganism with the potential for both colonization and pathogenesis. From the ecologic standpoint the governing feature of the ecosystem is the living animal/human. The host must be colonizable, i.e. it must be receptive to invasion by the particular disease agent. Not all higher animals are colonizable by all pathogenic bacteria, fungi or viruses. Three kinds of barriers underlie lack of receptiveness:

- the barriers of the nonreceptive host;
- the factors associated with the resistance of the receptive host prior to its first contact;
- the obstacles to further bacterial development or activity that appear as a consequence of infection.

In effect, there is an environmental feedback, a modification of the habitat resulting from the presence of one or more bacterial populations, a change that can affect the size, activity or survival of the invading population or one or more segments of the community. The production of antibody to colonizing species is an example of environmental feedback. This will be discussed in greater detail later in this chapter.

## Successful colonization

Success in colonization of a species depends on

- presence at the colonizable place at the correct time;
- possession of survival capability permitting prolonged viability in deleterious circumstances;
- ability to obtain all nutrients from the ecosystem;
- capacity to tolerate all of the ecologically significant nonmicrobial factors of the environment, e.g. pH, O<sub>2</sub> levels, temperature, osmotic pressure, oxidation reduction potential;
- possession of mechanisms to overcome or cope with environmental resistance attributable to viable hosts;
- ability to overcome or cope with environmental resistance attributable to species already in the habitat;
- capability to grow as rapidly as one's neighbors;
- ability to adhere to appropriate surfaces.

The role of some of these factors in the formation of microbial complexes was discussed in Socransky et al. (156).

## The climax community

The interaction between the microbial and nonmicrobial components of an ecosystem ultimately leads to a form of stabilization in which microbial and nonmicrobial forms exist in harmony and equilibrium with their environment. This is the climax community. This remains reasonably stable over time and reflects a dynamic situation in which cells are dying and being replaced. The climax is essentially a self-replicating entity that reproduces itself with remarkable fidelity. Given the same initial physical and chemical site characteristics or identical hosts, the same general successional sequences will be initiated and fostered, giving rise to remarkably similar climax communities. The climax can be modified from time to time by exogenous forces. The equilibrium tends to be restored as the habitat returns to its original state. At other times, environments may be irreversibly altered, leading to a different steady state and a different climax community. The climax contains many niches and the species occupying each is uniquely fit, at least among species having access to the locale, for the function associated with the niche. Inasmuch as there are numerous niches or potential functions, particularly when there is an intermeshing of food chains, many physiologically different groups of organisms can coexist indefinitely.

Most developed dental plaques represent climax communities. Minor perturbations probably result in a re-development of the same community, albeit with somewhat altered proportions of species. Preventative or therapeutic strategies probably encounter a tendency of the ecosystem to return to the original equilibrium after termination of treatment. This tendency can be frustrating to the clinician in that it necessitates a prolonged maintenance phase after therapy. Often, we wish for more profound changes in the periodontal microbiota after therapy. However, as the old adage states: 'be careful what you wish for, because you might get it'. More permanent changes can be brought about in the microbiota by employing potent exogenous forces of a persistent nature. The microbiota of a recent refractory patient is instructive. The subject is a dental professional who exhibited initial signs of periodontitis about 10 years ago. He was treated initially by scaling and root planing and later by surgery. The disease continued to progress, leading to the loss of five teeth in 2 years accompanied by a very painful symptomatology. He took in sequence: systemic tetracycline, ampicillin, amoxicillin + metronidazole, clindamycin, ciprofloxacin, long-

term low dose doxycycline and various local agents including Actisite, chlorhexidine, and triclosan over a period of about 4 years. When he was sampled, his microbiota had been simplified so that *Streptococcus oralis* made up over 95% of the cultivatable microbiota. Spirochetes, motile forms, *Fusobacterium*, *Veillonella*, *Prevotella*, *Capnocytophaga*, and *Eikenella* were undetectable by culture or DNA probe. A new climax population had been established in this subject that prevented establishment of additional taxa. Unfortunately, this climax population was not host-compatible. Each of the above treatments presumably lowered the numbers of the new climax population and relief was achieved for 5–6 weeks. However, the same species would re-emerge after therapy (the new climax community had no competition) and damage to the habitat would resume. This case reinforces the importance of controlling pathogens but without deleterious changes in the remaining ecosystem. It is essential to clearly define host compatible ecosystem(s) to provide desired therapeutic end points. The effects of various forms of therapy on the subgingival climax community will be described in Haffajee & Socransky (65).

## Current studies of oral microbial ecology

The 215 cm<sup>2</sup> surface area of the oral cavity (23) presents numerous surfaces for microbial colonization. The diverse mucosal surfaces are lined by different types of epithelium, while hard surfaces are presented by the teeth or various types of replacements. These surfaces are continuously bathed in a 'bulk fluid', primarily saliva, and thus provide excellent environments for biofilm development. The microorganisms that colonize these surfaces produce biofilms of differing complexities depending on intraoral location, genetic background and environmental factors individual to each subject. As many as 700 bacterial species may colonize the surfaces of the oral cavity (120). It has been shown that marked differences in microbial composition can occur from person to person, from one type of intraoral location to another in the same subject (e.g. tongue dorsum vs. supragingival plaque) and from similar types of locations in the same subject (e.g. two periodontal pockets). As complex as this microbiota may appear, the approximately 500 species that may be detected in subgingival plaque are a miniscule fraction of the estimated billion or more bacterial species present on

Earth that potentially could have colonized the oral cavity. Clearly, potent forces control the establishment of the oral microbiota, govern its composition and influence its re-development once the ecosystem has been disturbed. It is the intent of this section to describe some of the factors that influence the development and composition of human intraoral biofilms and that lead to the variation that may be seen from subject to subject and from site to site within an individual.

The study of host–microbial relationships is not easy, in part because of the complexity of the microbiota, and in part because of the multiplicity of influences that impact on the microbial composition of a biofilm in a given site. All biofilms consist of three components (Fig. 3): the surface needed for the attachment of the biofilm, the biofilm community itself, and the 'bulk fluid' that passes over the biofilm, providing nutrients to the colonizing organisms, removal of waste products and transport of cells to new colonization sites. Each of these 'components' may be altered by local or subject level factors that may influence the microbial composition of the colonizing biofilm. For example, the nature of the surface presented for colonization may be influenced by the type of tissue presented, the genetic background of the subject (which might alter surface

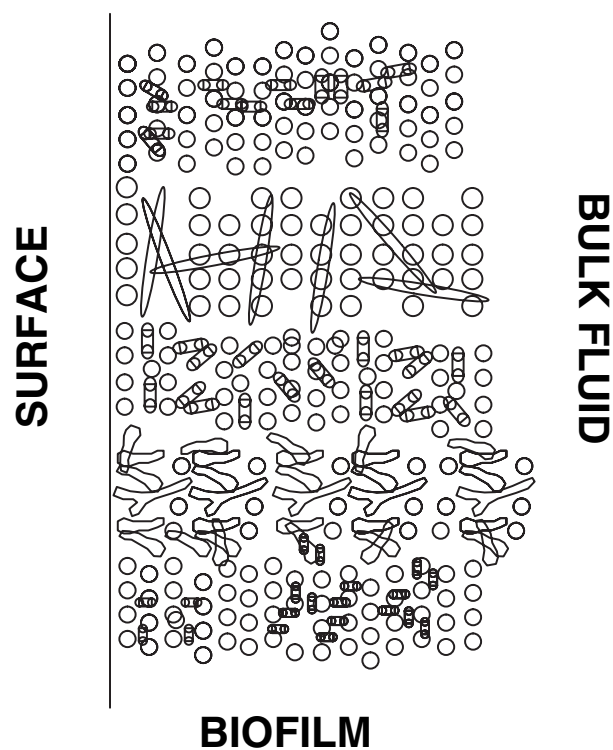


Fig. 3. Components of a biofilm.

receptors), the possible introduction of artificial surfaces, hygiene practices, etc. Given this heterogeneity, it is essential to examine large numbers of samples from similar and different locations to attempt to discriminate the local and host-level factors that govern the composition of human oral biofilms. Critical to our understanding of these factors is the recognition that these relationships are not one-way. The host may influence the microbiota, but in turn the microbiota influences the host on a local and perhaps systemic level. The bacterial colonization that elicits a local inflammatory response is in turn influenced by the inflammatory response which in turn... You get the picture. The interactions between bacterial species in a biofilm and between bacterial species and the nonbacterial habitat are dynamic. They reflect a back and forth interplay between host and colonizing species. When investigators sample a range of biofilms, they get a series of snapshots at individual points in time of this dynamic relationship, which they try to integrate into a coherent picture. Understanding of the ecologic relationships within intraoral biofilms and between biofilm composition and the host has been slowed by the difficulty in obtaining sufficient 'snapshots' of the microbial composition of biofilms taken from carefully monitored clinical situations. The major limiting factor has been the lack, until recently, of microbial techniques that are specific and rapid enough to permit evaluation of the large numbers of samples needed for meaningful *in vivo* studies. The next section will provide an overview of the techniques that have been employed to study the composition of intraoral biofilms and will describe the development of methodologies in recent years that permit meaningful ecologic studies to take place.

### Detection and enumeration of bacterial species in complex biofilm samples

The study of infectious diseases has traditionally focused on one or a small number of pathogens in a given infectious disease. Even when samples are taken from areas where complex mixtures of species coexist, emphasis has been placed on seeking a limited number of likely pathogens from that site. The remaining organisms are often considered to be 'normal flora'. In most instances such species might well be host-compatible, common residents of the sampled site; however, in some instances, these species might contribute to the pathogenesis of the observed condition. In addition, the absence of some host compatible species may be as important in

disease initiation or progression as the presence of one or more pathogenic species. Examination of complex mixtures of microorganisms has been hampered by at least two factors. The first is the tradition of focusing on a small number of species thought to be pathogenic. The second is the absence of useful, rapid identification techniques to evaluate large numbers of bacterial species in large numbers of samples taken from areas where complex microbiotas exist. This capability has been slow to develop and thus studies of the complex ecologic relationships among bacterial species and between bacterial species and the host as well as studies of the effects of different therapies on the subgingival ecosystem was delayed until recent years.

The earliest studies of subgingival biofilm composition involved the techniques of light microscopy. These techniques were reasonably rapid, but limited in the precision of identification of individual bacterial species. Thus, while about nine morphotypes could be conveniently recognized (88), there were actually as many as 500 bacterial species in oral biofilm samples (120). Light microscopic examination of wet mount preparations became popular for a number of years as part of 'monitored, modulated periodontal therapy' in which eradication of motile forms including spirochetes was employed as an aid to guide the intensity of periodontal therapy (70). The development of the electron microscope permitted examination of biofilm samples with greater resolution. Electron microscopic techniques allowed a somewhat finer distinction of microbial groups based on cell wall ultrastructure and the presence and arrangement of various appendages to the microbial cell such as axial filaments or flagella. Electron microscopy by itself could not precisely identify a cell to the species level; however, in combination with immunocytochemical techniques or *in situ* hybridization, the technique permitted precise localization of bacterial cells in relation to each other and the host when block sections were taken from the subjects (72, 110–113). The great strength of the microscopy techniques, including the promising confocal microscopy, is the delineation of spatial arrangements of the organisms. The great weakness of these techniques from an ecologic perspective is that they are slow and labor intensive and thus limit the number of samples that can be examined. In addition, precise speciation using immunologic or hybridization techniques can only be performed for a very limited number of species in any given sample.

For many years, the major technique available to researchers to identify plaque bacteria was to

cultivate the organisms and identify the species by their phenotypic traits; a rather time-consuming, labor-intensive, and expensive undertaking (Table 1). As a result, relatively few plaque samples in small numbers of subjects could be examined. For example, in studies performed at The Forsyth Institute between 1982 and 1988, 300 subgingival plaque samples from actively progressing periodontal lesions before and after therapy were evaluated using cultural techniques (37, 62). At the time, this was considered a large scale study, and indeed the characterization of approximately 15,000 isolates in a 6-year period was a major undertaking. Nonetheless, only two or three samples were taken per subject, at each time point, from a total of 88 subjects. The classic studies of Moore & Moore (105), in which they examined the composition of subgingival plaque

samples in periodontal health and different states of periodontal disease, employed cultural techniques to examine over 17,000 isolates from over 600 periodontal sites. This represented a huge body of work on, by current standards, a limited number of samples. Despite their limitations, these studies and other cultural studies of the 1970s and 1980s defined the species thought to be important in the initiation and progression of periodontal diseases as well as species thought to be host-compatible or beneficial. The major strength of culture is that, in theory, the majority of the bacterial species sampled will grow and be identified. However, difficult to grow species and uncultivable species, such as many of the spirochetes, will not be detected by this technique. Other species require special conditions for their growth. If these conditions are not met, their

**Table 1.** Methods to determine microbial composition of oral samples

Method	Strengths	Weaknesses	Application
Predominant cultivable	Can detect unrecognized species; provides cultures for further analysis.	Extremely time consuming and expensive; often difficult to speciate cultures.	Studies of new ecosystems
Selective media	Modest numbers of samples for modest numbers of species.	Few useful selective media available; often media are too selective or not selective enough; expensive.	Studies of limited scope involving 1–10 species in modest numbers of samples.
Immunofluorescence	Specificity; reasonably rapid.	Limited number of useful antisera; small numbers of samples may be run.	Maybe more useful for diagnostic than ecologic or treatment studies.
PCR	Sensitivity; specificity.	Not quantitative (presence/absence); expensive; dependent on amplification.	Detection of species in a subject (prevalence); detection of low numbers of species post therapy.
Real time PCR	Sensitivity; specificity; quantitative.	Comparatively slow; very expensive; limited in numbers of samples/species.	Studies where quantitation of a very limited range of species in low to modest numbers of samples is required.
DNA–DNA hybridization	Sensitivity; specificity; quantitative.	Confined to species for which probes are available; modest number of species for modest numbers of samples.	Seeking a specific set of species in modest numbers of samples.
Checkerboard DNA–DNA hybridization	Sensitivity, specificity, quantitative; can use entire sample; large numbers of species and samples; inexpensive.	Confined to species for which probes are available; possibility of cross-reactions.	Studies of large numbers of species in large numbers of samples, e.g. ecology and treatment studies.
16S rDNA amplification cloning	Detection of cultivable and uncultivable species; phylogenetic positioning of taxa.	Extremely expensive; extremely small numbers of samples.	Survey a range of species that may occur in specific habitats.

numbers will be severely underestimated. For example, the importance of the recognized periodontal pathogen, *Tannerella forsythia* (*Bacteroides forsythus*), was not confirmed by culture until the unusual growth requirements of this organism were determined (182). However, it took immunofluorescence techniques to initially indicate the relatively high prevalence of *T. forsythia* in subgingival plaque samples of chronic periodontitis subjects (52). Despite its drawbacks, culture still has a place in studies of periodontal microbiology, particularly for the identification of organisms in unusual clinical conditions and in situations where pure cultures are needed for additional analysis.

Light microscopy, electron microscopy, and cultural techniques have largely been supplanted for studies of microbial ecology and periodontal treatment by more rapid techniques. Antibody-based methods were among the first to be used to enumerate specific species of microorganisms without their cultivation. Immunofluorescence techniques and enzyme-linked immunosorbent assay (ELISA) techniques have been successfully employed to examine a limited range of bacterial species in larger numbers of plaque samples than had been examined using cultural techniques (8, 46, 51, 52, 56, 97, 187). These techniques are dependent on the specificity of the developed antibodies to specific taxa. Properly prepared and evaluated monoclonal or polyclonal antisera provide a sensitive and specific method of detecting specific bacterial species in dental plaque samples. These techniques have the advantage that samples do not have to be cultured for enumeration, and they are rapid and less expensive than culture. However, they are limited to species for which reagents have been developed. In addition, it is difficult to use these techniques, particularly immunofluorescence techniques, to evaluate large numbers of species in very large numbers of plaque samples. Recently, however, Singleton et al. (152) have described a fully automated microscopic bacterial enumeration method to permit larger numbers of samples to be evaluated. Another disadvantage of antibody-based techniques is the time required to develop and validate specific antisera to new species.

In the last decade, polymerase chain reaction (PCR) has been used to detect the presence of selected bacterial species in subgingival plaque samples (5, 82, 92, 101, 102, 172). Given the appropriate primers, this method is rapid and simple and is able to detect very small numbers of cells of a given species. It has the disadvantage of not providing

quantitative data (until recently) (69, 95), but usually indicates the presence or absence of a species in the sample. For some applications, this would be sufficient. For applications where the relative levels of species are important, PCR may not be ideal. In addition, examining large numbers of species in large numbers of samples is difficult and may not be cost effective. Real time PCR has been added to the potential methods for examining the composition of biofilm samples (69, 95). This method has the advantages of being specific, sensitive, and providing quantification of the level of selected species in biofilm samples. However, the technique suffers similar limitations to conventional PCR in that it is currently not suitable for screening large numbers of samples for large numbers of bacterial species.

DNA probes provide another approach to identification and enumeration of bacterial species in complex communities such as dental plaque. Oligonucleotide probes are short probes designed to identify unique regions of DNA within cells of a given bacterial species. It has been suggested that these probes are highly specific and the likelihood of cross-reactions with other species is very low. A number of studies have utilized these probes to identify periodontal bacteria (47, 103, 104, 106). Because they target a limited segment of the DNA of an organism, oligonucleotide probes tend to be less sensitive for the detection of low numbers of bacteria than whole genomic probes. However, oligonucleotide probes directed towards targets that have multiple copies in the cells such as 16S rRNA have the potential to be more sensitive. To date, there have been no large scale studies of the subgingival microbiota that have utilized oligonucleotide probes for the detection and enumeration of a wide range of bacterial species.

Whole genomic DNA probes have been used extensively in studies evaluating the composition of subgingival plaque (24, 61, 94, 116–118, 156, 183, 184). Whole genomic probes are constructed using the entire genome of a bacterial species as the target and thus can be quite sensitive. One of the criticisms of these probes is that the use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. Other concerns have been that the whole genomic DNA probes might not detect all strains of a given species and that the probes would have a low sensitivity in terms of the numbers of cells that they detect. Investigations at The Forsyth Institute, however, using whole genomic DNA probes have indicated that many of the



concerns regarding their use are unjustified or can be overcome (159).

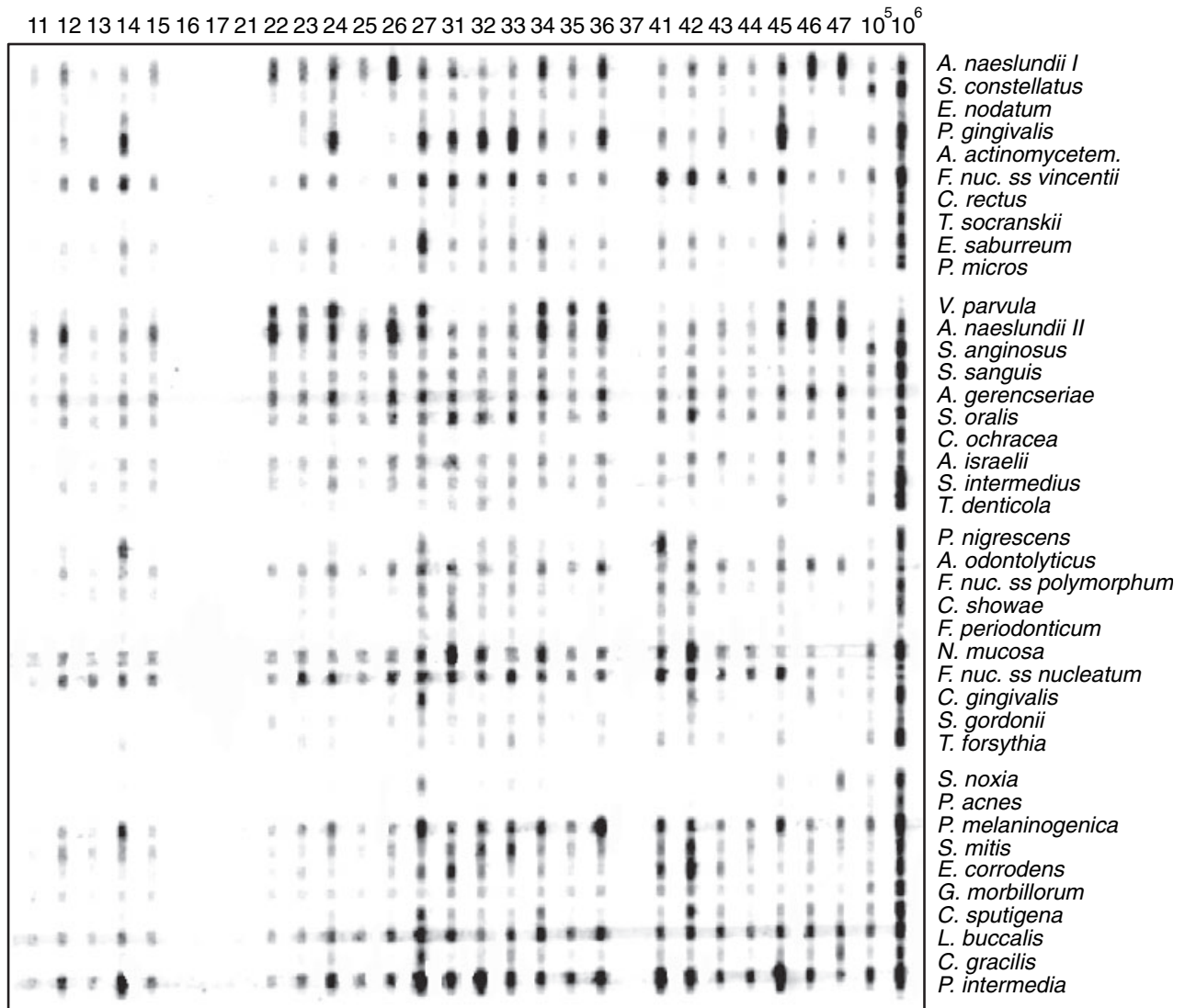
DNA probes can be very effective for the detection of bacterial species, but when employed in the typical format (one probe against multiple targets), only limited numbers of probes can be employed to enumerate relatively large numbers of samples. Checkerboard format procedures, whether employing direct or reverse hybridization procedures, can extend markedly the number of samples evaluated for a wide range of bacterial species. Paster et al. (9, 122) have combined the potential high specificity of the oligonucleotide probe approach with a 'reverse capture' checkerboard format. The investigators overcame the sensitivity issue of the oligonucleotide probe by amplifying the 16S rDNA region of DNA extracted directly from biofilm samples using 'consensus primers' (primers that will permit amplification of a wide range of bacterial taxa). Oligonucleotide probes specific for different species are fixed in parallel lanes on a nylon membrane and individual samples which were labeled by digoxigenin during the PCR amplification are hybridized in parallel channels at right angles to the probe lanes. This technique permits the detection of at least 28 taxa in 44 samples on a single nylon membrane (9). The technique has the advantages of being rapid, relatively inexpensive, and potentially specific to species, genus or family level (at the choice of the investigator). It also provides the advantage of detecting uncultivable species. It has the disadvantage of not permitting accurate quantification and being somewhat more expensive than the direct checkerboard technique, described below, because of the need to amplify the sample using PCR. In addition, amplification by PCR might introduce some bias into the results because of possible differences in amplification of target species in the biological sample.

The 'direct' checkerboard DNA-DNA hybridization technique (Fig. 4) offers a number of advantages for the study of multiple species of bacteria in large numbers of samples containing complex mixtures of microorganisms. As this technique provides the data for most of the examples provided in this chapter, it will be described in somewhat more detail. It must be emphasized that this technique is not the only method of value for the study of the composition of oral biofilms. However, the capability of studying large numbers of samples for large numbers of species does provide a major benefit for studies of oral microbial ecology. The checkerboard technique is rapid, sensitive, and relatively inexpensive. It overcomes many of the limitations of cultural

microbiology, including loss of viability of organisms during transport, the problem of enumerating difficult to cultivate (or even uncultivable) species, and the difficulty encountered in speciating certain taxa that are difficult to grow or exhibit few positive phenotypic traits. Another advantage is that the entire sample may be employed without dilution or amplification (with appropriate regard to total sample size) overcoming problems in quantification imposed by either serial dilution (used in culture) or PCR amplification procedures. The technique in its original format evaluates 28 samples for their content of 40 taxa (i.e. 1120 bacterial counts) on a single 15 × 15 cm nylon membrane. The membranes may be stripped and re-probed with a new set of 40 different DNA probes if desired. The technique is sensitive since it can routinely detect 10<sup>4</sup> cells of a given species in a sample and conditions of hybridization and detection can be altered to detect as low as 10<sup>3</sup> cells (31). For the most part, the whole genomic DNA probes have been found to be remarkably specific; 93.5% of probe : heterologous species cross-reactions were less than 5% of the homologous probe signal (159).

The checkerboard DNA-DNA hybridization technique does have limitations. The technique can detect only species for which DNA probes have been prepared. Thus, this method would not detect novel pathogens or environmentally important species that might be detected in culture or by other molecular techniques. The probes must be used to detect organisms in samples of the appropriate size. Probes optimized to detect species in the 10<sup>4</sup>-10<sup>7</sup> range often will provide cross-reactions if much larger samples are employed.

When properly employed, checkerboard DNA-DNA hybridization and other rapid microbiological techniques permit investigation of etiologic, therapeutic, and ecologic problems which could not be approached by other means. The data from clinical samples presented in this chapter demonstrate the feasibility of describing the microbiota in sites with different local clinical conditions as well as in subjects with different systemic backgrounds, different periodontal disease states or health. In addition, species abundance, species diversity, and community structure can be computed from data derived using this technique (156). As new and improved DNA probes are developed, and rapid microbiological techniques are employed, an understanding of the ecologic relationships of complex microbial communities can be developed at a level hitherto beyond our reach.



**Fig. 4.** Example of checkerboard DNA-DNA hybridization being used to detect 40 bacterial species in 28 subgingival plaque samples from a single patient. The vertical lanes are the plaque samples numbered from 11 (maxillary right central incisor) to 47 (mandibular right second molar). In this subject, teeth 16, 17, 21, and 37 were missing. The two vertical lanes on the right are standards containing either  $10^5$  or  $10^6$  cells of each test species. The horizontal lanes contained the indicated DNA probes in hybridization buffer. A signal at the intersection of the vertical and horizontal lanes indicates the presence of a species. The intensity of the signal is related to the number of organisms of that species in the sample. In brief, samples of plaque were placed into individual Eppendorf tubes and the DNA released from the

microorganisms by boiling in NaOH. After neutralization, the released DNA was transferred to the surface of a nylon membrane using the 30 channels of a Minislot device (Immunetics, Cambridge, MA). The DNA was fixed to the membrane by UV light and baking and placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at right angles to the 45 channels of the Miniblotter device. Whole genomic DNA probes labeled with digoxigenin were placed in hybridization buffer into 40 of the lanes and hybridized overnight. After stringency washing, the signals were detected using phosphatase-conjugated antibody to digoxigenin and chemifluorescence substrates. Signals were compared to the standards using a Storm Fluorimager and converted to counts. Reprinted with permission from (159).

## The first component: microbial composition of subgingival biofilms

### Total numbers of subgingival bacteria in periodontally healthy and diseased subjects

The first microbial ecology question that might be asked is how many bacterial cells are present in

subgingival biofilms in both periodontally healthy and diseased subjects? An estimate of total subgingival bacterial numbers may be derived from data from Sharawy et al. (149). These investigators carefully harvested all of the subgingival plaque (or as much as possible) from the subgingival tooth surfaces of half of the mouth in 14 periodontally healthy and 21 periodontitis subjects. The average

dry weight of the samples was  $1.6 \pm 0.8$  and  $8.3 \pm 3.9$  mg, respectively, for the two clinical groups. This translated to about 8.0 and 41.5 mg wet weight per half mouth or a total of 16 and 83 mg wet weight of plaque from periodontally healthy and periodontitis subjects, respectively. Since the average total microscopic count of organisms in subgingival plaque is  $2.1 \times 10^8$  per mg wet weight (153), the total number of microbial cells in subgingival plaque from periodontally healthy subjects may be estimated at  $33.6 \times 10^8$  and from periodontitis subjects at  $174.3 \times 10^8$ , with considerable subject to subject variation. It is interesting that the full-mouth mean counts differed by a factor of only 5, since clinicians sometimes can remove very large amounts of plaque from some subjects with periodontitis. However, it must be recognized that massive subgingival plaque accumulation occurs in only a small number of subjects and that most periodontal sites in most subjects with periodontitis are 'healthy', i.e. exhibit minimum disease and little subgingival plaque.

### Species abundance

One of the first steps that an ecologist would take in studying any ecosystem would be to survey the range and nature of the species that exist in the habitat of interest. This has been admirably described by Paster et al. (121). They indicated that approximately 500 species may be encountered in subgingival biofilms and they have summarized the nature of the taxa found. Figure 5 (from [121]) describes the 10 phyla that have been detected in subgingival plaque samples, provides examples of known species from these phyla, and demonstrates that more than half of the taxa detected were of novel species. However, it is clear that the approximately 500 taxa that may be encountered are not uniformly distributed in subgingival biofilm samples, i.e. some taxa are detected with far greater frequency and in greater numbers than others in any oral habitat of interest. The frequency of detection of the cultivable and as yet uncultivated taxa in samples from periodontal sites (121) is presented in Fig. 6. Twenty-six of the 306 taxa

Phylum	N known species	N novel phylotypes	Known species detected in at least 4 subjects
Obsidian pool	0	1	—
TM7	0	5	—
Deferribacteres	0	8	—
<i>Spirochaeta</i>	10	45	<i>T. medium</i> , <i>T. denticola</i> , <i>T. maltophilum</i> <i>T. lecithinolyticum</i> , <i>T. socranskii</i>
<i>Fusobacteria</i>	6	13	<i>F. naviforme</i> , <i>F. nuc ss nucleatum</i> , <i>F. nuc ss polymorphum</i> , <i>F. animalis</i> <i>L. buccalis</i>
<i>Actinobacteria</i>	12	18	<i>A. naeslundii</i> 2, <i>R. dentocariosa</i> , <i>C. matruchotii</i> , <i>Atopobium parvulum</i> <i>A. rimae</i>
<i>Firmicutes</i>	23	12	<i>S. oralis</i> , <i>S. mitis</i> , <i>S. sanguis</i> , <i>S. cristatus</i> <i>S. intermedius</i> , <i>S. constellatus</i> , <i>S. anginosus</i> , <i>Abiotrophia adiacens</i> <i>G. morbillorum</i> , <i>G. haemolysans</i>
"Clostridia"	19	40	<i>E. brachy</i> , <i>E. saphenum</i> , <i>Filifactor alocis</i> <i>P. micros</i> , <i>Catonella morbi</i> , <i>V. dispar</i> <i>V. parvula</i> , <i>Dialister pneumosintes</i> <i>Sel. sputigena</i> , <i>S. noxia</i> , <i>S. infelix</i>
<i>Proteobacteria</i>	26	19	<i>N. mucosa</i> , <i>H. parainfluenzae</i> , <i>C. gracilis</i> , <i>C. concisus</i> , <i>C. rectus</i>
<i>Bacteroidetes</i>	17	34	<i>Prev. denticola</i> , <i>P. oris</i> , <i>P. tanneriae</i> , <i>T. forsythia</i> , <i>P. gingivalis</i> <i>P. endodontalis</i> , <i>Capno gingivalis</i> <i>C. ochracea</i>
	<b>113</b>	<b>195</b>	

Fig. 5. Range of cultivable and as yet uncultivated species detected in samples of subgingival plaque. The 10 bacterial phyla detected are presented to the left along with the number of known species and novel phylotypes

detected in each phylum. On the right, example species for each phylum are presented. Each of these species were detected in at least four subjects. Data from (121).

N Clones of a taxon detected	N taxa		
1	103	103 taxa (34%) account for 0.04% each: total = <b>4.1%</b>	
2	52		
3	30	52 taxa (17%) account for 0.08% each: total = <b>4.1%</b>	
4	11		
5	11	242 taxa (79%) account for a total of 661/2522 = <b>26.2%</b>	
6	10		
7	6		
8	11		
9	5		
10	3		
11–19	38		
20–29	11		26 taxa (8.5%) account for > <b>50%</b> of the “microbiota”
30–39	4		
40–49	5		
50–99	2		
100+	4		

**Fig. 6.** Relative ‘species abundance’ in subgingival plaque samples from 31 subjects using PCR amplification of the 16S rDNA genes from plaque samples followed by cloning

detected accounted for > 50% of the sequenced clones, whereas 103 taxa (43%) were detected in only one of the 31 subjects.

The conclusion that species are not uniformly distributed in periodontal sites may also be drawn from other data. The 40 DNA probes utilized in Fig. 4 identified 85% of 3400 pure cultures from subgingival plaque samples from 62 subjects. The remaining 15% were identified by phenotypic testing or 16s rRNA sequencing. None of these isolates was a species in the test battery, indicating that the probes could pick up all isolates of that species. Since, on average, we recover about 70% of the cells enumerated by microscopic count, the 40 DNA probes would account for 55–60% of the bacteria in subgingival biofilms. This finding is in accord with the data from Paster et al. (121) and supports the notion that a limited set of species accounts for the vast majority of bacterial cells encountered in subgingival biofilm samples.

Species abundance may be expressed in a number of ways. Figure 7 depicts the mean counts  $\times 10^5$  of 40 taxa in 20,247 subgingival samples from 184 periodontally healthy subjects and 592 subjects with chronic periodontitis. The dominant species was *Actinomyces naeslundii* genospecies 2. This is not surprising given that the majority of sampled sites exhibited shallow pocket depths. If only deep pockets

and sequencing of the inserts. The figure indicates the number of taxa that were detected once, twice, three times, etc., in the samples from the test subjects. Data from (121).

were sampled, a somewhat different picture would emerge, as will be seen in a later section. Other prevalent species included *Prevotella nigrescens*, *Prevotella intermedia*, *Veillonella parvula*, *T. forsythia*, and *Prevotella melaninogenica*.

### Species diversity

Diversity indices are often used by ecologists to describe species richness and species evenness in diverse ecosystems (93). Examples of the use of some of these indices are presented in Fig. 8. For each index, diversity was computed at a site, averaged across sites in a subject, and then averaged across subjects in the periodontally healthy and chronic periodontitis groups described above. The first three indices (Hill’s N0, N1, and N2) were chosen because they are readily interpretable in terms of actual numbers of species. ‘Hill’s N0’, the number of species detected, averaged about 22 and 24 per site for the periodontally healthy and periodontitis subjects, respectively. The effective number of abundant species was about the same in both clinical groups, while the effective number of very abundant species was higher in samples from periodontal health than in samples from periodontitis. Hill’s evenness index ranges from 0 to 1, with 1 indicating a perfectly even distribution of species and 0 a very skewed distribution. There appeared

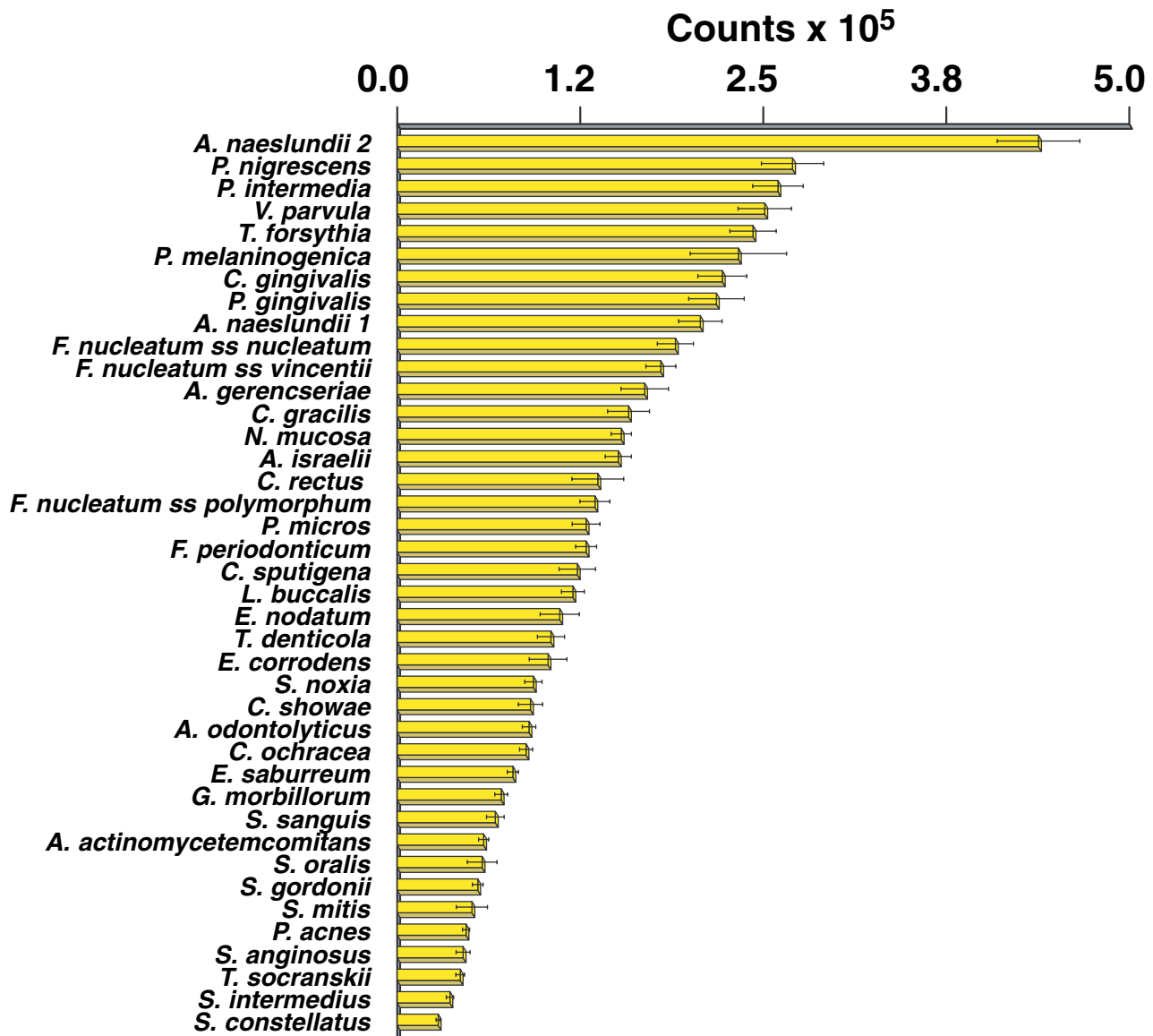


Fig. 7. Bar chart of the 'species abundance' of subgingival taxa as determined by checkerboard DNA-DNA hybridization. The mean counts of 40 species in samples from 184 periodontally healthy subjects and 592 adult subjects with chronic periodontitis are presented. The total number of

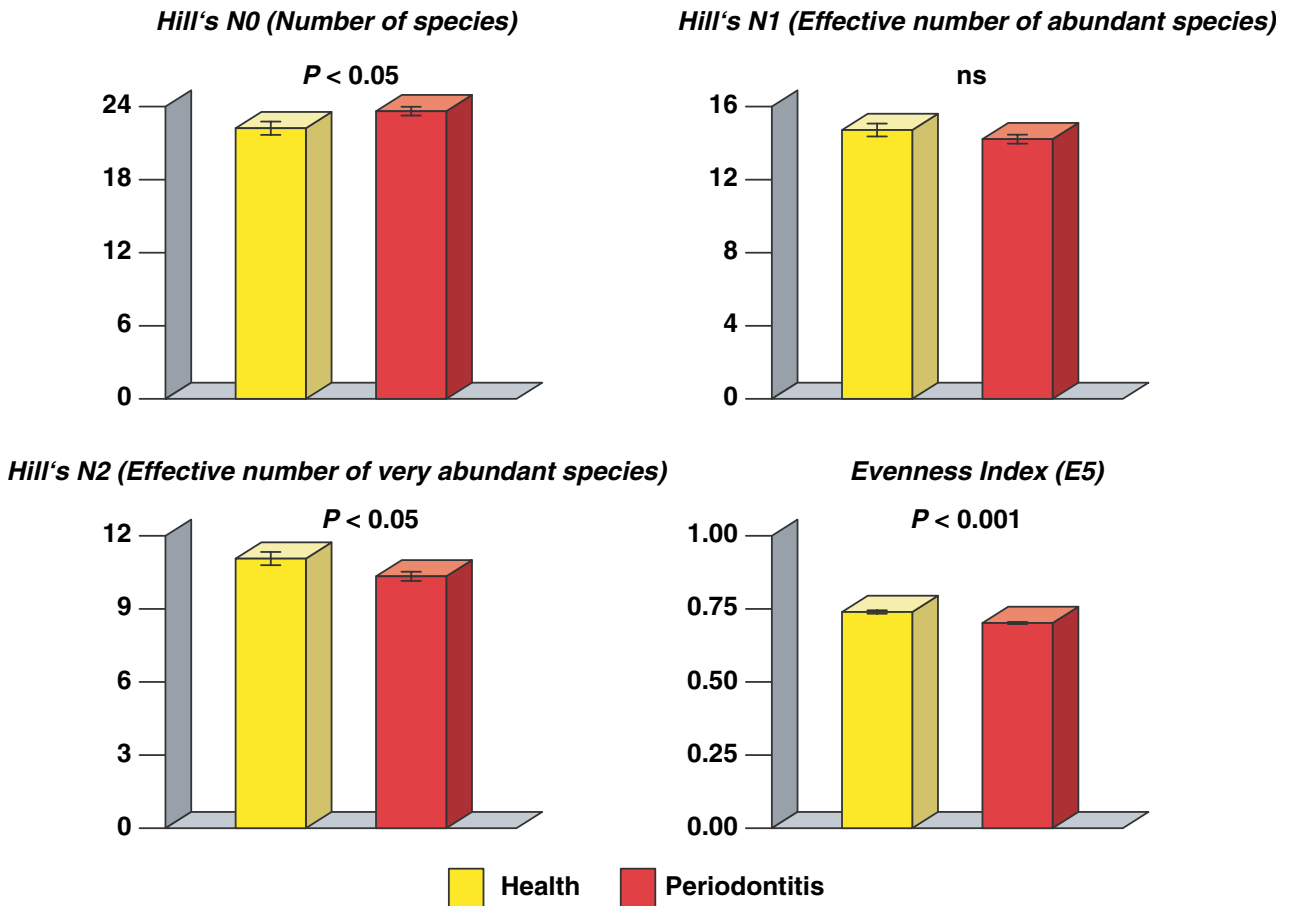
samples was 20,247 (mean 26.1 per subject). Counts of each species were averaged within a subject and then across subjects. The bars and whiskers represent mean counts  $\times 10^5$ ,  $\pm$  SEM.

to be greater 'evenness' in the samples from the periodontally healthy sites than in the samples from periodontitis subjects. As Ludwig & Reynolds (93) point out, diversity indices are often used in ecology but are difficult to interpret.

#### Microbial associations in subgingival biofilms

Casual examination of our macro environment indicates that there are easily discernible specific species associations in different habitats. Often these associations are habitat-driven. For example, a dry climate may select one set of plant species, a wet climate another, and a climate that has alternating

periods of snow and hot weather still another. Habitat is also a pre-eminent factor in determining species associations among microorganisms. Some of the selective pressures that determine bacterial species assemblages were briefly mentioned in the 'principles' section and include shared nutrient or environmental requirements, similar receptors for initial adhesion, shared mechanisms for protection from host and other species, and the dependence of one species on another for colonization. Attempts to define some of the principles of species association have been carried out *in vitro* and include efforts to define specific species pairs involved in coaggrega-



**Fig. 8.** Bar plots of four diversity indices used to compare subgingival samples from the 184 periodontally healthy subjects and 592 adult subjects with chronic periodontitis

presented in Fig. 7. The diversity indices were computed (93) for each site and averaged within a subject prior to averaging across subjects in the two groups.

tion (74, 75) as well as species interactions that may occur in *in vitro* mixed culture experiments (142, 164, 180). In 1998 (156), an effort was made to define species associations that occur in subgingival biofilms *in vivo*. The genesis of this approach was the expectation that specific species associations must occur in subgingival biofilms since they occur regularly in other ecosystems in nature. In addition, in the early years, checkerboard DNA–DNA hybridization membranes were read visually by the authors, who came to recognize that when certain species were detected at a site, certain other species were virtually certain to be there as well. This notion was tested by analysis of data from over 13,000 subgingival biofilm samples from 185 subjects with different states of periodontal health and disease (156). Associations between all pairs of species were measured by various similarity coefficients and the resulting similarity matrices subjected to cluster analysis. These analyses supported the hypothesis that there were distinct complexes of microorganisms in subgingival biofilms. The data were examined further using two

community ordination techniques, principal components analysis and correspondence analysis. These analyses were valuable in that they confirmed the complexes described by the pairwise cluster analyses and suggested the nature of the relationships of the complexes (communities) to each other. The publication of this paper was greeted by more enthusiasm than was anticipated. This appeared to be in large part because the delineation of the ‘colored’ complexes provided a framework for describing and understanding the subgingival ecosystem. The original synthesis of the cluster and community ordination analyses is presented in Fig. 9.

As will be seen in later sections, specific complexes relate to their habitat in terms of periodontal health/disease, local clinical characteristics and the systemic background of the host. The relationships among species described in Socransky et al. (156) paralleled the findings of *in vitro* coaggregation studies (74, 75). Species that make up the complexes also appear to be distributed in different regions of the periodontal pocket/gingival sulcus as determined



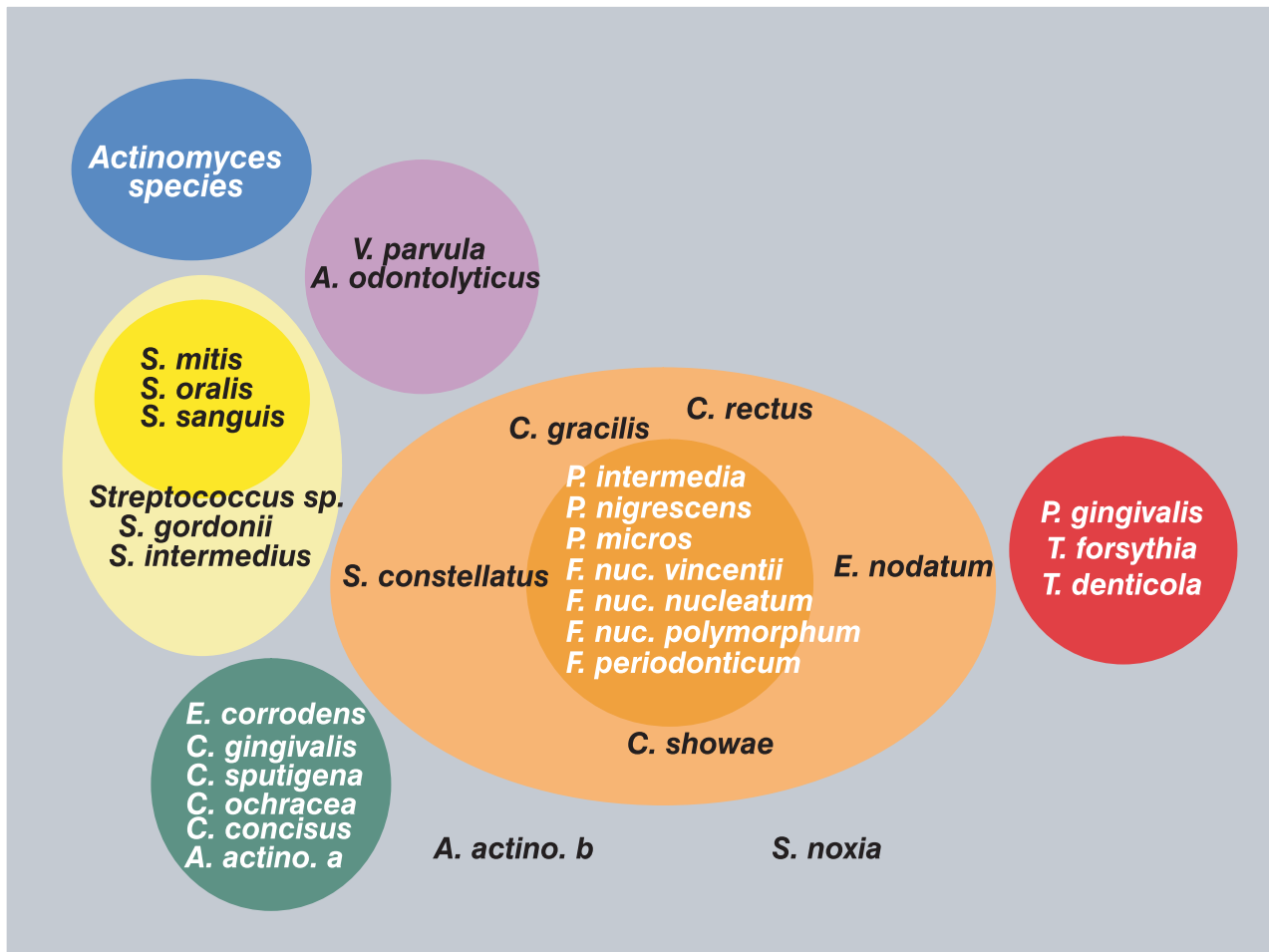


Fig. 9. Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes. Updated from (156) with *B. forsythus* changed to *T. forsythia*.

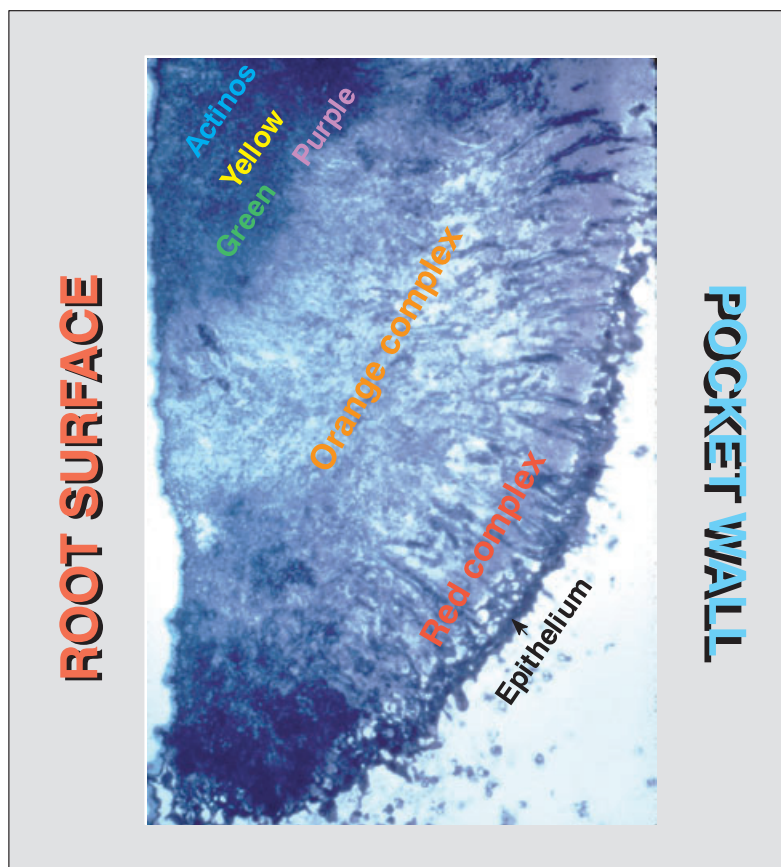
by *in situ* immunocytochemistry. Figure 10, which is based on publications by Kigure et al. (72) and Noiri et al. (110–113), indicates regions in the subgingival area that appear to be enriched for specific microbial complexes.

## The second component: influence of surface on the composition of intraoral biofilms

### Comparison of microbial composition of biofilms on teeth and soft tissues

One of the key determinants of biofilm composition is the nature of the surface on which the biofilm develops. Nowhere is this more easily demonstrated than by examining the composition of the biofilms that develop on the teeth and the various soft tissue surfaces of the oral cavity. Mager et al. (98) used checkerboard DNA–DNA hybridization to compare the composition of the microbiota taken from eight different soft tissue surfaces and saliva as well as supragingival and,

separately, subgingival plaque in the same individuals. The samples differed markedly in the proportions of the 40 test taxa examined. For example, much higher proportions of *Actinomyces* species were observed in samples from the tooth surfaces, high proportions of *P. melaninogenica* were observed in samples from saliva and the dorsal and lateral surface of the tongue, and high proportions of *Streptococcus mitis* and *S. oralis* were observed in samples from the remaining soft tissue surfaces (Fig. 11). Cluster analysis of the samples (Fig. 12) revealed that the samples from the dorsum and lateral surfaces of the tongue and from the saliva were similar to each other and confirmed an earlier notion that the source of most bacterial cells in saliva was the tongue surface. The microbiotas of the other soft tissue surfaces, including tongue ventral, floor of mouth, hard palate, cheek, vestibule/lip, and attached gingiva formed a second cluster. The two clusters formed by the soft tissue and saliva samples were more similar to each other than to the samples taken from supragingival and subgingival plaque, which made up a third cluster.



**Fig. 10.** Histologic section of human subgingival dental plaque stained with toluidine blue-methylene blue. The tooth surface is to the left and the epithelial lining of the periodontal pocket is to the right. Bacterial plaque attached to the tooth surface is evident towards the upper left of the

section, while a second zone of organisms can be observed lining the periodontal pocket wall. (Courtesy of Dr. Max Listgarten). The suspected location of species in the different complexes has been indicated on the section based on data from Kigure et al. (72) and Noiri et al. (110–113).

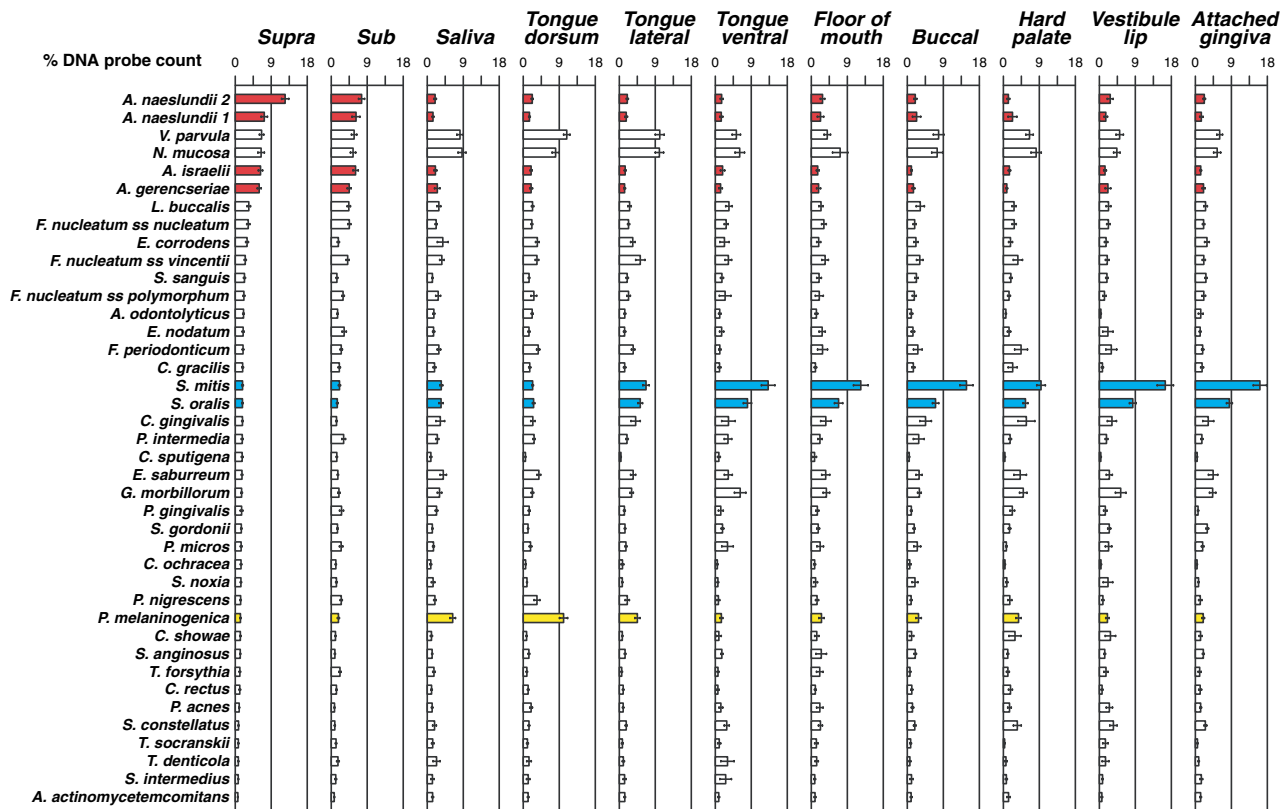
The findings of this study highlight the importance of the nature of the surface to be colonized on subsequent biofilm composition. In this study, each subject was hisher control, ruling out possible host background differences. The saliva ‘bulk fluid’ bathing the surfaces was essentially identical for all locations except for the subgingival samples. In spite of these identical background factors, the biofilms differed markedly due to distinct differences in the surfaces that became colonized. Nowhere was this more apparent than for the tongue dorsum vs. hard palate. These two soft tissue surfaces come in contact with each other hundreds of times each day and yet the microorganisms colonizing these surfaces were radically different in terms of both abundance and species proportions.

#### Comparison of microbial composition of biofilms on teeth and dentures

The replacement of all of the natural teeth by full dentures provides a direct method to assess the possible effect of change in intraoral hard tissue

surfaces on the composition of hard tissue and soft tissue biofilms. The microbiota of the edentulous subject is not well understood because there have been relatively few studies of the microbiota colonizing dentures or the mucous membranes or saliva in edentulous subjects. At one age extreme, studies of the edentulous oral cavity of infants prior to tooth eruption suggest that anaerobic species could be detected in the absence of teeth and that *P. melaninogenica* was the most frequently isolated anaerobic species, being found in 70% of infants (77). Other common anaerobes included *Fusobacterium nucleatum*, *Veillonella* species, and nonpigmented *Prevotella*. The source of the anaerobes appeared to be the mother since there was a correlation between maternal salivary concentration and the infant’s colonization by *P. melaninogenica* (78). At the other end of the age spectrum, the microbiota of 51 edentulous subjects (mean age 74 years) with complete dentures was studied (76). Samples were taken from the fitting surface of the denture as well as the palate, buccal mucosa, dorsum of the tongue and





**Fig. 11.** Mean percentage DNA probe count ( $\pm$  SEM) for samples from the 11 intraoral locations in 41 periodontally healthy and 39 subjects with chronic periodontitis. Species are ordered according to their mean proportions in supragingival plaque. Significance of differences among sample locations was determined using the Kruskal–Wallis test and adjusted for multiple comparisons (157). All

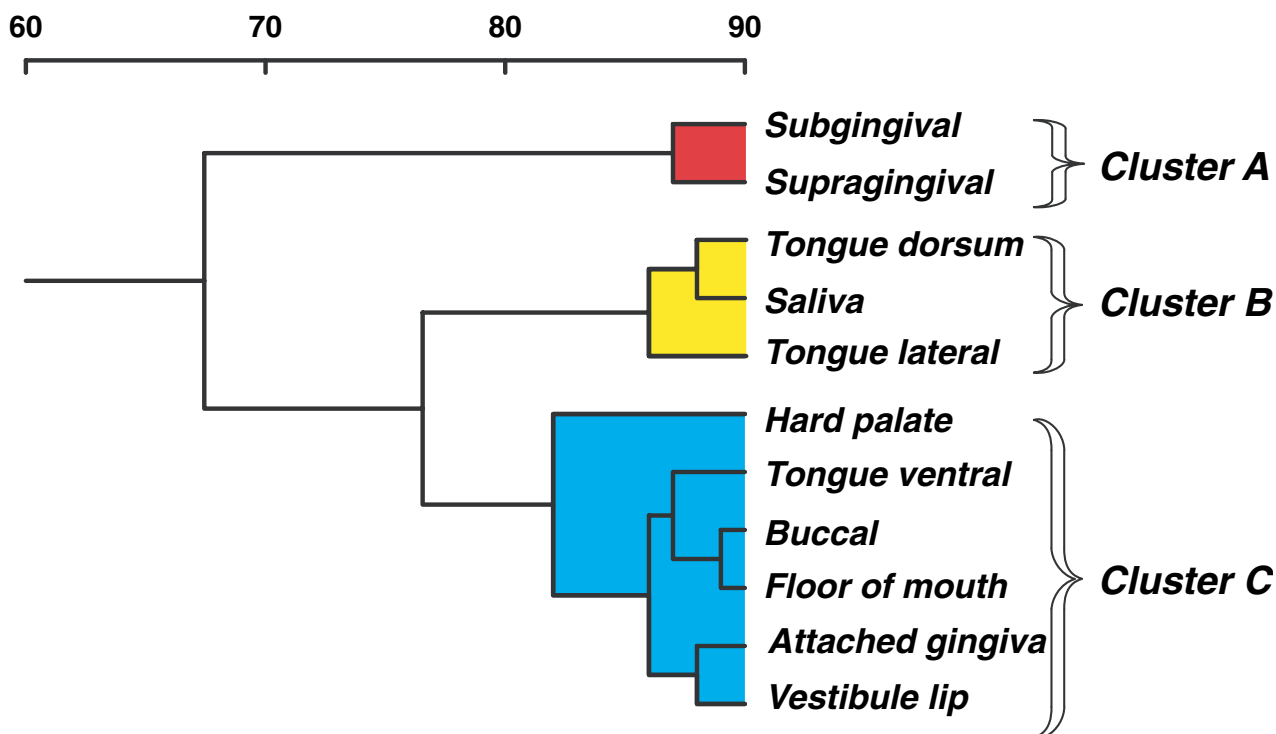
species were significantly different among groups at  $P < 0.001$  with the exception of *Streptococcus constellatus* and *Gemella morbillorum*. The colored bars represent species that showed particularly marked differences among sample locations. Updated from (98) by the addition of 36 subjects.

saliva. ‘Black pigmented *Bacteroides*’ were found in 96% of subjects, and yeasts in 49% of subjects. Of the saliva samples, 84% yielded mutans streptococci and 92% lactobacilli. Data in the literature suggest that certain species such as *Streptococcus mutans* require hard surfaces for sustained colonization (18, 43, 91, 169), although they may be detected in dentate subjects at low levels on the soft tissues. Studies have suggested that *S. mutans* essentially disappears from the oral cavity when all the teeth are extracted and reappears if hard surfaces are provided in the form of full dentures (18, 43, 91, 169). Other studies have suggested that *A. actinomycetemcomitans* and *P. gingivalis* disappear from the oral cavity after extraction of all the teeth and do not re-appear even when hard surfaces such as full dentures are provided (26). *S. mutans* as well as lactobacilli can be found in the mouths of edentulous subjects wearing dentures, suggesting that dentures provide a surface for colonization (18, 43, 169). Theilade et al. (168, 169) examined the predominant cultivable microbiota on dentures in patients with healthy mucosa or denture-

induced stomatitis. They found that the microbiota in both instances was dominated by gram-positive organisms, primarily members of the genera *Streptococcus*, *Actinomyces*, and *Lactobacillus*. *Veillonella* species made up about 10% of the microbiota, whereas gram-negative rods constituted  $< 1\%$ .

The data from the above studies are intriguing in that they suggest that the teeth may be essential for colonization of species such as *A. actinomycetemcomitans* and *P. gingivalis*. Further, hard surfaces appear essential for the colonization of *S. mutans*. The data of Theilade et al. (168) suggest that the gingival crevice as well as the fluid passing through the gingival crevice may also be essential for the colonization of most gram-negative rod species including common species such as *F. nucleatum*, *P. intermedia*, and *P. nigrescens*.

Thus, the findings in the literature suggest the need for hard surfaces for the colonization of some species and the gingival crevice fluid from gingival sulci or periodontal pockets for the colonization of others. This notion prompted us to compare the microbiota

**% similarity (minimum similarity coefficient)**

**Fig. 12.** Dendrogram of a cluster analysis of the mean species proportions from the 11 sample locations described in Fig. 11. A minimum similarity coefficient was

employed and an average unweighted linkage sort. Three clusters were formed at >80% similarity. Cluster B was more similar to Cluster C than Cluster A.

on dentures of individuals who were fully edentulous and had been wearing dentures for at least 1 year with that present in samples of supragingival plaque from subjects who were periodontally healthy or who had chronic periodontitis. Supragingival plaque samples were taken from the mesial aspect of each tooth (or denture tooth) in 28 periodontally healthy, 8 periodontitis, and 18 fully edentulous denture-wearing subjects and individually analyzed for their content of 41 bacterial species (the standard 40 + *S. mutans*) using checkerboard DNA–DNA hybridization. The counts were averaged within a subject and then across subjects in the 3 clinical groups. The mean ( $\times 10^5$ ,  $\pm$  SEM) total DNA probe counts were  $45 \pm 7$ ,  $66 \pm 13$ , and  $52 \pm 11$  in healthy, periodontitis, and edentulous subjects, respectively. The mean microbial profiles differed quite markedly among clinical groups (Fig. 13). Noteworthy were the lower levels of *Capnocytophaga ochracea*, *Capnocytophaga sputigena*, *Campylobacter rectus*, *Campylobacter showae*, *T. forsythia*, *Neisseria mucosa*, and *P. melaninogenica* in samples from the subjects with dentures. These data, although limited in the number of subjects studied to date, support the concept that the nature of the hard tissue surface impacts on the composition of the biofilms that form on hard tissue surfaces.

Soft tissue and saliva samples from the same group of subjects were also examined by checkerboard DNA–DNA hybridization (Fig. 14). In general, the mean microbial profiles at the different sample locations were more similar between the healthy and periodontitis subjects than the edentulous subjects. Proportions of *F. nucleatum* subspecies, *Eikenella corrodens*, and *V. parvula* were significantly lower for most sample locations in the edentulous subjects. In contrast, proportions of *S. mitis* and *Streptococcus gordonii* were significantly higher at all soft tissue sites, except the tongue dorsum and tongue lateral in the edentulous subjects. *Fusobacterium periodonticum* was consistently detected in significantly higher proportions on all soft tissue surfaces and in the saliva of the edentulous subjects. In addition, total DNA probe counts were higher on the soft tissues of edentulous subjects. These data suggest that the oral soft tissue microbiotas of edentulous subjects also differed markedly from that observed in healthy or periodontitis subjects and suggest that the nature of the hard tissue surface in the oral cavity not only impacts on the nature of colonizing species on hard tissue surfaces, but also on the soft tissue surfaces. One caveat to the interpretation of the findings of this section is that the different methods used by dentate

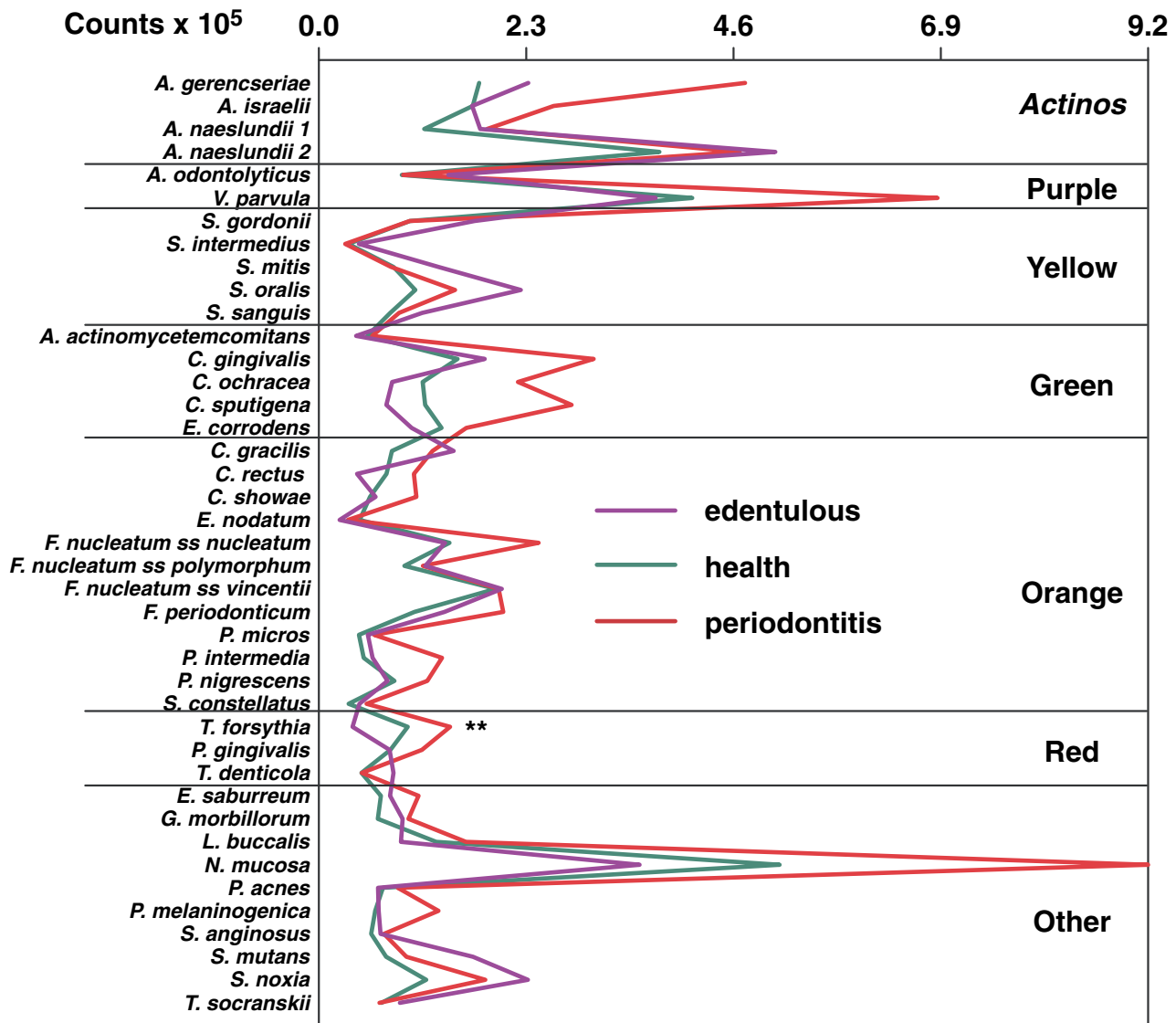


Fig. 13. Profiles of mean counts of 41 taxa in supragingival plaque samples from 28 periodontally healthy, 8 chronic periodontitis, and 18 fully edentulous subjects. Plaque samples were taken from the mesial aspect of each tooth or denture tooth and analyzed separately for their content of 41 species. Data for each species were averaged

within a subject and then across subjects in the three clinical groups separately and significance of differences among groups sought using the Kruskal–Wallis test and adjusted for multiple comparisons (157); \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Species were ordered according to the complexes described by Socransky et al. (156).

and edentulous subjects to clean their ‘teeth’ may also have an impact on the colonizing microbiota.

### The third component: influence of bulk fluid on the composition of intraoral biofilms

#### The critical role of ‘bulk fluid’ – saliva

‘Bulk fluid’ is critical to the survival of species in a biofilm. This fluid provides nutrients, removes waste products, and acts as the vehicle for transport of bacterial cells from site to site in the oral cavity and

probably facilitates person to person transfer. The major bulk fluid in the oral cavity is saliva, which is essential to the survival of almost all oral biofilms with the exception of biofilms in subgingival areas or areas within pathologic lesions such as root canal infections or oral abscesses. Examination of the existing literature suggests a number of intriguing, seeming inconsistencies. For example, the total viable counts of bacteria in saliva average about  $10^8$  per ml (13, 66, 139). The total daily amount of saliva secreted has been variously estimated at 500 ml (178, 179), 1000 ml (138) or 1500 ml (32, 45). Thus, theoretically, about  $1 \times 10^{11}$  bacteria would be

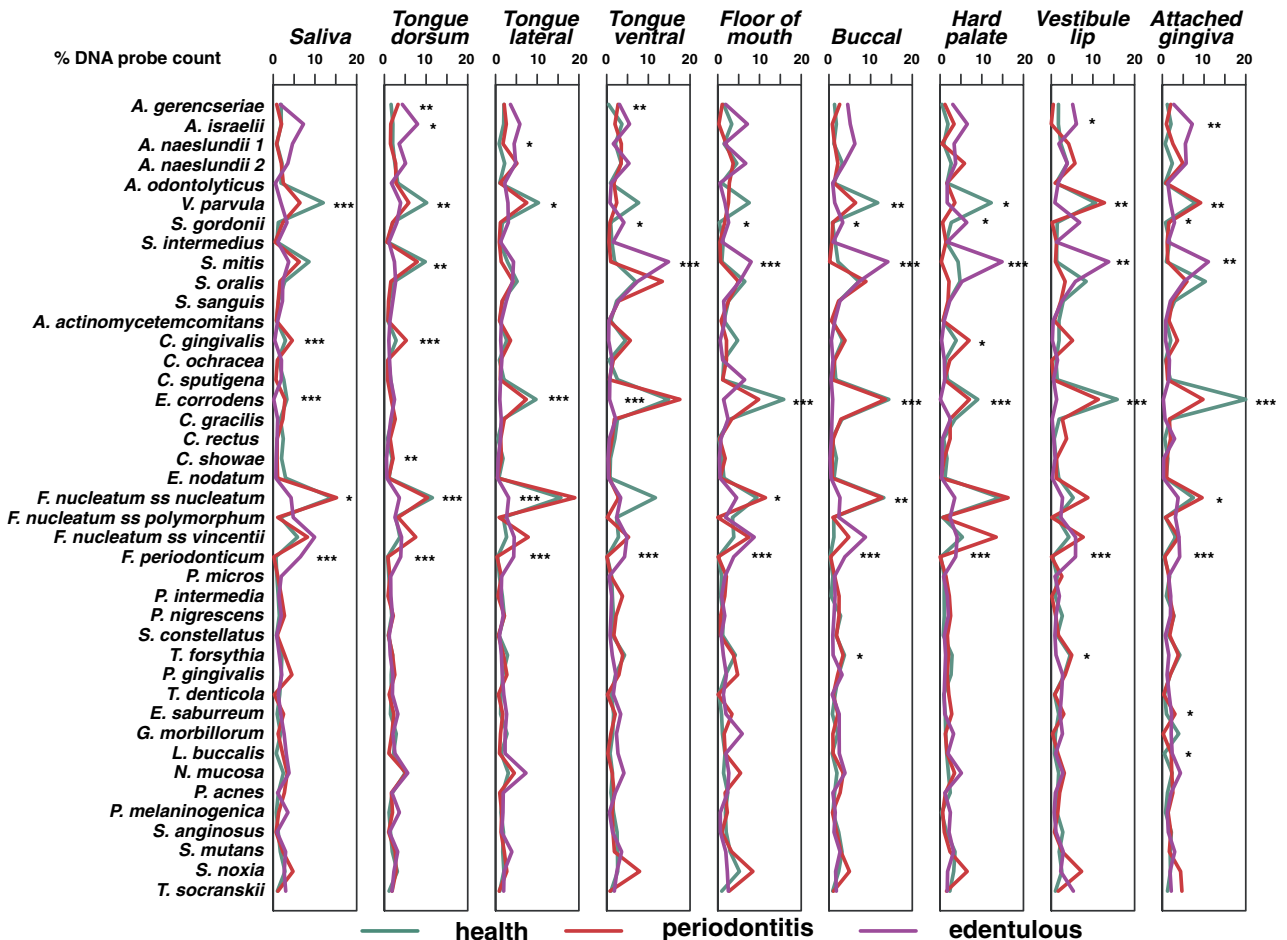


Fig. 14. Profiles of mean percentage of the DNA probe count of 41 taxa in samples from 8 soft tissue surfaces and saliva obtained from 28 periodontally healthy, 8 chronic periodontitis, and 18 fully edentulous subjects. Samples of saliva were obtained by expectoration and samples from the indicated soft tissue surfaces were obtained using a

buccal brush and analyzed separately for their content of 41 species. Significance of differences among groups for each species was sought using the Kruskal-Wallis test and adjusted for multiple comparisons (157); \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Species were ordered according to the complexes described by Socransky et al. (156).

swallowed each day. This translates to about 1 g wet weight of bacterial cells. Where do these bacteria come from? The average total mass of bacteria on the teeth of a healthy subject has been estimated to be about 16 mg, while an average value for periodontitis subjects was about 83 mg (149). The mass and number of organisms that colonize the soft tissues are not known. Collins & Dawes (23) measured the surface area of the oral cavity and found that teeth made up about 20% of the available surface for microbial colonization. If organisms colonized at the same density on the soft tissues as on the teeth, then the mass of organisms might be estimated to be four times as high or perhaps 400 mg. Thus, one might estimate about 500 mg of bacteria might colonize the oral cavity. Even if the total mass were 1 g of bacteria, this would suggest that the entire mass of organisms, on average, would duplicate each day. Generation

times would have to be shorter if smaller masses of bacteria colonized the oral cavity or if only a limited segment of the microbiota was capable of replicating. One possible explanation for the large numbers of bacteria in saliva is that the saliva is supporting multiplication while the bacteria are in saliva (14, 28–30, 174). This is possible but the high turnover rate of saliva in the oral cavity makes this unlikely. It has been estimated that the volume of saliva within the oral cavity typically ranges from 0.77 to 1.07 ml (81). If the flow rate were, on average, 1 ml per min, then the turnover of saliva would be very short and the likelihood of bacterial multiplication in saliva explaining the numbers of bacteria observed would be rather small.

These observations set the stage for a set of intriguing questions. If the flow rate of saliva were markedly diminished, what would happen to the

counts of bacteria in saliva, to the microbiotas that colonize the soft tissues, and to the microbiotas that colonize the tooth surfaces? On the one hand, saliva might remove or limit bacteria on the hard and soft tissues and perhaps affect microbial composition. On the other hand, saliva may provide nutrients to surface colonizing species and provide a means of transport from one surface to another, increasing bacterial load. Thus, a decrease in saliva could alter the microbial composition in both beneficial and harmful ways. Studies of xerostomia, a common clinical condition that affects a large proportion of the adult, particularly senior, population, could help to answer some of these questions. Xerostomia occurs as a result of certain medications, radiation or chemotherapy or as part of an autoimmune syndrome called Sjogren's syndrome. The last condition affects, among other things, saliva production, decreasing the mean unstimulated flow rate to values well below 0.1 ml per min (177). This condition provides an excellent model to ask critical biofilm-related questions: does the composition of the microbiota differ in Sjogren's subjects from that observed in age-matched controls? Will the re-development of soft tissue biofilms differ in subjects whose saliva production is markedly diminished? These questions are being addressed in ongoing studies carried out in association with Dr. Athena Papas at Tufts School of Dental Medicine. The purpose of the first phase of investigation was to compare the supra- and subgingival microbiotas of Sjogren's subjects with those of periodontally healthy and periodontitis subjects. Separate supra- and subgingival plaque samples were taken from the mesial aspect of each tooth at baseline in 24 Sjogren's, 30 periodontally healthy, and 53 periodontitis subjects and individually analyzed for their content of 40 bacterial species using checkerboard DNA-DNA hybridization.

The mean ( $\times 10^5$ ,  $\pm$  SEM) total DNA probe counts in Sjogren's, health, and periodontitis were supragingivally  $13 \pm 3$ ,  $44 \pm 7$ , and  $43 \pm 4$  ( $P < 0.001$ ). This suggests that saliva may foster growth of bacterial species in supragingival biofilms or that Sjogren's subjects were far more fastidious in their home care than subjects with normal salivary flow. Subgingivally, mean counts were  $11 \pm 1$ ,  $15 \pm 2$ , and  $25 \pm 2$ , respectively ( $P < 0.001$ ). The significant difference among groups in the subgingival samples was due to higher mean counts in the periodontitis subjects than in the other two groups. This suggests that the subgingival domain was less affected in the Sjogren's subjects, possibly due to gingival crevice fluid provi-

ding the bulk fluid to the area rather than saliva. In supragingival samples, mean counts of 30 species (Fig. 15) and proportions of 10 species differed significantly among groups after adjusting for multiple comparisons. In subgingival samples, mean counts of 19 species (Fig. 16) and proportions of 16 species differed significantly among groups. The species with the highest mean counts in both supra- and subgingival samples from Sjogren's subjects were *V. parvula* and *N. mucosa*. Although the mean supragingival counts of *V. parvula* did not differ significantly among the three clinical groups (Fig. 15), the mean proportions of this species did. The mean percentage ( $\pm$  SEM) in Sjogren's, health, and periodontitis was  $17 \pm 2$ ,  $9 \pm 1$ , and  $10 \pm 1$  ( $P < 0.05$ ), respectively. *N. mucosa* also exhibited significantly different proportions;  $14 \pm 2$ ,  $11 \pm 2$ , and  $4 \pm 1$  ( $P < 0.001$ ) in Sjogren's, health, and periodontitis, respectively. The subgingival microbiota of Sjogren's exhibited low counts of most species compared with periodontitis and healthy subjects. *T. forsythia*, *P. gingivalis*, *Treponema denticola*, and *Treponema socranskii* were markedly higher in subjects with periodontitis than in healthy or Sjogren's subjects. These data indicate that there were many significant differences in the supra- and subgingival microbial composition of periodontally healthy, chronic periodontitis and Sjogren's syndrome subjects. However, differences in mean microbial profiles among the three clinical groups for subgingival plaque were not the same as those observed for supragingival plaque.

The microbiota of the oral soft tissues and saliva was compared in Sjogren's subjects and subjects with normal salivary flow. Biofilm samples from soft tissues were taken in 24 Sjogren's subjects and 11 controls at baseline. The samples were taken using a buccal brush from the tongue dorsum, lateral and ventral surfaces, floor of mouth, buccal, hard palate, vestibule/lip and attached gingiva. These samples as well as an unstimulated saliva sample were individually analyzed for their content of 41 bacterial species using checkerboard DNA-DNA hybridization. Levels and percentage DNA probe counts of each species were determined for each sampled site and averaged across subjects in the two clinical groups.

There were significantly lower mean total DNA probe counts ( $\times 10^5$ ,  $\pm$  SEM) for Sjogren's subjects compared with controls (Fig. 17) in the samples from the tongue dorsum ( $47 \pm 10$  vs.  $98 \pm 22$ ), buccal ( $6 \pm 1$  vs.  $16 \pm 4$ ), and hard palate ( $8 \pm 2$  vs.  $33 \pm 15$ ). For these locations, species that were significantly elevated at multiple sample locations in the control

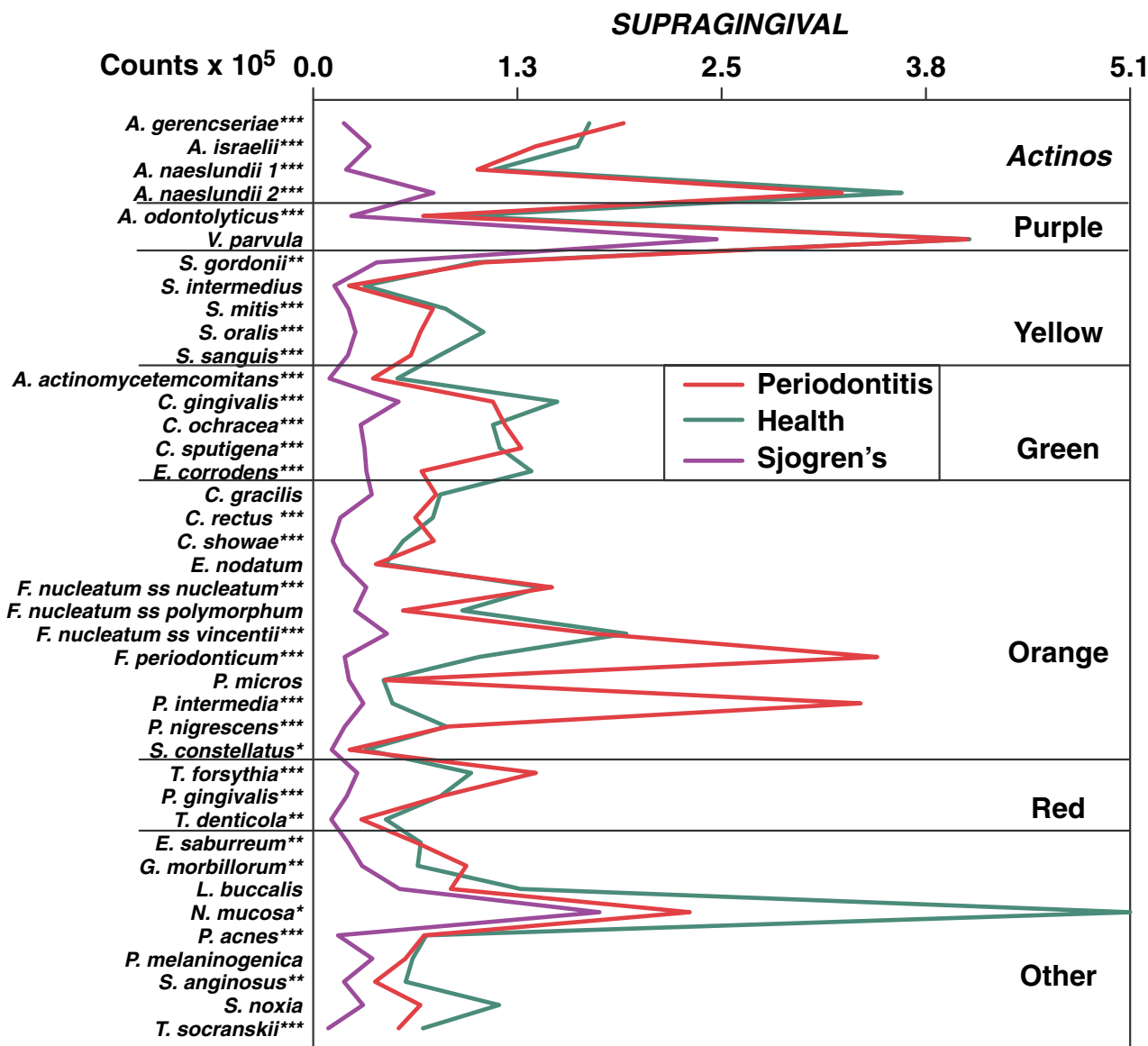


Fig. 15. Profiles of mean counts of 40 taxa in *supragingival* plaque samples from 28 periodontally healthy, 53 chronic periodontitis, and 24 Sjogren's subjects. Plaque samples were taken from the mesial aspect of each tooth and analyzed separately for their content of 40 species. Data for each species were averaged within a subject and

then across subjects in the three clinical groups separately and significance of differences among groups sought using the Kruskal-Wallis test and adjusted for multiple comparisons (157); \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Species were ordered according to the complexes described by Socransky et al. (156).

subjects were *S. oralis*, *E. corrodens*, *N. mucosa*, *Propionibacterium acnes*, and *P. melaninogenica* (Fig. 18). Using cluster analysis (Fig. 19), the mean microbial profiles for Sjogren's and control subjects clustered separately. Further, in each clinical group, two clusters were formed consisting usually of saliva and tongue dorsum samples vs. the remaining surfaces. These data indicate that microbial species on the soft tissues and in saliva of Sjogren's subjects differed in quantity and proportions from species detected in control subjects and suggest that the decreased levels of saliva in Sjogren's syndrome

subjects may have affected the amount and nature of the species exposed to this 'bulk fluid'.

#### The critical role of 'bulk fluid' – gingival crevice fluid

The major bulk fluid that affects most of the exposed surfaces of the oral cavity is saliva. A second locally, and possibly generally, important bulk fluid is gingival crevice fluid, which emanates from the sulcus or periodontal pocket in dentate subjects. Some studies suggest that this fluid may be critical for sustained colonization of certain taxa (26). It has also been suggested that the inflammatory state of the gingival



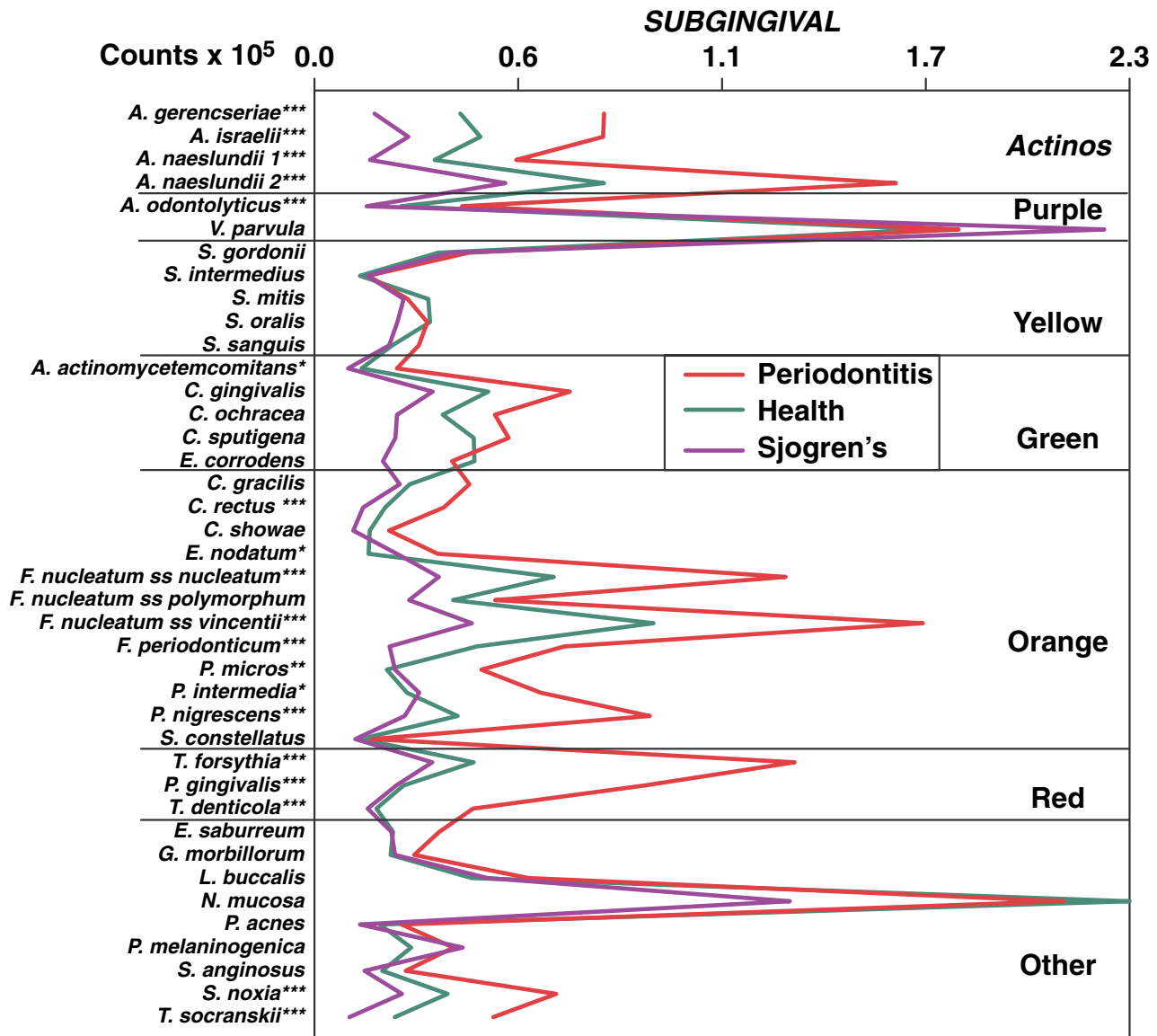


Fig. 16. Profiles of mean counts of 40 taxa in *subgingival* plaque samples from 28 periodontally healthy, 53 chronic periodontitis, and 24 Sjogren's subjects. The methodology and statistical analysis are as described for Fig. 15.

sulcus or periodontal pocket is important to the rate and perhaps nature of microbial colonization at or below the gingival margin (54, 67, 129, 131, 133, 146). Sites that exhibit natural gingivitis (131) or experimentally induced gingivitis (67, 129, 133) exhibit far more rapid plaque re-growth than periodontally healthy sites. The explanation for this has, at least in part, been a greater flow rate of the bulk fluid, gingival crevice fluid. The flow rate of gingival crevice fluid is related to surrogate measures such as the Gingival Index or other measures of gingival inflammation (115, 163, 171).

It was shown above that the composition of the biofilms present on natural teeth differs from that observed in samples from dentures. Some of the differences may have been due to the nature of the

surface present for initial colonization. Some, however, may have been due to the availability of gingival crevice fluid in the dentate but not the edentulous subjects. It has been suggested that *A. actinomycetemcomitans* and *P. gingivalis* disappear from the oral cavity after extraction of all the teeth and do not re-appear even when hard surfaces such as full dentures are provided (26). Is this true? Or is this finding due to the less sensitive cultural techniques that were used? This issue is of major consequence because it has been recognized for some time that *A. actinomycetemcomitans* is a common colonizer of the soft tissues and supragingival plaque (39, 44, 107–109, 160). However, if the removal of the natural teeth leads to the eradication of this species as well as *P. gingivalis*, then this strongly implicates gingival

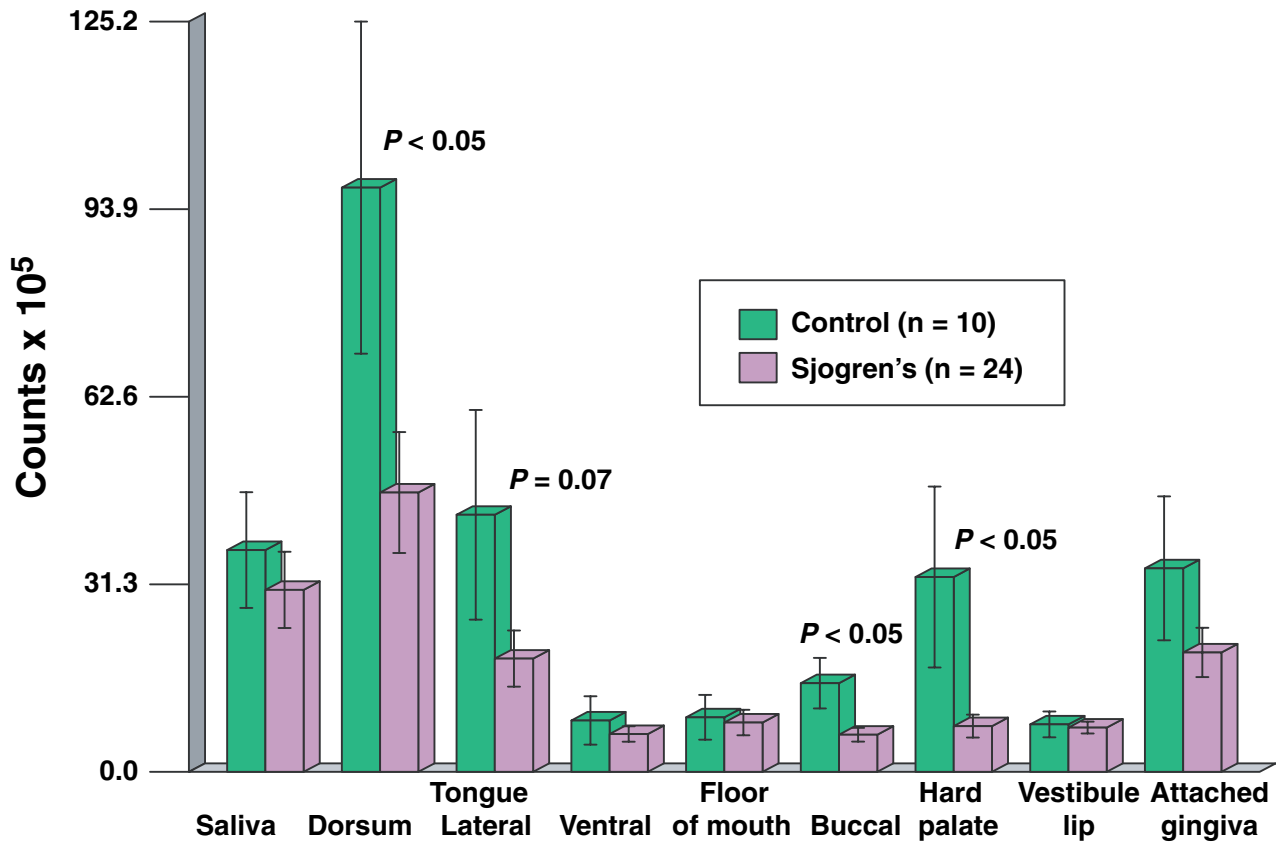


Fig. 17. Bar chart of the mean total DNA probe counts in 24 Sjogren's and 10 matched control subjects in saliva and at 8 intraoral soft tissue locations. Saliva samples and microbial samples taken from the soft tissue surfaces were individually analyzed for their content of 40 bacterial species. The total DNA probe counts were computed for

crevice fluid or the periodontal pocket environment as essential to sustained colonization by these consensus periodontal pathogens. In the studies of edentulous subjects described above, both *P. gingivalis* and *A. actinomycetemcomitans* as well as *T. forsythia* were detected on the dentures and soft tissues of some but not all subjects. Since this was at odds with existing literature, we examined the composition of split microbial samples from the dorsum of the tongue and the denture of 10 fully edentulous subjects using checkerboard DNA-DNA hybridization, conventional culture techniques, and PCR. *P. gingivalis* and *T. forsythia* were detected by hybridization in nine and eight samples each from the dentures. However, the species were not detected in pure culture on enriched media. PCR using primers directed to the 16S rRNA genes (5) did detect the species in aliquots of the same samples, suggesting that culture was less sensitive for the detection of low numbers of these species in biofilm samples than the molecular methods. The data indicate that *P. gingivalis*, *A. actinomycetemcomitans*, and *T. forsythia* can

each subject for each sample site and then averaged across subjects in the two groups for each sample location separately. Significance of differences in total counts between Sjogren's and control subjects were determined using the Mann-Whitney test.

be detected in edentulous subjects 1 year or longer after all teeth have been extracted.

### Comparison of supragingival and subgingival biofilm composition

Three components are described above as making up a biofilm; the surface to be colonized, the colonizing microbiota, and the bulk fluid. These 'components' differ for supra- and subgingival biofilms and impact on the microbial composition of biofilm samples from the two locations. Figures 20 and 21 compare the mean counts and mean proportions of species in supragingival and subgingival biofilms, respectively. Because periodontal disease status impacts so markedly on biofilm composition (to be discussed below), data from 50 periodontally healthy subjects (left panels) and 89 periodontitis subjects (right panels) are presented separately. The mean counts of most species are significantly higher in supragingival than in subgingival biofilms of periodontally healthy subjects (Fig. 20, left panel). This should come as no



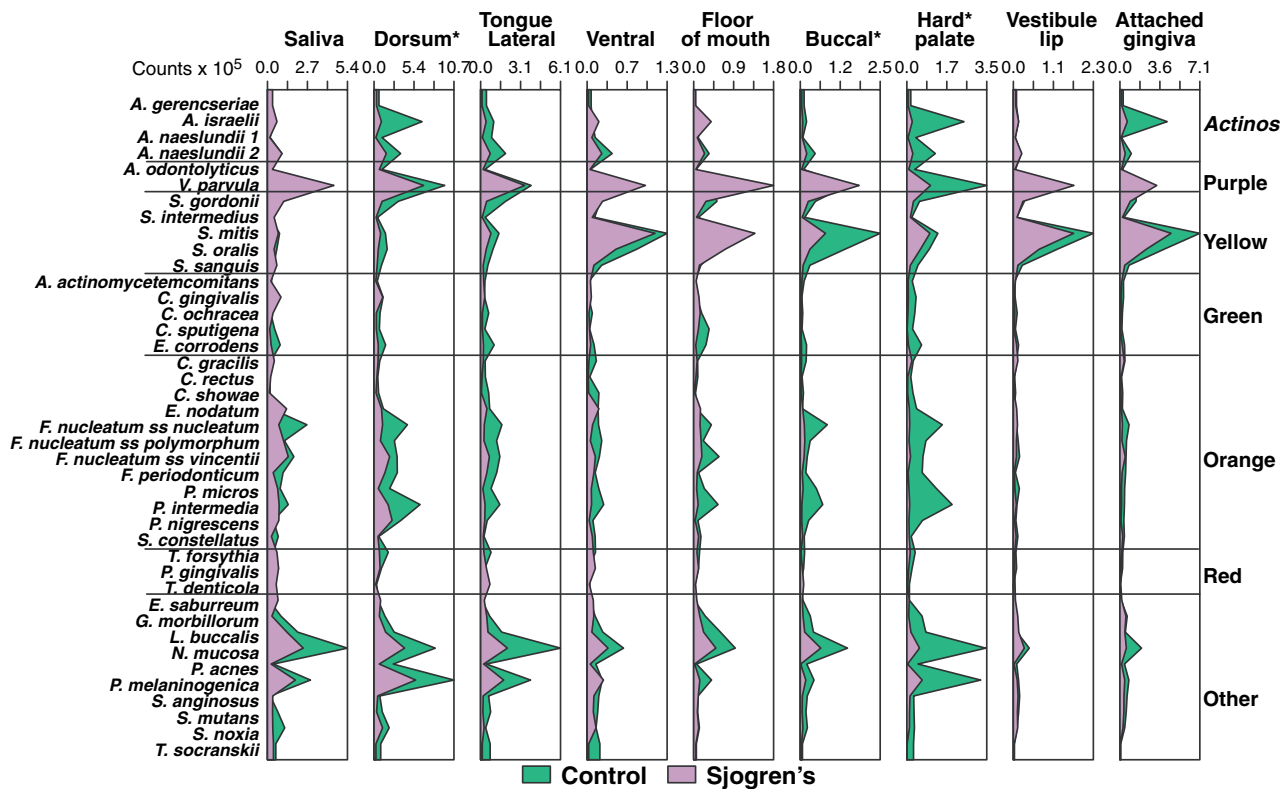


Fig. 18. Mean microbial profiles of saliva and 8 oral soft tissue surfaces in 24 Sjogren's and 10 matched control subjects. The samples were collected and analyzed as described in Fig. 17. Significance of differences in bacterial counts between Sjogren's and control subjects was

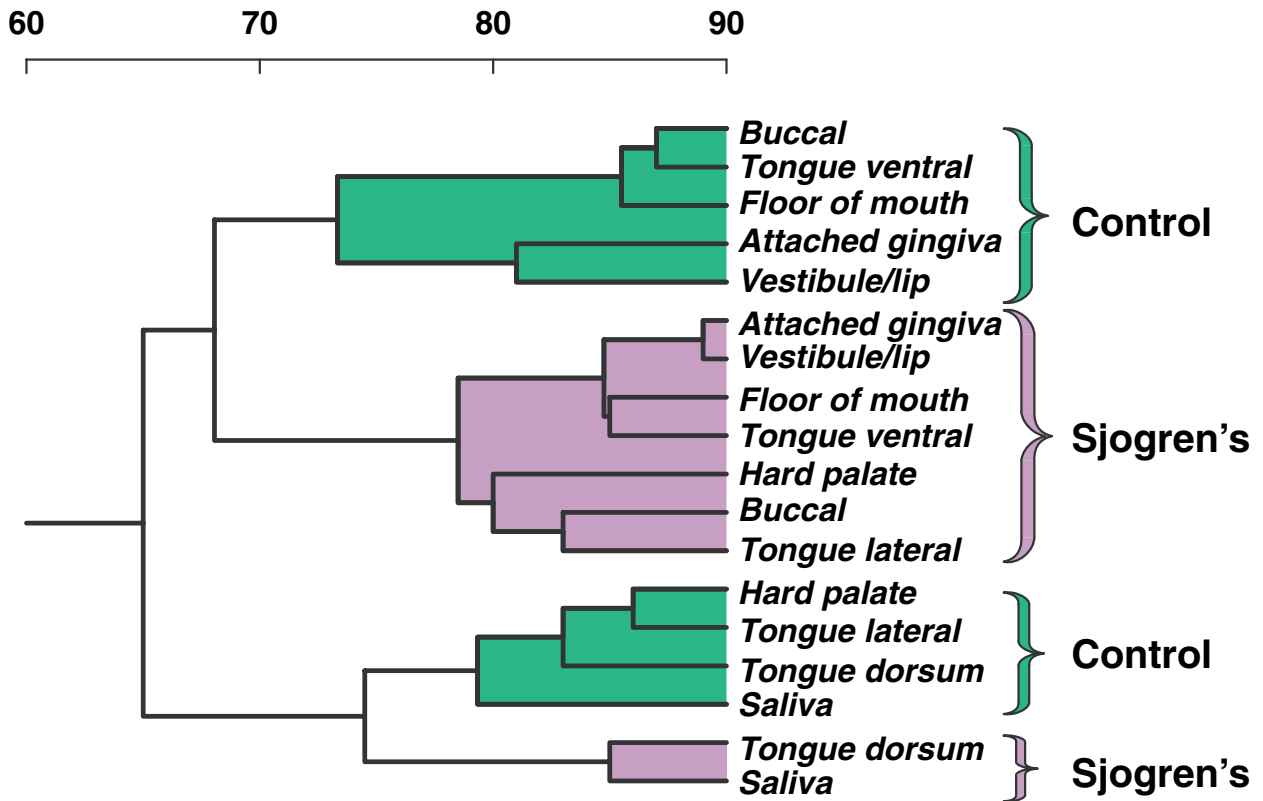
determined for each sample location using the Mann-Whitney test. Significant differences between Sjogren's and control subjects were seen in the microbial profiles of the tongue dorsum, buccal mucosa and hard palate.

surprise to anyone who has ever treated a periodontally healthy subject. The major difference appears to be in much higher counts of *Actinomyces* species and members of the purple, yellow, green, and 'other' complexes. The proportions of species were not markedly different from supragingival to subgingival plaque in periodontally healthy subjects (Fig. 21, left panel). There tended to be somewhat higher proportions of *Actinomyces* and *Streptococcus sanguis* in supragingival sites, whereas orange complex species were in somewhat higher proportions in subgingival sites.

The mean counts of supragingival and subgingival plaque were higher in subjects with periodontitis (Fig. 20, right panel) than in periodontally healthy subjects. Comparison of the left and right panels of Fig. 20 indicates that there was a marked increase in many taxa in the subgingival biofilms of periodontitis subjects; particularly in orange and red complex species. There was also an increase in many of the species in supragingival plaque of the periodontitis subjects (Fig. 20, right panel). There were many more significant differences between the proportions of bacterial species in supragingival compared with subgingival

biofilms in the subjects with periodontitis (Fig. 21, right panel) than in the periodontally healthy subjects. There were increased proportions of the *Actinomyces* species and green complex species along with *N. mucosa* in supragingival biofilms, and significantly higher proportions of members of the orange complex and all red complex species in subgingival biofilms.

The generally higher counts of many species in supragingival than in subgingival biofilm samples, particularly in periodontally healthy subjects, probably reflects, in large part, the space available for colonization, i.e. a physical barrier for colonization. The gingival sulcus in periodontally healthy sites provides little space for the habitation of large numbers of microorganisms. And while the periodontal pocket in diseased subjects provides a greater space for microbial colonization, counts in the supragingival area are still somewhat higher than in the adjacent subgingival site for most species. This varied from subject to subject in the periodontitis group. Some subjects showed far higher counts in supra- than in subgingival samples, while others exhibited higher counts in the subgingival samples. This may have reflected individual oral hygiene practices.

**% similarity (minimum similarity coefficient)**

**Fig. 19.** Dendrogram of a cluster analysis of the mean species proportions from saliva and 8 oral soft tissue surfaces in 24 Sjogren's and 10 matched control subjects. A minimum similarity coefficient was employed and an

average unweighted linkage sort. Four clusters were formed at >70% similarity. Two clusters consisted of Sjogren's subjects and two of the control subjects.

The differences in the microbial composition of the supra- vs. subgingival ecosystem so strongly demonstrated in the periodontitis subjects is probably due to multiple factors. The supragingival biofilm presents a single surface for colonization, the tooth, whereas the subgingival area provides two surfaces, the tooth and the epithelium lining the gingival sulcus or periodontal pocket. Indeed, the periodontal pocket or gingival sulcus appears to be unique in the oral cavity in that there appears to be two biofilms in a single anatomic space. The anatomy of this area also permits the establishment of a third 'zone' within the pocket, the 'loosely adherent' plaque zone with less intracellular matrix (86). This zone occurs between the biofilm attached to the tooth and the biofilm attached to the epithelium in deeper periodontal pockets. How tightly organisms in this zone are attached to either biofilm is not clear, but its existence is possible because the organisms in this area are surrounded by confining tissues on all sides except the orifice of the pocket. Without this physical confinement, weakly adherent species would prob-

ably be washed away from colonized surfaces. A second major difference between the supra- and subgingival habitats is the nature of the bathing bulk fluid; for supragingival biofilms this is saliva, and for subgingival biofilms, gingival crevice fluid. The composition of the gingival crevice fluid differs from that of saliva and the composition also differs from periodontal health to disease. These differences in composition can markedly affect the nature of species that colonize this area. For example, species of spirochetes are very much favored by the gingival crevice fluid emanating from periodontal disease sites (41).

## Development of intraoral biofilms

### Biofilm development – the earlier studies

Given the critical role that the microorganisms in biofilms play in the etiology of dental diseases, it is astonishing that the *in vivo* microbial changes that occur in the development of these biofilms are not

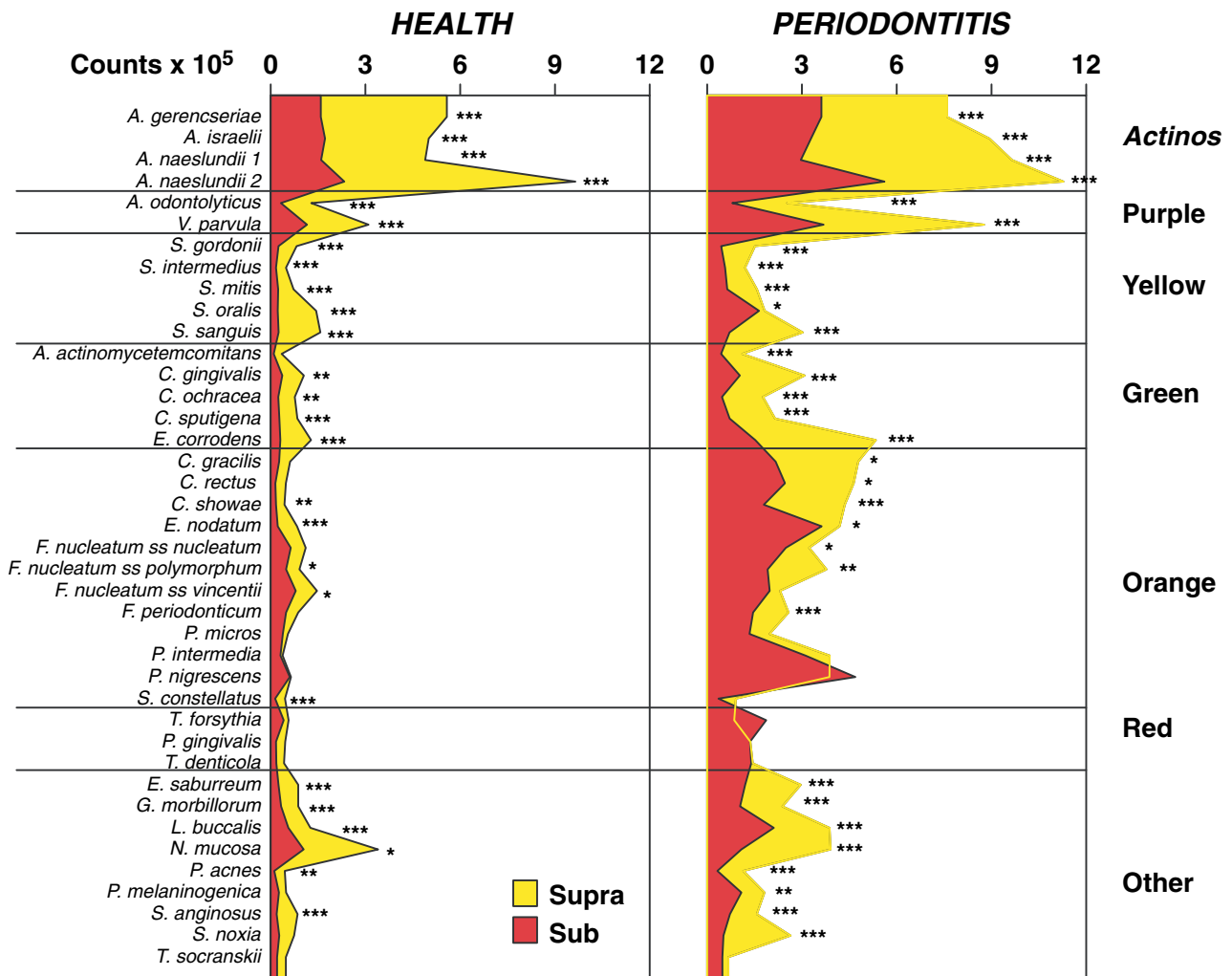


Fig. 20. Profiles of the mean counts of 40 taxa in supra- and subgingival plaque samples from 50 periodontally healthy subjects (left panel) and 89 chronic periodontitis subjects (right panel). Supragingival plaque samples and, separately, subgingival plaque samples were taken from the mesial aspect of each tooth and individually analyzed for their content of 40 species using checkerboard DNA–DNA hybridization. The supra- and subgingival counts of each species were averaged

separately within a subject and then averaged across subjects for the two clinical groups and two sample locations. The species were ordered according to the complexes described in (156). Significance of differences in species counts between supra- and subgingival samples was determined using the Wilcoxon signed ranks test and adjusted for multiple comparisons (157): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

known in exquisite detail. Elaborate scenarios of plaque development have been laid out, based on light or electron microscopic observation (86, 87, 89), *in vitro* adhesion and coaggregation models (35, 48–50, 74, 75, 100, 147, 150, 165, 166), and *in vitro* continuous culture studies (142, 164, 180). From these studies and others detailed in (155), the putative steps in the development of intraoral biofilms have been postulated as outlined in Fig. 22. However, virtually no studies have comprehensively examined *in vivo* the microbial shifts that occur during supra- or subgingival plaque development and no published studies have examined changes in soft tissue biofilm composition over time.

Ritz (140) described the changes that occurred in plaque that formed on the labial surfaces of the six maxillary and six mandibular anterior teeth. He used selective media to enumerate seven ‘genera’ of organisms – streptococci, *Actinomyces*, corynebacteria, *Neisseria*, fusobacteria, *Veillonella*, and *Nocardia* – in two pooled samples from each of six adult subjects at 1, 3, 5, 7, and 9 days. Streptococci were predominant at 1 day, constituting an average of 46% of the colonies detected. *Neisseria* (9%) and *Nocardia* (6%) were also high in mean proportions at 1 day but decreased in counts and proportions over time (2% and 0.1%, respectively, at 9 days). *Actinomyces* were initially low in proportion (0.2%) but rose to 23% of the

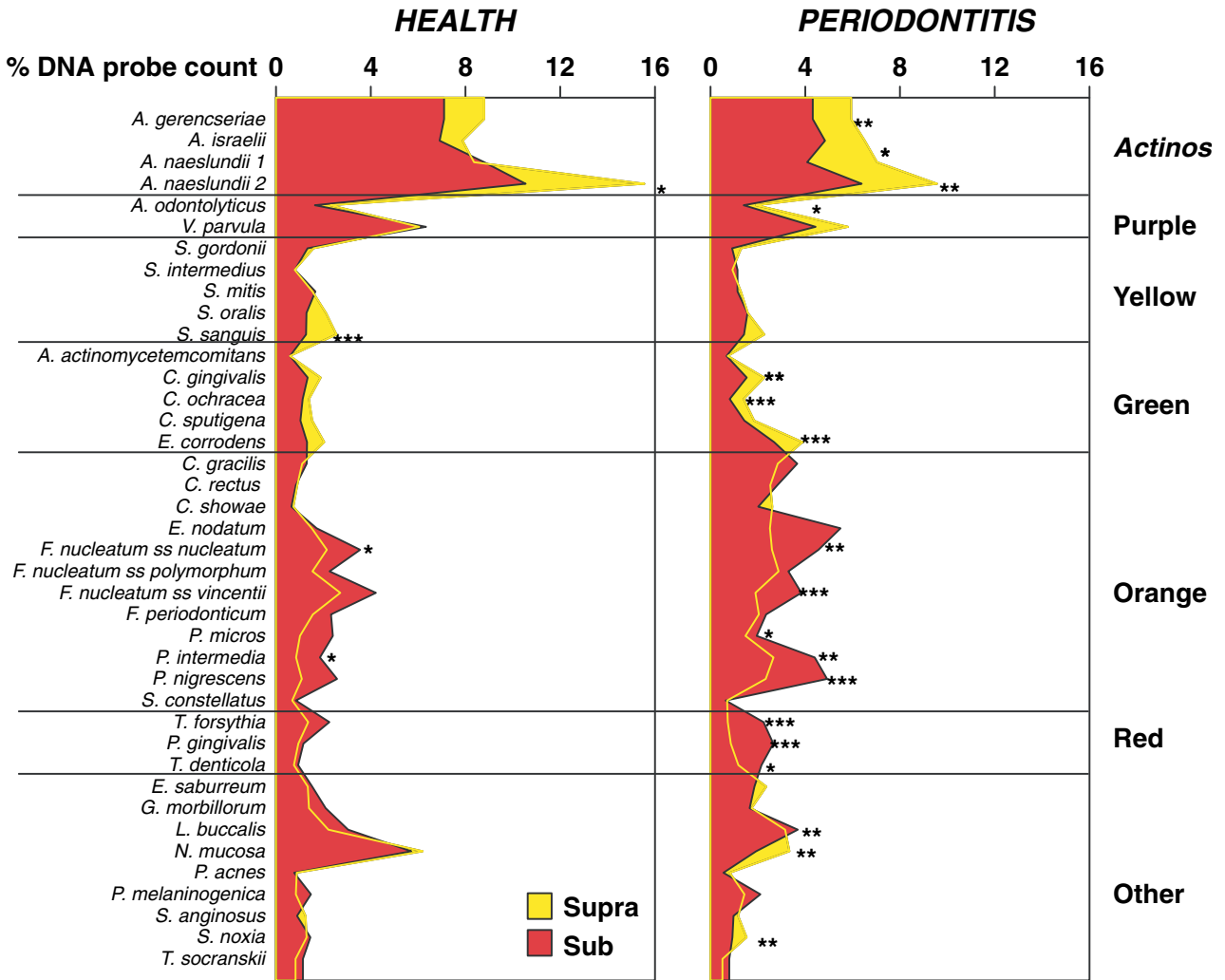


Fig. 21. Profiles of the mean percentage of the DNA count of 40 taxa in supragingival and separately subgingival plaque samples from 50 periodontally healthy subjects and 89 chronic periodontitis subjects. The methodology and statistical analysis were as described in Fig. 20.

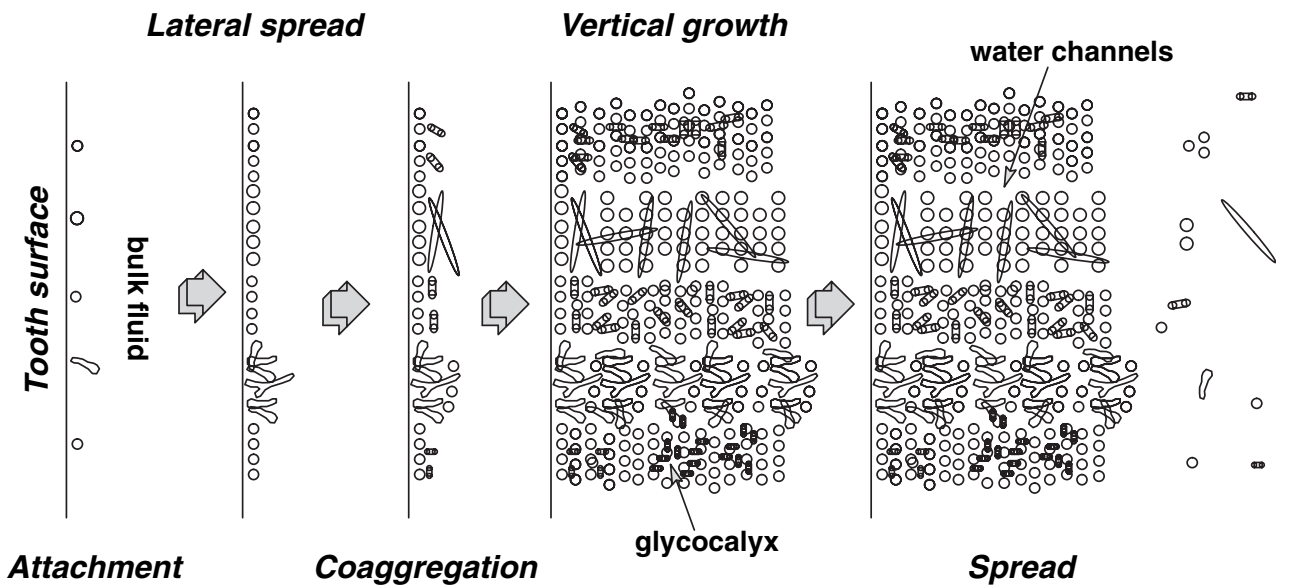


Fig. 22. Diagrammatic representation of the events thought to occur during the development of dental biofilms. These stages are discussed in (155).

microbiota by 9 days. Ritz (140) felt that there was microbial succession in plaque development with aerobic or facultative species reducing the environment for the subsequent growth of anaerobic species. The study was a 'classic' in its time, but by today's standards suffered from the fact that few subjects and samples were evaluated, that the test organisms were not speciated, and that the anaerobic culture techniques employed were flawed due to the lack of H<sub>2</sub> in the anaerobic gas mixture.

Socransky et al. (161) used predominant cultivable microbiota techniques to study shifts in the microbial populations that occurred in supragingival plaque forming on the buccal surface of a single tooth in one subject. The data indicated that few shifts occurred in microbial composition from 5 min to 8 h. There was a marked increase in total counts and counts of specific species that occurred at 1 day but this leveled off from 2 to 16 days. *Actinomyces* species were high in proportion from 5 min to 8 h but declined in proportions to 1 day, increased in proportions from 1 to 2 days, leveling off by 16 days. *S. sanguis* was detected at all time points, increased in proportion at 1 day and declined thereafter. This study speciated organisms to the level possible at that time, but was limited in that only one sample site in one subject was studied.

The most comprehensive microbiological assessment of plaque development over time in the human was carried out by Zee et al. (188). In that study, a single plaque sample was taken from each of five 'rapid' plaque-forming subjects and six 'slow' plaque-forming subjects at days 1, 3, 7, and 14. Samples were anaerobically dispersed, diluted and plated, and 20–30 colonies identified per subject. Gram-positive bacteria were the predominant cultivable species in both clinical groups, but gram-negative species increased in proportion more rapidly in the 'rapid' plaque formers. At 14 days, the 'rapid' plaque formers had a mean of 38% gram-negative rods compared with 17% in the 14-day samples from the 'slow' plaque formers. The majority of cultivable gram-negative rods were in the genera *Fusobacterium* and *Capnocytophaga*. A striking finding in Zee et al.'s study was the decrease in the proportion of gram-positive cocci from 50–60% at day 1 to <15% at day 14. This decrease was accompanied by an increase in the proportion of *Actinomyces* species and gram-negative rods. A similar study was performed in monkeys by Radford et al. (130). The labial surfaces of the central incisors of 15 monkeys were cleaned and plaque samples were then taken at 0.5, 1, 2, 4, 7, 14, and 28 days. They found a high proportion of *Streptococcus* species at 1 day (35%) which fell to 7%

at day 7. The *Actinomyces* increased throughout the period of plaque formation and constituted 15% of the total colony forming units at 28 days.

## Supragingival biofilm development

The very early stages of supragingival plaque development on buccal/labial surfaces in 15 periodontally healthy subjects were studied by Li et al. (84). A saliva sample was collected by expectoration prior to cleaning the tooth surfaces with pumice. Pooled samples of supragingival plaque were taken 0, 2, 4, and 6 h after cleaning using a PVDF membrane. After each sampling, the teeth were re-cleaned and samples were allowed to form for the next time period. The counts and proportions of 40 bacterial species were determined using checkerboard DNA–DNA hybridization. The data indicated that the distribution of species in saliva was different from that observed in the plaque samples from the same subjects, indicating the selectivity of the initial adhesion process (Fig. 23). *Actinomyces* species were observed to increase from 0 to 2 h but remained essentially constant to 6 h. The major increases that were observed were for *S. mitis* and *S. oralis*, which increased markedly up to 6 h. Other species remained at negligible or low levels during this time period.

A second study of supragingival biofilm regrowth for a longer period of time also used molecular techniques (134). The authors demonstrated that 4-day growth of plaque in the absence of home care was quite limited in 10 subjects who had had a 'preparatory period' during which the supragingival plaque and gingival inflammation was brought to minimal levels by professional and self-performed hygiene measures. In the absence of gingival inflammation, the microbial shifts in these subjects were also minimal.

Another study was initiated at The Forsyth Institute to compare early microbial changes in supragingival biofilm formation in periodontally healthy, periodontitis and edentulous subjects. Supragingival plaque samples were taken from the mesial aspect of each tooth (or denture tooth) in 30 periodontally healthy, 8 periodontitis, and 18 denture-wearing subjects, at entry, and individually analyzed for their content of 41 bacterial species using checkerboard DNA–DNA hybridization. The teeth (or dentures) were cleaned and immediately re-sampled to provide a '0 time' baseline. Subjects refrained from oral hygiene for 7 days. Plaque samples were taken from seven teeth in randomly selected quadrants at 1, 2, 4, and 7 days and analyzed by checkerboard DNA–DNA hybridization. Counts of each species were determined for each

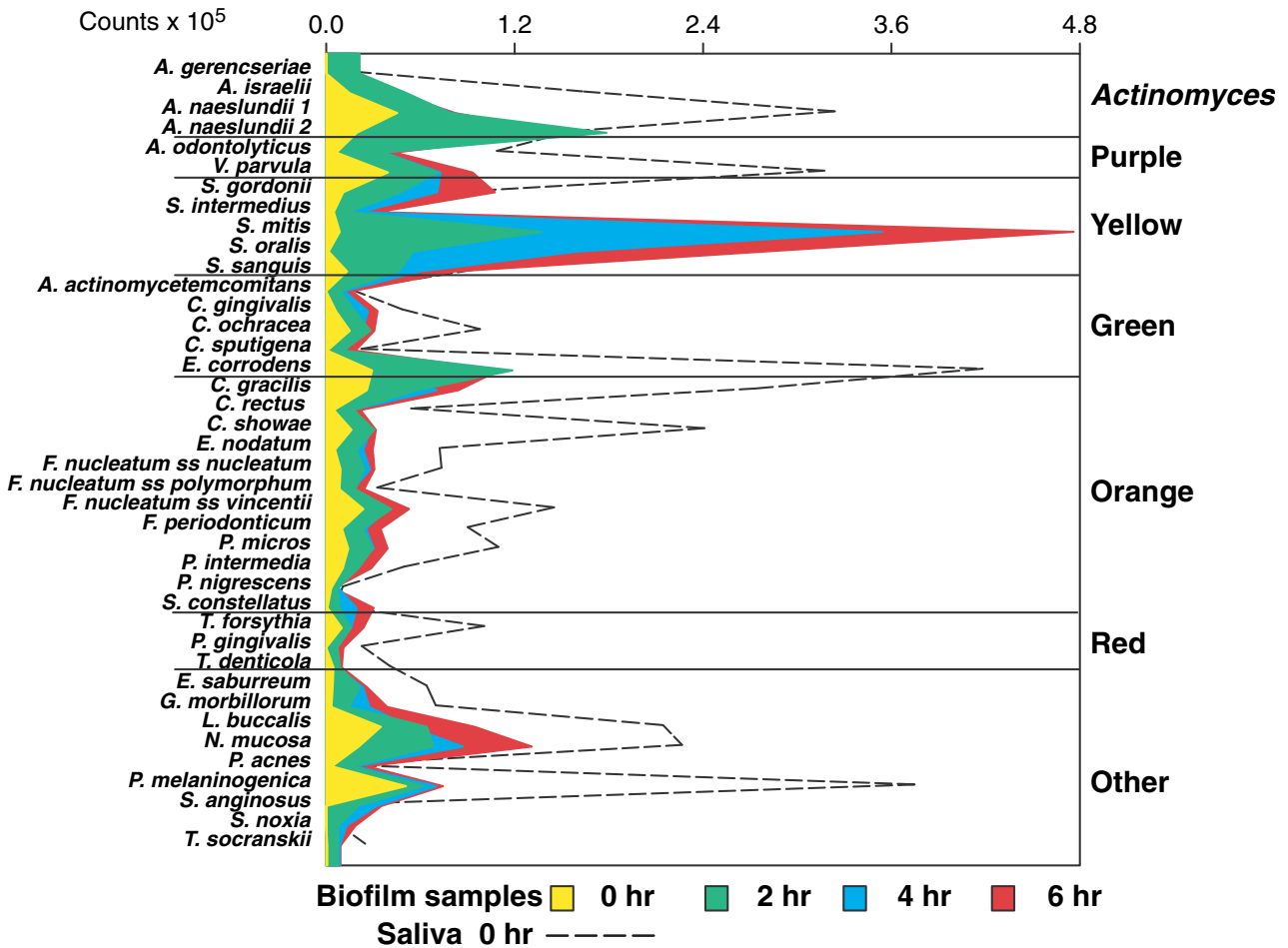


Fig. 23. Microbial profiles of the mean counts ( $\times 10^5$ ) of 40 taxa in saliva samples and plaque biofilm samples pooled from at least 20 buccal surfaces immediately postcleaning (0), 2, 4, and 6 h in 15 subjects. Samples were analyzed for their content of 40 bacterial species using

checkerboard DNA-DNA hybridization. Counts of individual species were computed in each subject and then averaged across subjects for each time point. The species are ordered according to the complexes described by Socransky et al. (156).

sampled site and averaged in each subject at each time point.

The mean ( $\times 10^5$ ,  $\pm$ SEM) total DNA probe counts were  $45 \pm 7$ ,  $66 \pm 12$ , and  $52 \pm 11$  on entry and  $6 \pm 1$ ,  $9 \pm 4$ , and  $6 \pm 1$  immediately after cleaning in healthy, periodontitis and edentulous subjects, respectively (Fig. 24). Total counts in healthy and periodontitis subjects exceeded baseline values ( $61 \pm 11$  and  $64 \pm 13$ , respectively) at 2 days, but took 4 days in edentulous subjects ( $57 \pm 18$ ). Baseline mean counts of most species were highest in periodontitis and lowest in edentulous subjects (Fig. 24). Counts of streptococci, *A. actinomycetemcomitans*, *Peptostreptococcus micros*, *E. corrodens*, and *V. parvula* returned to baseline levels by 2 days (Fig. 25). The remaining species returned to baseline levels by 4–7 days. The streptococci showed a tendency to plateau between 2 and 7 days. The pattern of supragingival re-colonization in periodontitis and health was virtually identical and more rapid than that

observed in edentulous subjects. However, individual species differed in the rate and maximum level of re-colonization achieved during 7 days of no home care.

Two points about home care emerged from Fig. 24 and 25. First, home care is of benefit to the individual, since the total counts of bacteria in supragingival plaque samples were markedly increased after 7 days of no home care compared with precleaning (entry) levels. Second, the fact that professional cleaning markedly lowered mean total counts from the precleaning sampling to the postcleaning 'baseline' sampling indicates that there is a lot of room for improvement in self-performed home care procedures.

### Subgingival biofilm development

Microbial changes that occurred in subgingival biofilm formation from baseline to 7 days were compared in the periodontally healthy and periodontitis



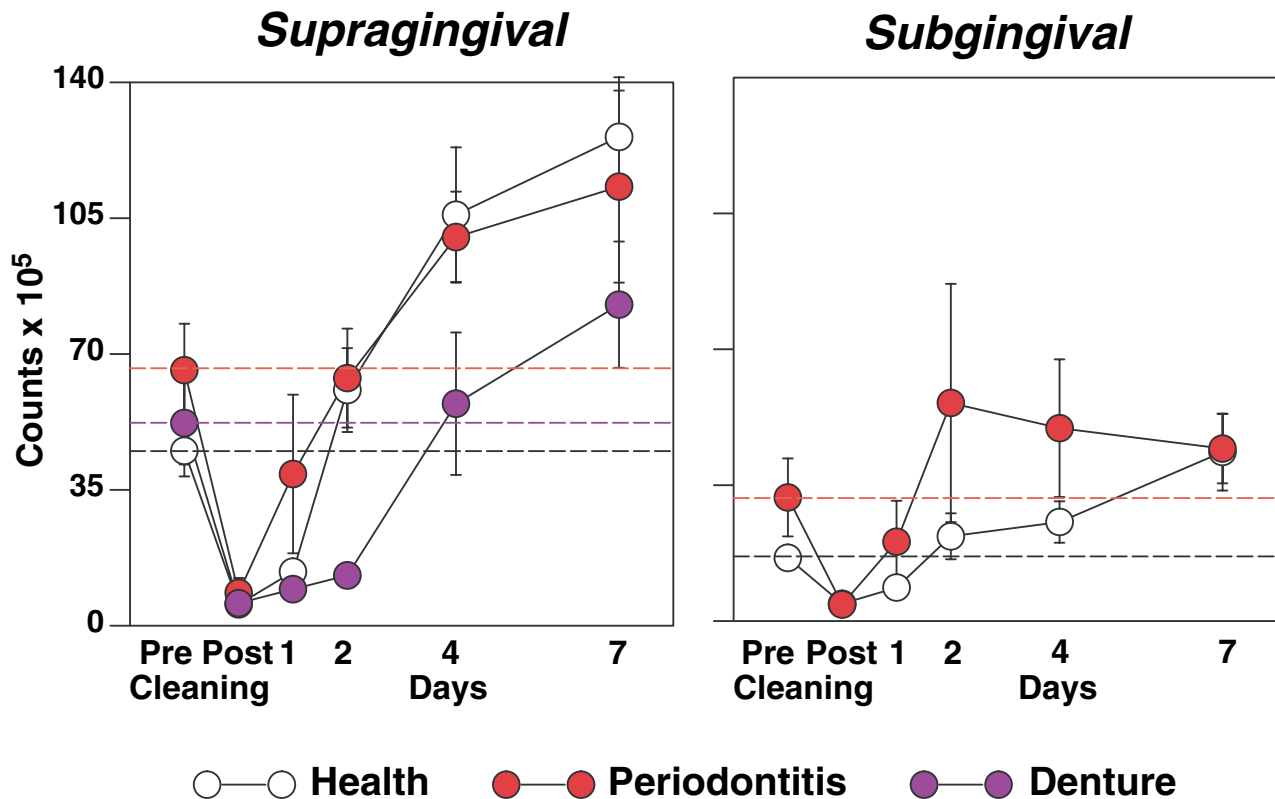


Fig. 24. Mean total DNA probe counts ( $\times 10^5$ ,  $\pm$ SEM) of plaque samples taken prior to, immediately postcleaning, and after 1, 2, 4, and 7 days of no oral hygiene in subjects

who were periodontally healthy, had periodontitis or wore full dentures. The dashed horizontal lines are provided to indicate the precleaning levels for each clinical group.

subjects described above. The mean ( $\times 10^5$ ,  $\pm$ SEM) total DNA probe counts were  $16 \pm 3$  and  $32 \pm 10$  on entry and  $5 \pm 1$  and  $4 \pm 1$  immediately postcleaning in healthy and periodontitis subjects, respectively (Fig. 24). At 2 days, total counts in healthy and periodontitis subjects exceeded baseline values ( $22 \pm 6$ ,  $56 \pm 31$ ). There were striking differences between health and periodontitis in the patterns of re-colonization (Fig. 26). In periodontitis subjects, counts of most species increased rapidly to well above baseline values by 2 days and 17 species, including the *Actinomyces* and *Streptococcus*, decreased to about baseline levels by 7 days. For other species, the counts plateaued or increased. Some species did not reach baseline levels by 7 days, including *C. gracilis*, *Eubacterium nodatum*, *P. gingivalis*, and *T. denticola*. For healthy subjects, the counts of most species increased slowly over time, reaching or exceeding baseline values by 7 days. These data indicate that the kinetics of subgingival plaque re-development in subjects with periodontitis or periodontal health differs markedly, at least in the absence of oral hygiene procedures.

As one examines the kinetics of hard tissue associated biofilm re-development, one is impressed

firstly by the speed of this process. In the absence of oral hygiene, re-growth as measured by total counts appeared to be achieved on average in a 2–4-day period for the three clinical groups. However, it must be stressed that there was great variability in the rate of biofilm re-development from subject to subject. This has been recognized for years from clinical observation and from studies that quantified plaque re-growth (149). The reason for these subject-to-subject differences is not known. In an earlier section, the contribution of gingival inflammation and increased gingival crevice fluid flow was described. However, it may not be the total answer. Differences in the rate of biofilm formation may be observed in subjects or at periodontal sites with comparable levels of clinical gingival inflammation. Differences are also observed in the rate of biofilm formation in subjects or sites where gingival inflammation is not clinically evident. Since gingival inflammation only partially explains differences in the rate of biofilm formation, there is a gap in our knowledge of the ecologic factors that foster or limit biofilm development in different individuals.

The data of this section support the notion of microbial succession discussed in the 'principles'

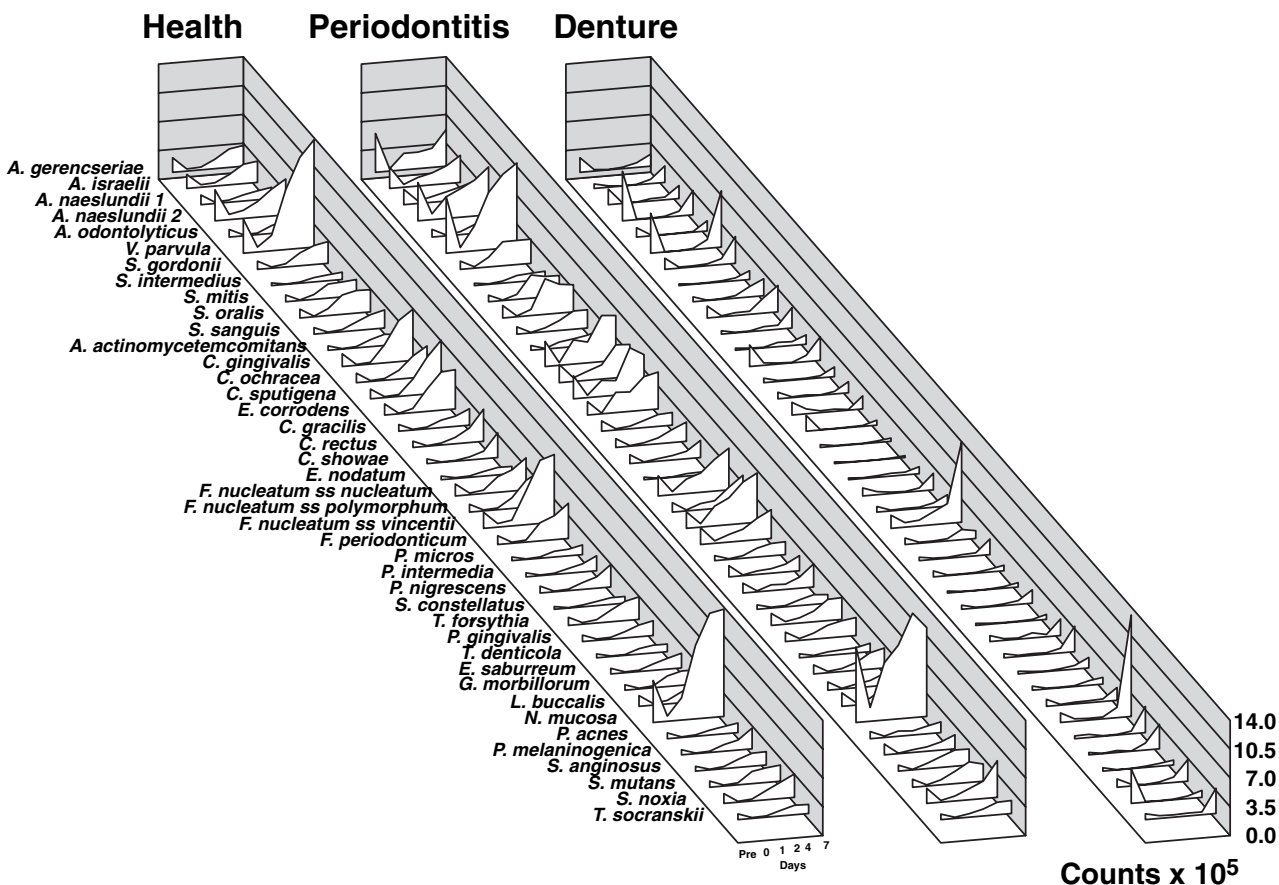


Fig. 25. Mean counts ( $\times 10^5$ ) of 41 taxa in supragingival plaque samples taken prior to, immediately postcleaning, and after 1, 2, 4, and 7 days of no oral hygiene in subjects who were periodontally healthy, had chronic periodontitis, or were wearing full dentures. Each 'slice' represents

the mean counts of an individual species at the various time points. The left edge of the 'slice' provides the mean count precleaning. The rapid decline to its right represents the immediate postcleaning mean values.

section of this paper in that species appeared to multiply in 'waves' (Fig. 27). For example, in the supragingival plaque samples of periodontally healthy subjects, *S. oralis* had reached its precleaning level by 1 day and 'peaked' by 2 days. *E. corrodens* had exceeded its precleaning value by 2 days and plateaued by 4 days. By contrast, it took 4–7 days for *A. naeslundii* genospecies 2 to reach precleaning levels. This species and *C. showae* exhibited a slower rate of growth initially and appeared to be continuing to increase at the 7-day termination of the study. These data are in accord with patterns observed by culture (161, 188). In those studies, supragingival biofilm development in the periodontitis subjects showed similar 'waves' of succession. *S. oralis* and *E. corrodens* had peaked well above their precleaning levels by 2–4 days while *A. naeslundii* genospecies 2 and *C. showae* reached precleaning levels between 4 and 7 days and appeared to be continuing to increase at 7 days.

Microbial succession could also be observed in samples of subgingival plaque from periodontally

healthy and periodontitis subjects (Fig. 27). However, there were astonishing differences in the pattern of re-growth in samples from these clinically different habitats. In samples from periodontally healthy subjects, there was a similar pattern to that observed in supragingival samples in that counts of *S. oralis* and *E. corrodens* increased to precleaning levels by days 1–2 and more or less plateaued, whereas counts of *A. naeslundii* genospecies 2 and *C. showae* increased more slowly and exceeded precleaning levels only by 4–7 days. In sharp contrast, in the subgingival samples from periodontitis subjects, there was very rapid increase of *A. naeslundii* genospecies 2, which peaked at 2 days and declined at 4 and 7 days. The rapid increase in this species was accompanied by a rapid increase in *S. oralis* and *E. corrodens*, which also decreased in mean counts after 2 days. *C. showae* showed a slow increase in growth reaching precleaning levels by 4 days. The reason for this pattern of explosive subgingival growth of certain species followed by a decline is unknown.



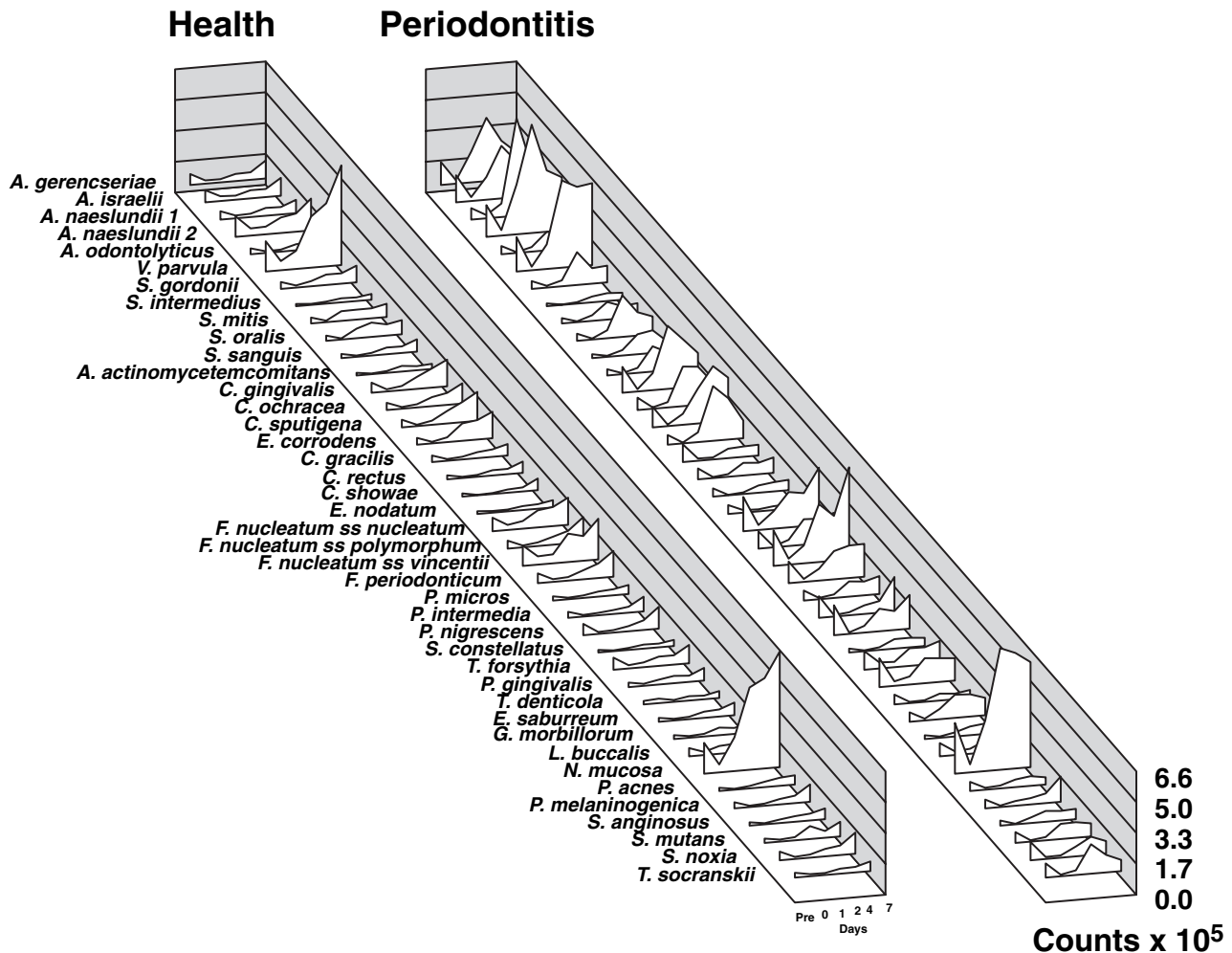


Fig. 26. Mean counts ( $\times 10^5$ ) of 41 taxa in subgingival plaque samples taken prior to, immediately postcleaning, and after 1, 2, 4, and 7 days of no oral hygiene in subjects who were periodontally healthy or had chronic periodontitis. Each 'slice' represents the mean counts of an

individual species at the various time points. The left edge of the 'slice' provides the mean count precleaning. The rapid decline to its right represents the immediate postcleaning mean values.

## Relation of periodontal disease status to subgingival biofilm composition

### Microbial composition of biofilms in periodontal health and disease

For over 100 years, each generation of oral microbiologists has come to the same conclusion; that the subgingival microbiota of periodontally healthy subjects differs from that found in subjects with periodontitis. This conclusion was drawn initially from light microscopy (88) and later from cultural microbiology (for review, see 63), electron microscopy (86), antibody-based techniques (33, 185) and molecular techniques (73). About a half century ago, Ted Rosebury, the pre-eminent oral microbiologist of his

era, wondered (in conversation with the old author), whether 'everybody had everything (all species), and the difference between health and disease was simply in the amount'. This question could not be clearly answered by the techniques available at that time, but can be partially answered today. Figure 28 provides mean microbial profiles of subgingival samples taken from 184 subjects who were periodontally healthy and 592 subjects with chronic periodontitis. The species are arranged according to the complexes described earlier. The Figure indicates that, on average, subgingival counts are higher in the subjects with periodontitis than in the subjects exhibiting periodontal health and that the major differences between health and disease occur primarily among the species of the red and orange complexes. Indeed, 11 of 12 species in the orange complex and all three members of the red complex were significantly

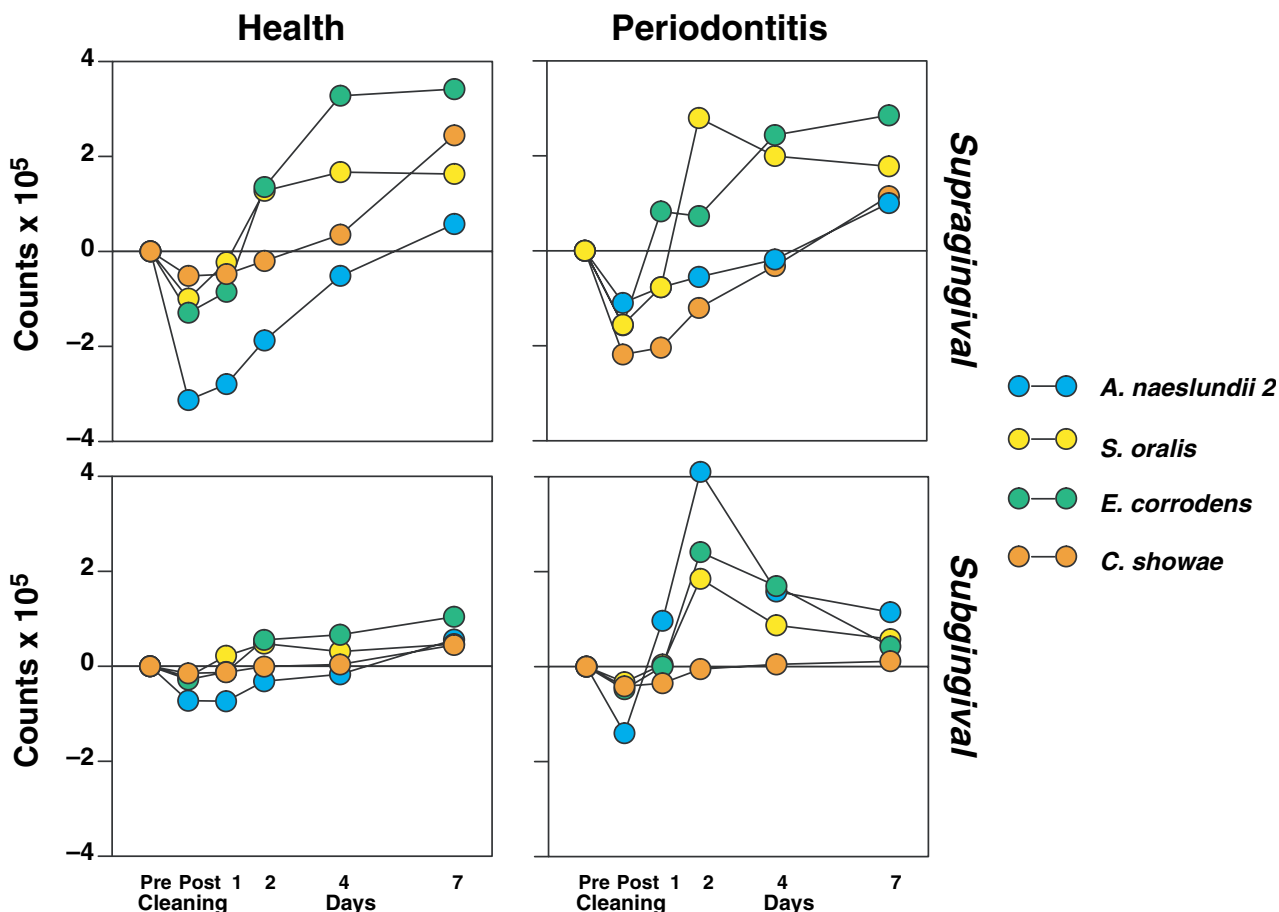


Fig. 27. Change in mean counts ( $\times 10^5$ ) of four species in supra- and subgingival plaque samples from precleaning to immediately postcleaning, and after 1, 2, 4, and 7 days

of no oral hygiene in subjects who were periodontally healthy or had chronic periodontitis.

elevated in the subjects with periodontitis even after adjusting for multiple comparisons. These data are in accord with studies in the literature that have used PCR to examine frequency of detection in health and disease (5, 22, 55, 73, 80, 83, 99, 167), real time PCR (69, 95), culture (for review, see 63), DNA probes (1), and antibody-based techniques (20, 33, 53, 151, 185). At this point, there can be little doubt that certain species such as *P. gingivalis*, *T. forsythia*, and *T. denticola* can be detected more frequently, in higher proportions and in higher numbers, in subjects with periodontitis than in periodontally healthy subjects. Other species, including 'uncultivable' species, have also been suggested to be more frequently detected in disease than in health using PCR techniques (80).

The difference in the composition of the subgingival microbiota between health and disease is instructive from an ecologic point of view. While the disease was initiated by the colonizing species, it is not clear whether the initial steps were brought about by some change in one or more species in a sub-

gingival site or by some change in the habitat (host), either locally or systemically. Some of the changes in the host induced by the colonizing species in the early stage of periodontitis might be in the local inflammatory status and some might lead to net loss of periodontal tissue. In either event, an alteration was made in the habitat (host tissue) which then affected the composition and metabolic activities of the colonizing microbiota. These back and forth modifications of host and microbiota eventually led to a new equilibrium, a new 'climax community', which in turn might result in an uneasy host-parasite equilibrium for a prolonged period of time. It is this new climax community that we sample in the subject with periodontitis. It did not achieve this new microbial composition overnight. The sampled climax community might be many years and myriads of back and forth iterations between host and parasites from the climax community that was initially present when the site was periodontally healthy. To change this climax community from that adapted to the disease state back to that present in the original

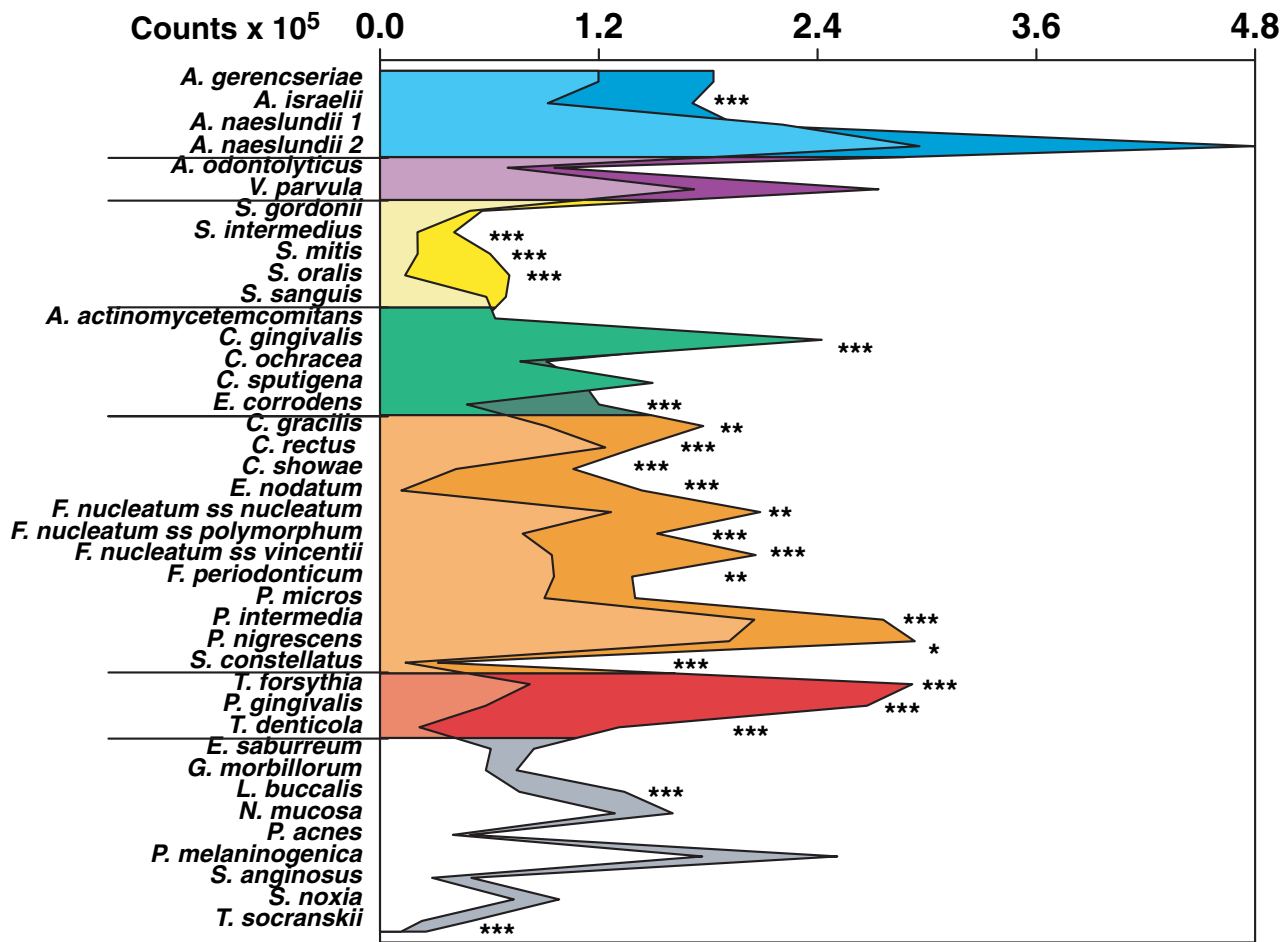


Fig. 28. Microbial profiles of the mean counts ( $\times 10^5$ ) of 40 microbial taxa in subgingival plaque samples taken from 184 periodontally healthy and 592 subjects with chronic periodontitis at baseline. The profiles represent the mean counts derived by averaging the counts of each species within a subject and then across subjects for both clinical groups. The species are ordered and color coded accord-

ing to the microbial complexes described in (156). The darker shade of each color represents the periodontitis subjects, while the lighter shade represents the periodontally healthy subjects. Significance of difference for each species between groups was determined using the Mann-Whitney test and adjusted for multiple comparisons (157): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

healthy situation might require more than a few well-placed strokes with a curette, as discussed elsewhere (65).

In the above section (and throughout this manuscript) mean values have been used to describe the microbial communities in various habitats. Mean values, while helpful in making incredibly complex data understandable, can be somewhat misleading. They fail to reflect the subject to subject variability in both the diseased and healthy subject groups. They also fail to represent the transition states from health to disease. Despite these limitations, it is clear that periodontal disease status has a major impact on the composition of the subgingival microbiota and that, on average, disease status affects certain species, particularly members of the red and orange complexes, more than others.

Some of the changes that may occur as an individual progresses from periodontal health to periodontal disease may be inferred by examining mean microbial profiles in subjects with different severities of periodontitis. The 592 subjects with chronic periodontitis in Fig. 28 were divided into subsets at the quartile values of mean baseline full-mouth pocket depth (Fig. 29). There is a striking change in the shape of the mean microbial profiles with increasing disease severity. This shift is particularly marked for the red complex species, which increase with increasing mean pocket depth, and to some extent, for some of the orange complex species. The change in mean microbial profile may be related directly to the increased numbers of deep periodontal pockets in the most diseased groups, since pocket depth at a site relates so strongly to the composition of the

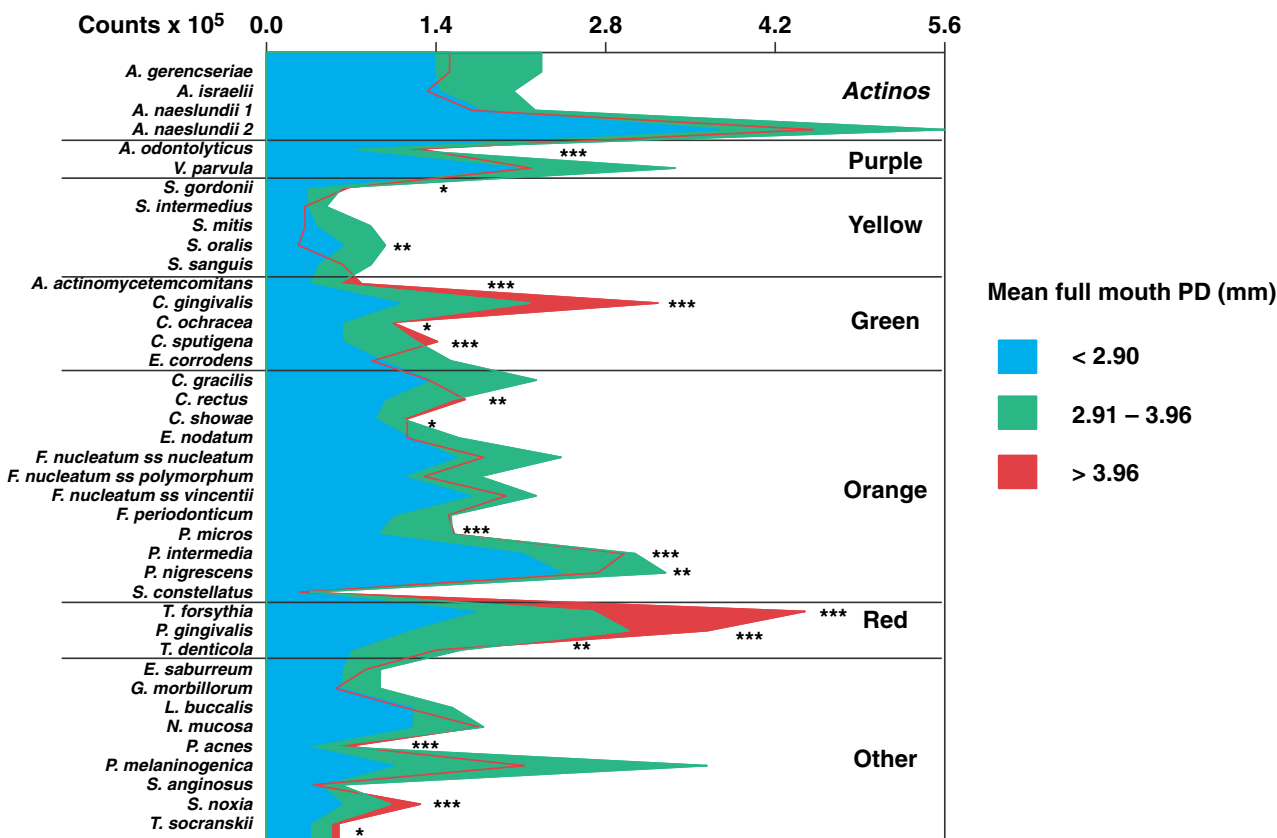


Fig. 29. Microbial profiles of the mean counts ( $\times 10^5$ ) of 40 microbial taxa in subgingival plaque samples taken from the 592 subjects with chronic periodontitis in Fig. 28. Subjects were divided into subsets according to mean full-mouth baseline pocket depth at the quartile values of 2.90 and 3.96 mm. The profiles represent the mean counts derived by averaging the counts of each species within a

subgingival microbiota, as discussed in the next section.

### Relationship of clinical parameters at a periodontal site to composition of the subgingival microbiota

Arguably the most important factor influencing the subgingival microbiota is the clinical status of the adjacent periodontal tissues. Two factors appear to be critical; the inflammatory status of the pocket and the periodontal pocket or gingival sulcus depth. The relationship of these factors to microbial composition has been recognized for quite some time (157). The relationship between pocket depth at a site and the counts of specific species is shown in Fig. 30. The data indicate that there is little relationship to pocket depth for the majority of microbial species; however, most species of the orange complex and all species of the red complex increased significantly with increasing pocket depth. A number of possible rea-

sons for the relationship of increased pocket depth with increased levels of red and orange complex species may be suggested. Deeper pockets have a greater epithelial surface area to which red complex species such as *P. gingivalis* and *T. denticola* may attach (72). The zone of loosely adherent bacteria which appears to contain large proportions of orange complex species (113) is increased in deep pockets relative to shallow pockets. In addition, local factors may limit the growth of the 'tooth-associated' species while promoting the growth of loosely adherent or epithelial-associated bacteria. For example, availability of fermentable carbohydrate might limit the growth of saccharolytic bacteria in the tooth-associated biofilm, while a relative abundance of non carbohydrate energy sources such as hydrogen, formate, acid end products, and protein degradation products might sustain better growth of some of the asaccharolytic species in the orange and red complexes.

The presence of gingival inflammation at a site also markedly affects the composition of the microbiota at

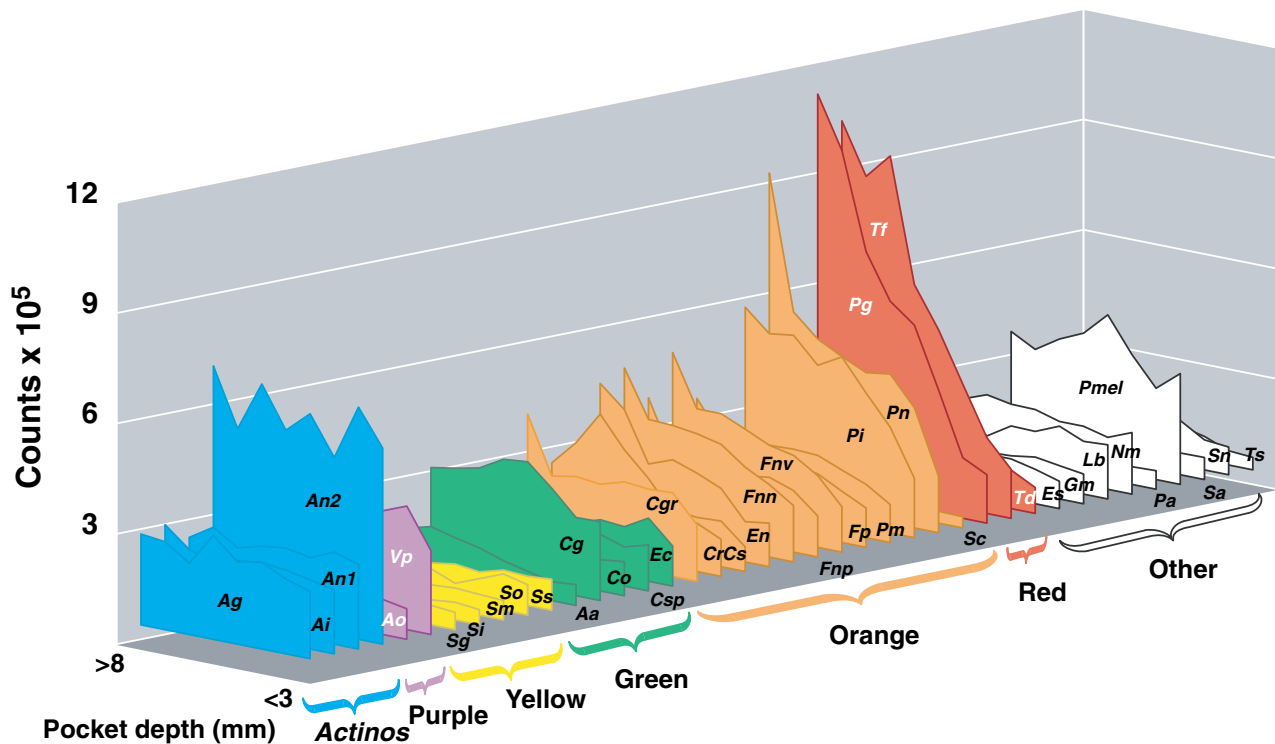


Fig. 30. Mean counts ( $\times 10^5$ ) of 40 species in subgingival plaque samples from different pocket depths in subjects with periodontitis. Subgingival plaque samples were taken from 14,577 sites in 588 subjects with chronic periodontitis at baseline and analyzed for their content of 40 subgingival species using checkerboard DNA–DNA hybridization. Counts of each species were averaged in each subject in the pocket depth categories of <3, 3, 4, 5,

6, 7, 8, and >8 mm and then averaged across subjects for each category. The species were ordered in the microbial complex species order presented in Fig. 28. The height of each 'slice' represents the mean counts for each species at each pocket depth category. Only species of the red and orange complexes were significantly related to pocket depth after adjusting for multiple comparisons.

that site (Fig. 31). The members of the red and orange complexes are significantly elevated at sites that exhibit bleeding on probing, which was used as a clinical indicator of periodontal inflammation. The species that were elevated in inflamed sites may have benefited from the inflammation in part because they were closest to the source of the increased gingival crevice fluid (first to the feeding trough) and in part because this fluid might be enriched with tissue degradation products that might favor the growth of many of the species that require proteins or peptides (e.g. *P. gingivalis* and *T. denticola*) or can use the metabolic end products of other species for their growth (e.g. *Campylobacter* species).

### Comparison of the microbiota in diseased and healthy sites of periodontitis subjects with the healthy sites in periodontally healthy subjects

Figure 32 compares the baseline mean subgingival microbial profiles of periodontal sites that all periodontists would agree were diseased, i.e. sites with

pocket depths  $\geq 6$  mm that bled on probing with the microbiota in 'periodontally healthy' sites in the same individuals that had a pocket depth <4 mm and did not bleed on probing. Major significant differences were found in the mean subgingival counts of many species, particularly for the species of the red and orange complexes, between the healthy and diseased sites in subjects with advanced periodontitis. Superimposed on the profiles from the periodontitis subjects is the mean microbial profile derived from samples from the periodontally healthy subgingival sites of periodontally healthy subjects. There were marked differences between 'periodontally healthy' sites in periodontally healthy subjects compared with 'periodontally healthy' sites in subjects with chronic periodontitis for virtually all species examined. The differences were most marked for orange and red complex species. These data are in accord with the findings of Riviere et al. (141) who demonstrated a greater frequency of detection of spirochetes and *P. gingivalis* in the healthy sites of periodontitis subjects compared with the healthy sites of subjects who were periodontally healthy (Fig. 2).

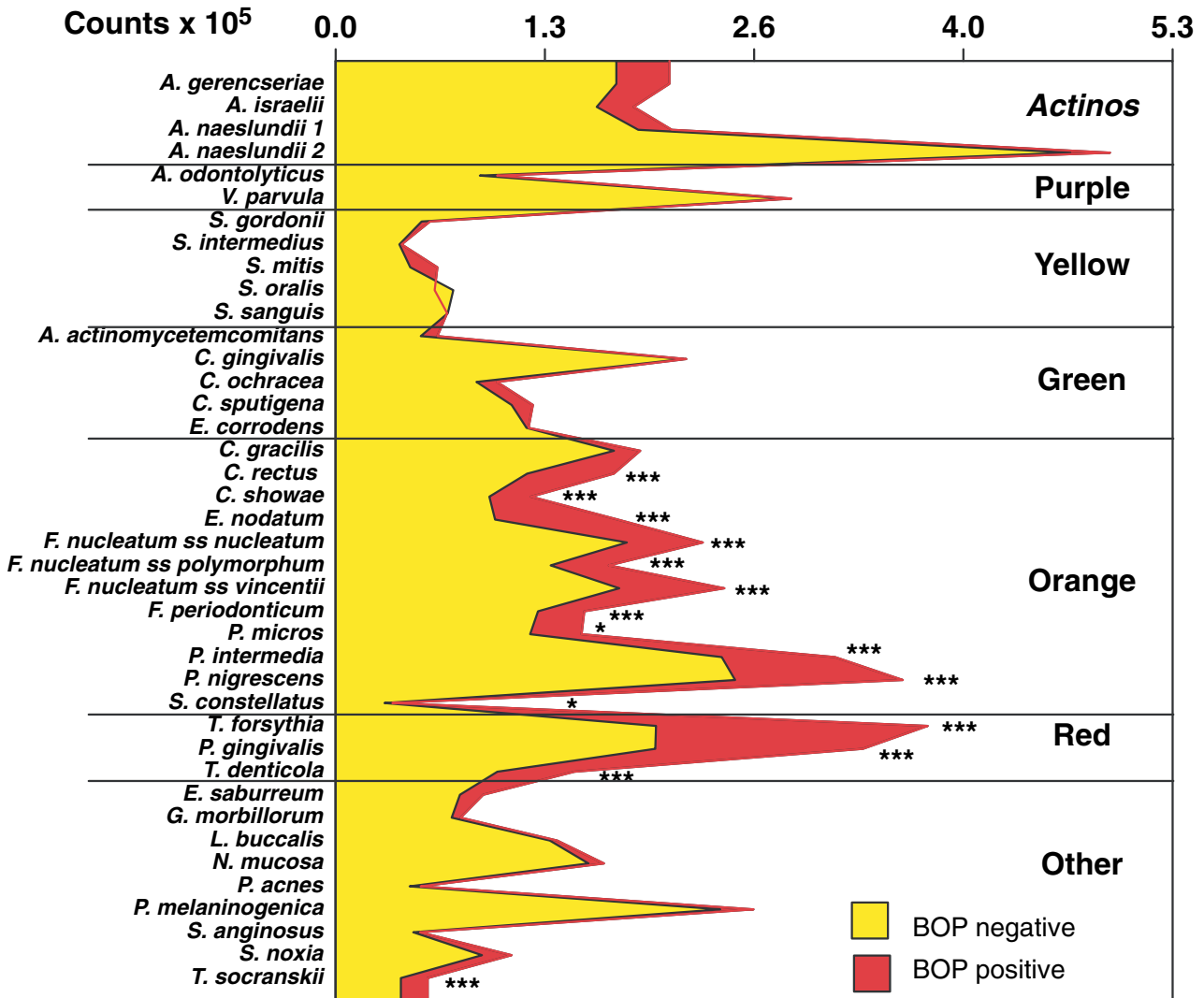


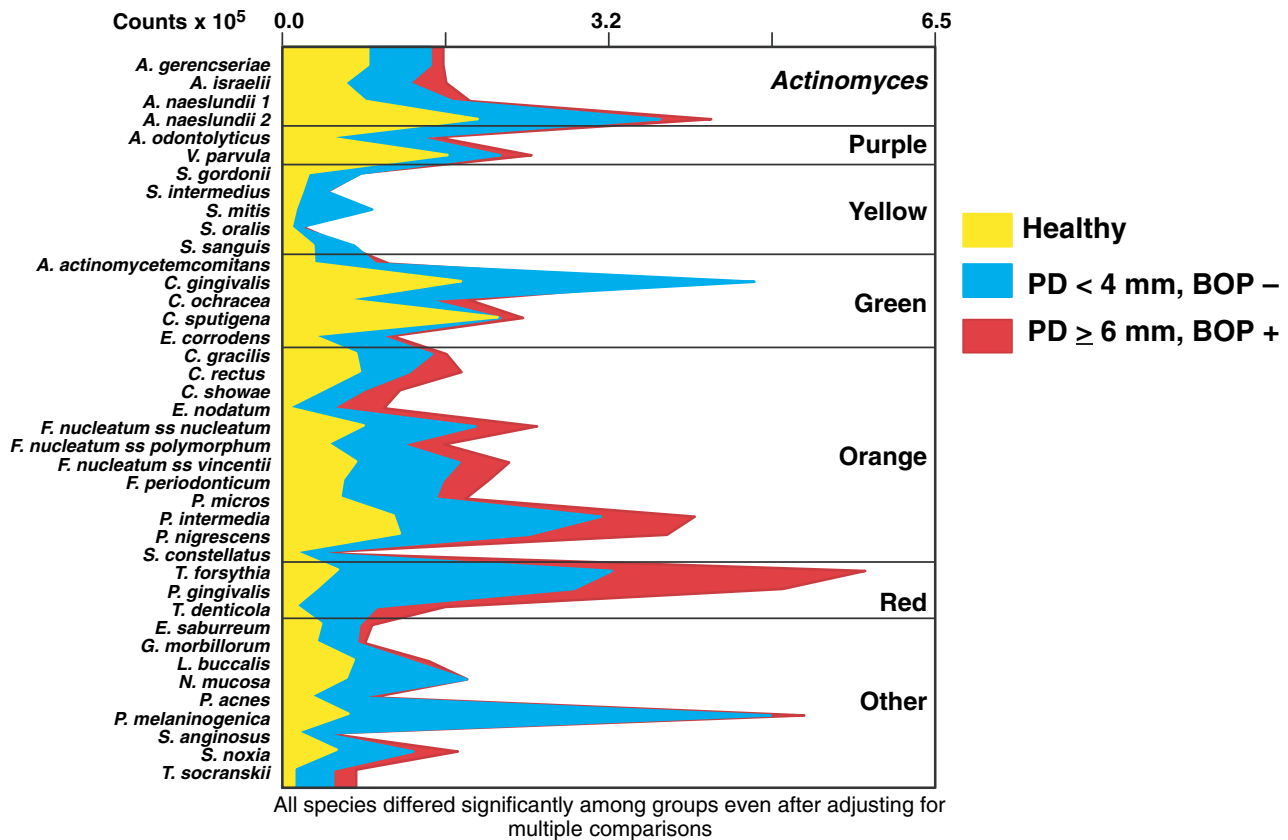
Fig. 31. Mean counts ( $\times 10^5$ ) of 40 species in subgingival plaque samples from sites that did or did not bleed on probing in subjects with periodontitis. Subgingival plaque samples were taken from 6920 sites that bled on probing and 7656 sites that did not bleed on probing in 588 subjects with chronic periodontitis at baseline and analyzed for their content of 40 subgingival species using checkerboard DNA-DNA hybridization. Counts of each

species were averaged in each subject in sites that did or did not bleed on probing and then averaged across subjects for each category. The species were ordered according to the microbial complexes (156). Significance of difference for each species between groups was determined using the Mann-Whitney test and adjusted for multiple comparisons (157): \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

From a clinical perspective, these data suggest that healthy sites in periodontitis subjects may be at far greater risk for future periodontal breakdown than healthy sites in periodontally healthy subjects. From an ecologic point of view, the differences between the two sets of healthy sites are difficult to explain. The local factors considered to be important in the previous section, pocket depth and bleeding on probing, were essentially the same in the two groups. The question then becomes, why in spite of local habitat similarities, were the microbiotas so different in the periodontitis and periodontally healthy subjects? The accurate answer is

that we don't know. One could speculate that the larger numbers of organisms in the reservoirs of deeper periodontal pockets may have continually seeded adjacent sites so that low level colonization at these sites eventually occurred. Another possibility is that the subject was already colonized at many shallow periodontal sites before disease was initiated at some of the sites. Whatever, the reason for the difference in levels in the periodontally healthy sites, the presence of increased levels of periodontal pathogens in 'periodontally healthy' sites of subjects with periodontitis is a serious treatment issue as will be discussed elsewhere (65).





**Fig. 32.** Mean counts ( $\times 10^5$ ) of 40 species in subgingival plaque samples from 'periodontally diseased' and 'periodontally healthy' sites in 209 subjects with advanced periodontitis and 'periodontally healthy' sites in 78 periodontally healthy subjects. Periodontally diseased sites in periodontitis subjects had baseline pocket depths  $\geq 6$  mm and bled on probing, while 'periodontally healthy' sites had pocket depths  $< 4$  mm and did not bleed on probing in both the periodontitis and periodontally healthy

groups. Subgingival plaque samples were taken at baseline and analyzed for their content of 40 subgingival species using checkerboard DNA-DNA hybridization. Counts of each species were averaged in each subject in a given site category and then averaged across subjects for each category. The species were ordered according to microbial complex (156). All species differed significantly among groups using the Kruskal-Wallis test even after adjusting for multiple comparisons (157).

## Relationship of host level parameters to subgingival biofilm composition

The local factors that impact on the composition of the oral microbiota, the nature of the surface to be colonized and the clinical status of the habitat, were described above. Of clinical importance, the supra- and subgingival plaque associated with a periodontally healthy site were quite different from plaque associated with inflamed and bleeding gingival tissues and deep periodontal pockets. There are also factors unique to each host that markedly affect subgingival plaque composition. Not all of the potential host-influencing factors will be discussed here, but the effect of certain key factors, including genetic background, obesity and habits such as cigarette smoking, on the subgingival plaque com-

position will be described below. In addition, it will be suggested that the nature of the subgingival microbiota can be influenced by the geographic location of the individual.

As discussed in the 'Principles' section, the host can modify the level of colonizing species and possibly modulate disease outcome by a mechanism known as environmental feedback. An example of this is illustrated in Fig. 33. Serum samples and samples of subgingival plaque were taken from 48 subjects with chronic periodontitis. Serum antibody levels to *P. gingivalis* were determined by ELISA and levels of *P. gingivalis* in subgingival plaque were determined using cultural techniques. The data indicated a significant inverse relationship between antibody levels to *P. gingivalis* and levels of this species in subgingival plaque, i.e. high levels of antibody to *P. gingivalis* were associated with lowered levels of *P. gingivalis* in the subgingival microbiota. Given

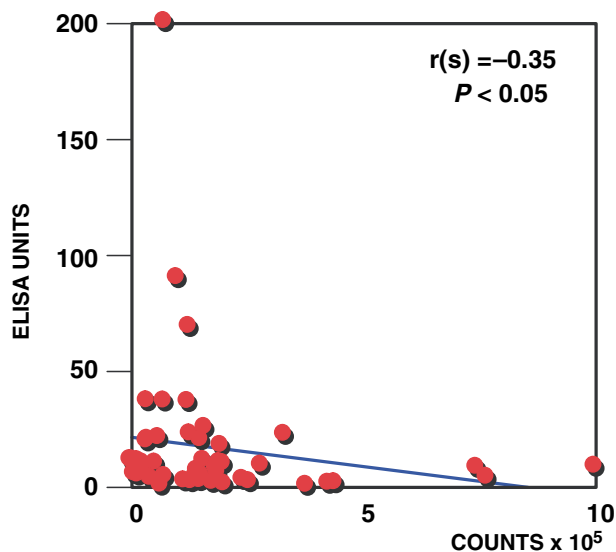


Fig. 33. Plot of mean counts of *P. gingivalis* ( $\times 10^5$ ) and serum antibody levels to *P. gingivalis* in 48 chronic periodontitis subjects. Each circle represents values for a single subject. Regression analysis indicated a significant negative relationship between counts of *P. gingivalis* in subgingival plaque samples and serum antibody to that species.

the significant association of this species with periodontal disease severity, decreasing its levels by environmental feedback should help to limit disease progression and augment therapeutic strategies.

### Cigarette smoking

Numerous studies in the literature have examined the relationship between cigarette smoking and the clinical parameters of periodontal diseases. These studies have indicated an increased level of periodontal disease in terms of more alveolar bone loss (11, 56, 114, 123), deeper periodontal pockets (10, 11, 59, 126), and greater attachment level loss (3, 6, 7, 12, 15, 34, 42, 57, 58, 96, 97, 148) in subjects who currently smoke cigarettes compared with those individuals who are past smokers or who have never smoked cigarettes. The same studies also indicated that cigarette smokers have less bleeding on probing compared with nonsmokers. While multiple studies are consistent in showing strong relationships between clinical periodontal data and cigarette smoking, the effect of cigarette smoking on the composition of the subgingival microbiota is less clear. Some studies suggest that cigarette smoking has little impact on subgingival plaque composition. Preber et al. (127) in a study of 142 adult periodontitis subjects showed that counts of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* were not

significantly different between smokers and nonsmokers based on a single plaque sample per subject taken from a site with pocket depth  $\geq 6$  mm. Stoltenberg et al. (162) evaluated eight subgingival plaque samples taken from a randomly selected posterior sextant in each of 615 adults. Using immunoassay, they found no significant differences between smokers and nonsmokers in the prevalence of test species *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *E. corrodens*, and *F. nucleatum*. In a group of 25 young adults with gingivitis, Lie et al. (85) found no difference between smokers and nonsmokers in the counts of nine subgingival species or groups in pooled supra- and subgingival plaque samples, with the exception of higher counts of *Streptococcus* species in the nonsmokers. Renvert et al. (137) evaluated the effect of periodontal therapy on the subgingival microbiota in smokers and nonsmokers and suggested that the post-therapy microbial change was consistent with clinical change rather than the influence of cigarette smoking.

Other studies have found differences between the subgingival microbiota of smokers and nonsmokers. Eggert et al. (40) using immunoassay demonstrated that *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* were found more frequently in shallow periodontal sites ( $\leq 5$  mm) in smokers than in similar sites in nonsmokers. Kamma et al. (68) used cultural techniques to examine two pooled plaque samples from each of 60 early onset periodontitis subjects. They found that proportions and/or prevalence of *P. micros*, *Campylobacter concisus*, *T. forsythia*, *C. rectus*, *Campylobacter gracilis*, *Selenomonas sputigena*, and *P. gingivalis* were significantly elevated in smokers, whereas *Streptococcus intermedius*, *A. naeslundii*, *Actinomyces israelii*, and *Eubacterium lentum* were significantly higher in nonsmokers. Zambon et al. (187) found that smokers had significantly higher levels of, and were at greater risk of infection from, *T. forsythia* than were nonsmokers, and smokers were 2.3 times more likely to harbor this periodontal pathogen than former or nonsmokers after adjusting for disease severity. Umeda et al. (173) found that past smokers had a decreased risk of harboring *A. actinomycetemcomitans* in saliva (OR = 0.23), while current smokers had an increased risk of harboring *T. denticola* subgingivally (OR = 4.61), although the risk of colonization by *T. forsythia*, *P. intermedia*, *P. gingivalis*, and *P. nigrescens* did not differ among smoking groups. Data from our study that examined the subgingival microbiota using checkerboard DNA-DNA hybridization, in 124 non smokers who had



never smoked, 98 past smokers, and 50 current smokers indicated that there were no significant differences in levels and proportions of the 29 test species among the different smoking groups (64). However, the prevalence (% of sites colonized) of several orange, *E. nodatum*, *F. nucleatum* ss. *vincentii*, *P. intermedia*, *P. micros*, and *P. nigrescens*, as well as all three red complex species *P. gingivalis*, *T. forsythia*, and *T. denticola* was significantly greater in smokers compared with past smokers or those who had never smoked (Fig. 34). The difference in prevalence of subgingival species among smoking groups was particularly marked at pockets  $\leq 4$  mm, whereas no significant differences were observed at pockets  $>4$  mm. The more widespread colonization of periodontal pathogens in current smokers compared with nonsmokers, particularly at shallow sites, may account in part for the difficulty in successfully treating periodontal infections in these individuals.

## Obesity

In the last decade, there has been an explosion of concern in the popular press and medical literature

about the increasing proportion of overweight and obese individuals in first world populations. The reason for this concern is that the risk for many medically important conditions such as diabetes, coronary heart disease, cancer and osteoarthritis is increased in overweight or obese individuals (16, 19, 38, 71, 135, 136, 176). Indeed, it has been determined that obesity will soon be the number one cause of preventable disease and death, replacing smoking as the major modifiable cause of disease (27).

Data from a number of cross-sectional studies have suggested that there may be a relationship between being overweight or obese and periodontitis. Data from the NHANES III study indicated that there was a relationship between an increased BMI and the extent of periodontal attachment loss (181). A second examination of the NHANES III data supported the relationship of BMI and also abdominal obesity to periodontal status and indicated that the relationship occurred primarily in younger subjects (aged 18–34 years) with less of a relationship in middle-aged and older adults (4). An increased BMI was also associated with an increased risk of periodontitis in Japanese subjects; particularly those with a high waist–hip ratio (144).

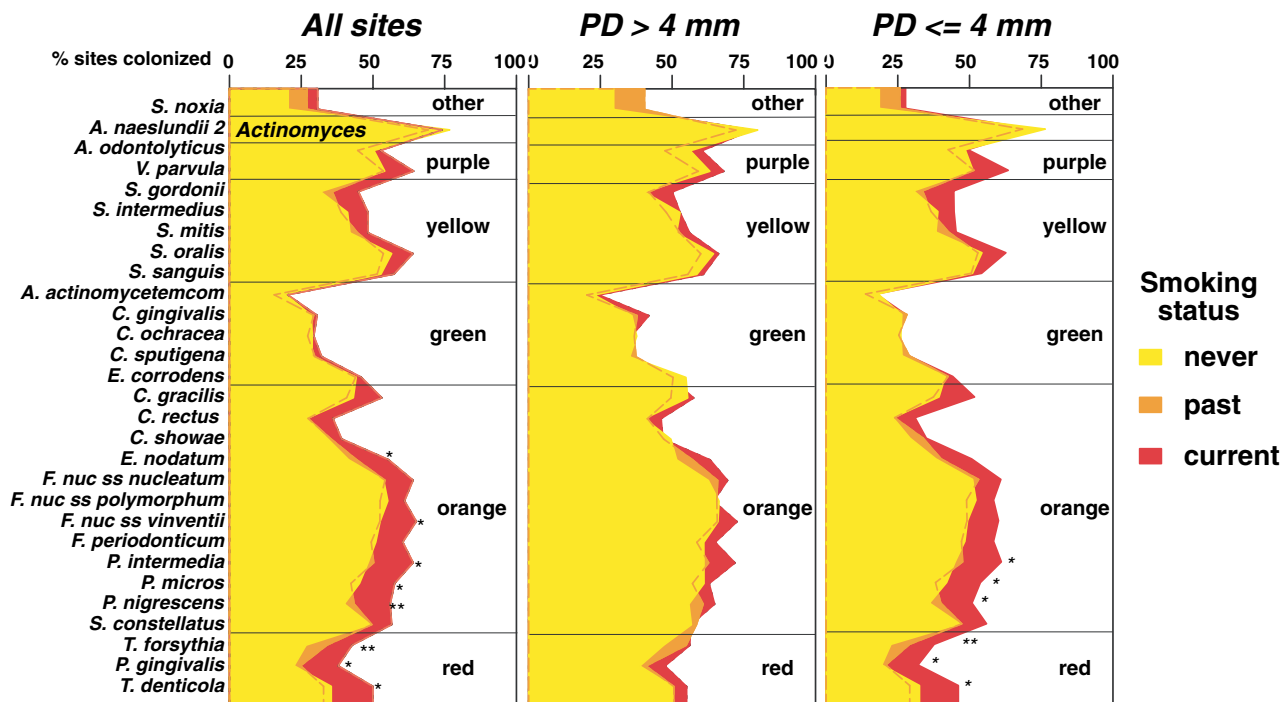


Fig. 34. Microbial profiles of the mean prevalence of 29 microbial taxa in subgingival plaque samples taken from 124 nonsmokers who had never smoked, 98 past smokers, and 50 current smokers. The profiles represent the mean prevalence (% of sites colonized) derived by averaging the prevalence of each species across subjects in each smoking group. The left panel represents all sites, the center

panel sites with pocket depth  $>4$  mm and the right panel sites with pocket depth  $\leq 4$  mm. Significance of differences among groups was sought using ANCOVA adjusting for pocket depth and attachment level; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The species were ordered and grouped according to the complexes described by Socransky et al. (156). Data adapted from (64).

In ongoing studies at The Forsyth Institute, height and weight were measured in 415 systemically healthy subjects with chronic periodontitis ( $n = 329$ ) or periodontal health ( $n = 86$ ). Clinical periodontal parameters were measured at six sites per tooth. Subgingival plaque samples were collected from the mesial aspect of each tooth and were individually analyzed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. Levels and percentage DNA probe counts of each species were determined for each site and averaged across sites in each subject. BMI was computed for each subject and subjects were divided into three subsets: normal weight (BMI < 25,  $n = 168$ ), overweight (BMI 25–30,  $n = 136$ ), and obese (BMI  $\geq 30$ ,  $n = 111$ ). Then significance of differences in clinical and microbial parameters among groups was tested using ANCOVA, adjusting for age and smoking status. The results indicated that the percent of normal, overweight, and obese subjects was 63%, 24%, and 13% in periodontally healthy subjects and 30%, 35%, and 35% in periodontitis subjects, respectively (Fig. 35). The percentage of obese subjects was significantly higher in the periodontitis subjects ( $P < 0.001$ ), and the percentage of normal weight subjects was significantly higher in the healthy subjects ( $P < 0.001$ ). Subjects with elevated BMI exhibited a significantly higher percentage of sites with plaque and bleeding on probing as well as significantly greater mean pocket depth and mean attachment level compared with non-obese subjects (Fig. 36). Obesity was also associated with increased counts and proportions of certain periodontal pathogens, including *T. forsythia*.

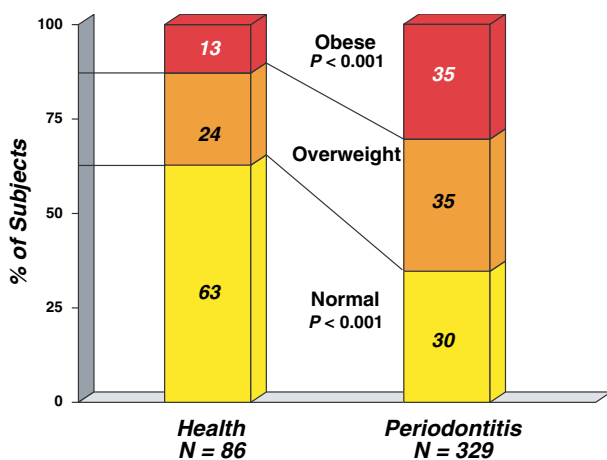


Fig. 35. Stacked bar chart indicating the percentage of periodontally healthy or periodontitis subjects who had a BMI < 25 (normal), 25–30 (overweight) or > 30 (obese). The association between BMI category and periodontal status was tested using Chi-squared analysis.

(Fig. 37). The increase in the proportion of *T. forsythia* was particularly marked in extremely obese subjects whose BMI was > 35. These data suggest that obese subjects were more likely to exhibit periodontitis, had greater mean pocket depth and percentage sites with plaque, and higher proportions of *T. forsythia* than overweight or normal weight subjects.

### Effect of genetic background on subgingival plaque composition

In 1997, Kornman et al. (79) provided data suggesting that polymorphisms in the interleukin (IL)-1 gene cluster appeared to relate to periodontal disease status in nonsmokers. Subjects who exhibited allele 2 of polymorphisms in the genes for both the IL-1 $\alpha$  and IL-1 $\beta$  gene were far more likely to exhibit severe periodontitis than subjects who did not exhibit these polymorphisms. These genes are associated with overexpression of cytokines associated with inflammation, IL-1 $\alpha$  and IL-1 $\beta$ . The question was asked whether subjects exhibiting different IL-1 polymorphisms would exhibit differences in subgingival biofilm composition. Socransky et al. (158) examined the effect of IL-1 polymorphisms on clinical and microbiological parameters of periodontitis. Six sites per tooth were examined clinically and subgingival plaque samples taken from each tooth were examined for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. In addition, finger-stick blood samples were taken from each subject and the extracted DNA used for genotyping of IL-1 polymorphisms (79). They found that the major difference in the subgingival microbiota between genotype positive and negative subjects was at sites with a pocket depth > 6 mm. Twelve of 29 species were significantly elevated in pockets > 6 mm in the genotype positive subjects compared with the genotype negative subjects (Fig. 38). These species included all three members of the red complex, *P. gingivalis*, *T. forsythia*, *T. denticola*, seven members of the orange complex, *C. gracilis*, *E. nodatum*, *F. nucleatum* ss. *nucleatum*, *F. nucleatum* ss. *polymorphum*, *F. nucleatum* ss. *vincentii*, *F. periodonticum*, and *Streptococcus constellatus* as well as *S. gordonii* and *S. intermedius*. In contrast, there were no significant differences in the subgingival microbiota between genotype positive and negative subjects at sites with pocket depths  $\leq 6$  mm. These data suggest that the increased levels of primarily orange and red complex species at deep sites of genotype positive subjects may be due to increased production of inflammatory cytokines producing an

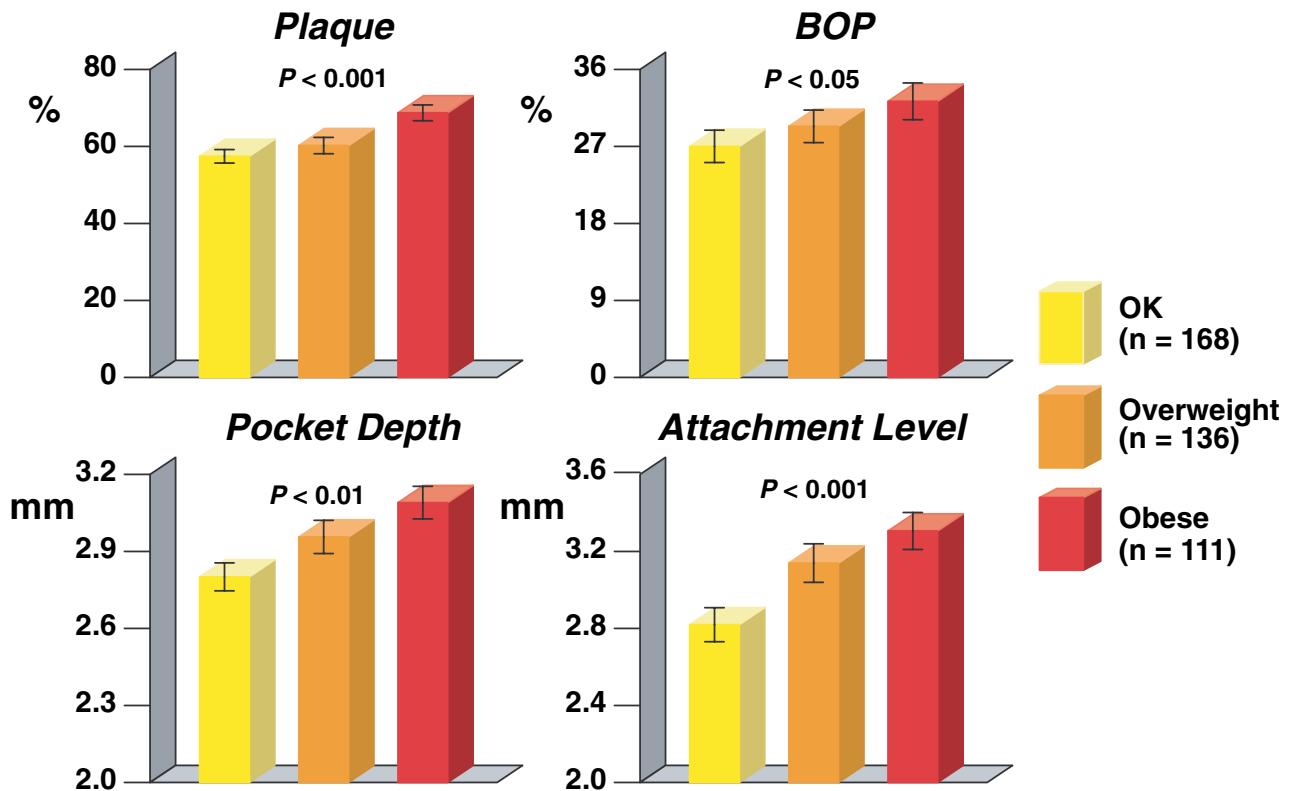


Fig. 36. Bar plots of mean periodontal clinical parameters in subjects who had a BMI <25 (normal), 25–30 (overweight) or > 30 (obese). The bars represent values for each clinical parameter averaged within each subject and then

averaged across subjects in each BMI group separately. The whiskers represent the standard error of the mean. Significance of differences among BMI groups for each parameter was sought using the Kruskal–Wallis test.

increased inflammatory response to the microbial challenge. The increased inflammatory response would lead to increased gingival crevice fluid, the bulk fluid bathing the subgingival biofilm, which provides nutrients to the bacteria in this biofilm. The proximity of the orange and particularly the red complex species to the epithelial lining of the periodontal pocket would insure that these species receive the maximum benefit from the increased gingival crevice fluid. Increased levels of these taxa would affect the local tissues leading to increased inflammation and pocket deepening, which in turn would foster growth of these species. In genotype positive subjects, the interplay between the host and colonizing subgingival species was altered, favoring increased gingival inflammation and subgingival colonization by periodontal pathogens.

### Effect of geographic location on subgingival plaque composition

In previous sections, significant differences in subgingival plaque composition between periodontally healthy and periodontitis subjects were described.

These differences were not surprising given the quite different clinical manifestations in these two populations. It has been generally assumed, however, that subjects with similar clinical conditions, for example chronic periodontitis, would exhibit, on average, similar subgingival microbial profiles irrespective of the geographic location in which the subjects reside. Several studies have indicated that the subgingival species examined to date are commonly found in many different populations throughout the world (17, 36, 119, 145, 186). However, studies that have directly compared the microbiotas of subjects residing in different geographic locations suggest that marked differences in the range, levels, and prevalence of subgingival species may exist (119, 145, 186).

A recent study (60) examined the composition of the subgingival microbiota in subjects with chronic periodontitis from four different geographic locations. Fifty-eight subjects from Brazil, 26 subjects from Chile, 101 Swedish subjects, and 115 subjects from Boston, USA, were clinically monitored and subgingival plaque samples taken. The samples were individually analyzed for their content of 39 bacterial species using checkerboard DNA–DNA hybridization

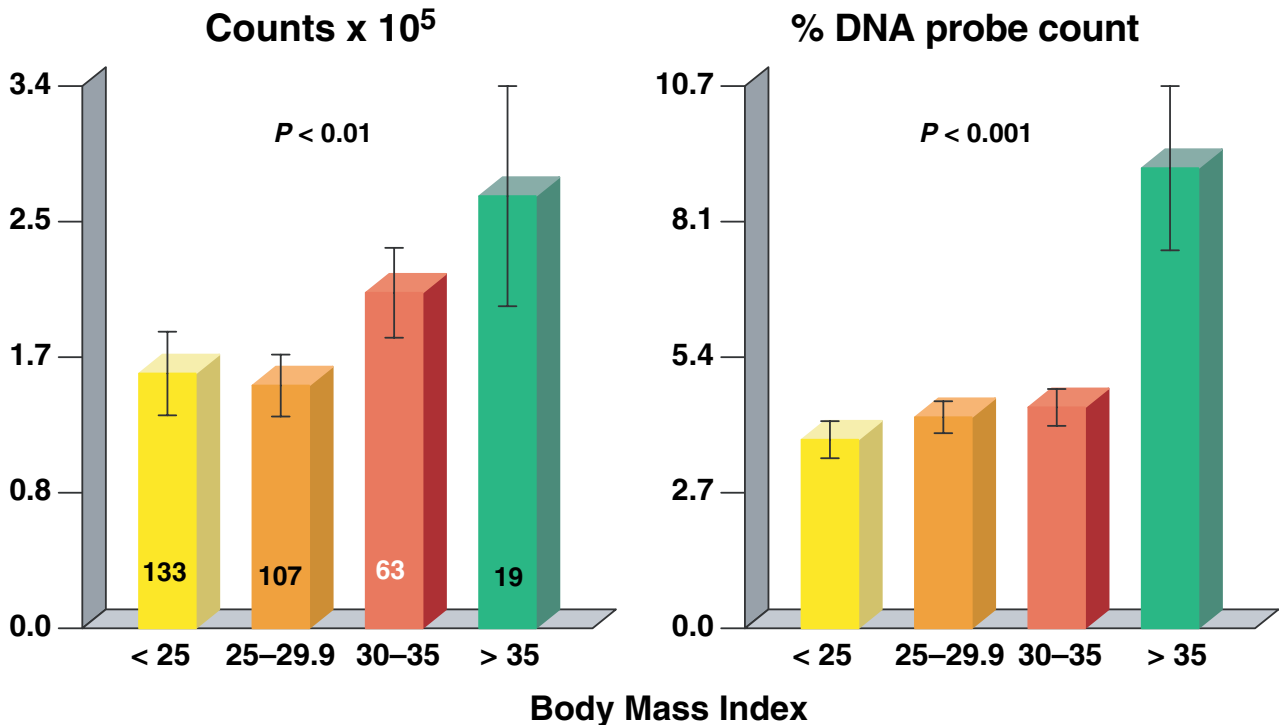


Fig. 37. Mean counts and proportions of *T. forsythia* in subjects who had a BMI < 25 (normal), 25–30 (overweight), 30–35 (obese) or > 35 (extremely obese). Counts of *T. forsythia* were determined using checkerboard DNA–DNA hybridization, averaged within each subject, and then averaged across subjects in each BMI group sepa-

rately. The bars represent the mean values and the whiskers the SEM. The numbers in the left hand set of bars indicate the number of subjects examined in each group. Significance of differences in *T. forsythia* counts and proportions was determined using the Kruskal–Wallis test and *P*-values were adjusted for 40 comparisons (157).

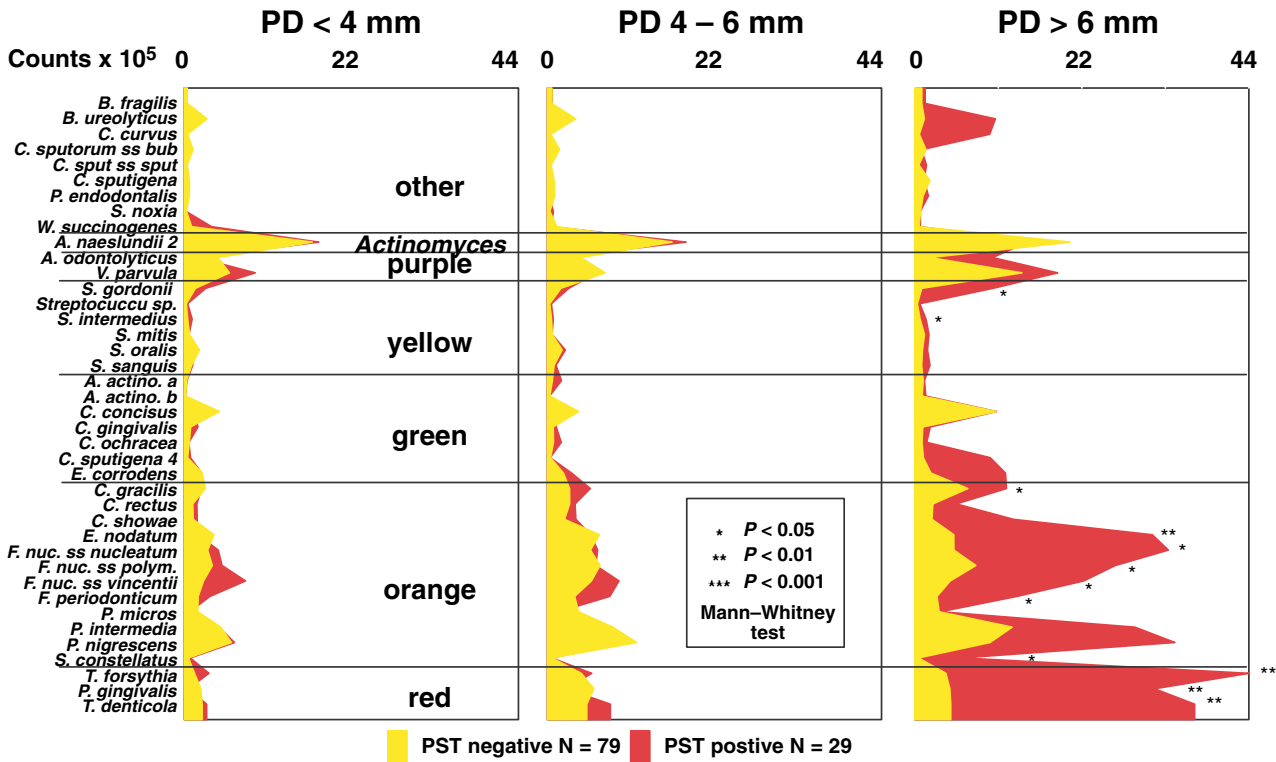
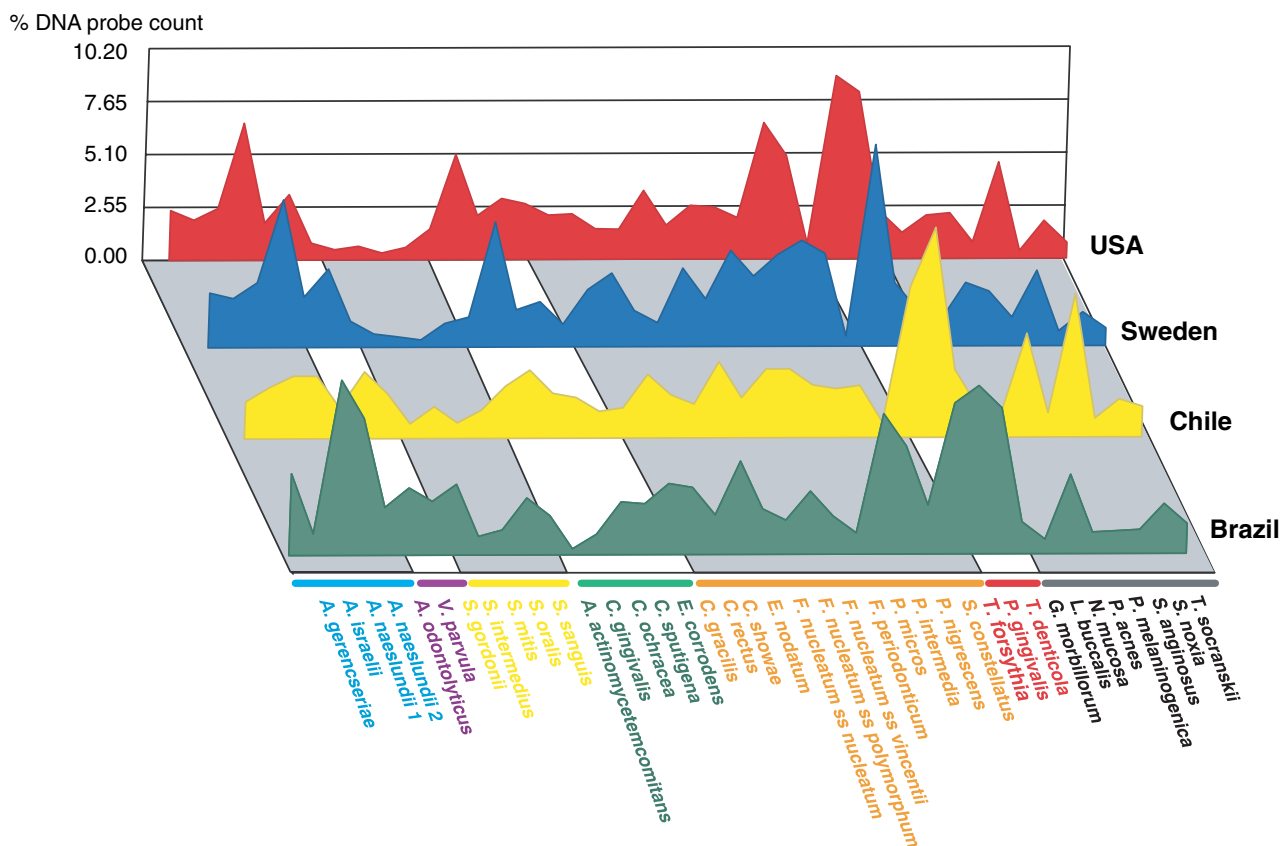


Fig. 38. Mean microbial profiles for chronic periodontitis subjects who were PST negative (*n* = 79) or positive (*n* = 29). The mean counts were derived by averaging the counts of

each species within a subject and then across subjects in the two clinical groups. Significance of differences for each species was determined using the Mann–Whitney test.

and the mean proportion of each species in each subject computed. The data indicated marked differences among the four populations in the proportions of the test species (Fig. 39), with 12 of 39 test species differing significantly among groups. The Brazilian subjects exhibited the highest mean proportions of *A. naeslundii* genospecies 1, several streptococci, including *S. gordonii*, *S. sanguis*, *S. intermedius*, and *S. constellatus*, as well as *E. nodatum* and *T. denticola*. The highest mean proportions of *P. gingivalis* and *F. periodonticum* were seen in the subjects from Chile, whereas the Swedish subjects exhibited the highest mean proportions of *Capnocytophaga gingivalis*, *C. gracilis*, *P. micros*, and *Leptotrichia buccalis*. Of particular interest was the difference in the proportions of the red complex species among the four subject groups (Fig. 40). No significant difference was detected among the four groups in the proportions of *T. forsythia*, although this species was the dominant

red complex species in the Swedish subjects. Significant differences among groups were seen for both *P. gingivalis* and *T. denticola*. The Chilean subjects had the highest proportions of *P. gingivalis* compared with the other groups, and the Brazilians had the highest proportions of *T. denticola*. It should be noted that there were no significant differences among groups in terms of clinical features, although the racial/ethnic background of the subjects differed and the percentage of current smokers ranged from 2% in the Brazilians to 62% in the Swedish subjects. Differences in the subgingival microbiotas of subjects with comparable levels of disease in different geographic locations could impact on therapeutic outcomes. It is likely that subjects with different microbial profiles will respond differently to a given periodontal therapy. In addition, these microbial differences among subjects may partly explain the differences in disease severity noted in different regions of the world.



**Fig. 39.** Adjusted mean percents of the total DNA probe count of 39 bacterial species in baseline subgingival plaque samples taken from 114 American, 101 Swedish, 26 Chilean, and 58 Brazilian subjects with chronic periodontitis. The top of each panel represents the mean percents after adjusting for age, mean pocket depth, gender, and smoking status. Mean percents of each species were computed by averaging samples from the four deepest

and three shallowest pocket depths sampled in each subject, and then averaging across subjects in the four countries. Significance of differences among groups was sought using ANCOVA adjusting for age, mean pocket depth, gender, and smoking status; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  after adjusting for multiple comparisons (157). The species were ordered and grouped according to the complexes described in (156). Data derived from (60).

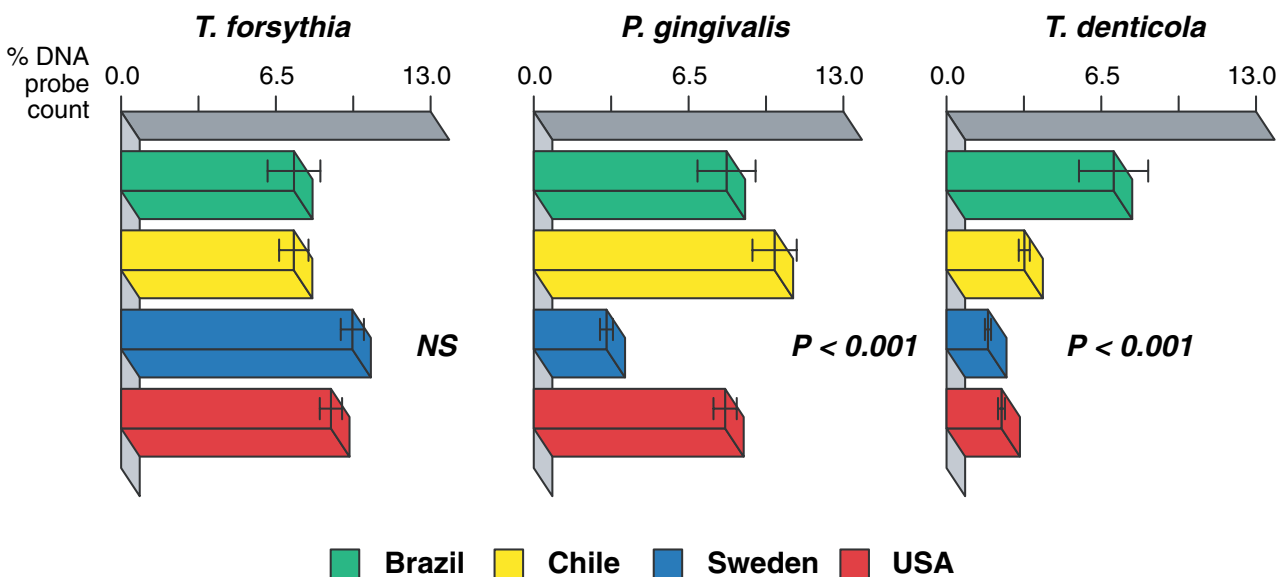


Fig. 40. Bar charts of adjusted mean percents ( $\pm$  SEM) of the total DNA probe count of the red complex species, *T. forsythia*, *P. gingivalis*, and *T. denticola* in baseline subgingival plaque samples taken from the four deepest and three shallowest, sampled periodontal pockets in 58 Brazilian, 26 Chilean, 101 Swedish, and 114 American chronic periodontitis subjects. The bars represent the

mean percents after adjusting for age, mean pocket depth, gender, and smoking status. The whiskers indicate the standard error of the mean. Significance of differences among groups for each species was sought using ANCOVA adjusting for age, mean pocket depth, gender, and smoking status. The  $P$ -values were adjusted for 40 comparisons (157). Data derived from (60).

## Concluding remarks

By now the reader's mind has probably turned to Jello. The multiplicity of factors that appear to impact on the nature of the oral microbiota may appear to be overwhelming. For this reason, we have decided to summarize some of the key elements that we feel are essential to our understanding of the ecology of the oral microbiota and the way that we may control oral infections. The first is not exactly a news flash. The mouth is not sterile. Whether we like it or not, the oral cavity will be colonized by microorganisms. The best that we can do is to guide the composition of the microbiota to one that leads to sustained freedom from the effects of oral infections. In order to undertake this process, we must understand the factors that govern microbial composition and biological activity in order to 'nudge' the microbiota to one that is host compatible.

## Habitat

One key recognition is that while the microbiota affects its habitat, the habitat affects the composition and metabolic processes of the colonizing species. The habitat starts even outside the individual (human) and may encompass factors controlled by

the geographic location of the individual, including genetic background, race/ethnicity, social customs, socioeconomic status, dietary practices and, importantly, the nature of the organisms colonizing other individuals in the same community. Once you move from the human community to the human individual, there are unique host-level factors that play a major role including his or her genetic makeup, health status, diet, oral hygiene procedures, smoking habits, drug use, and the individuals with whom he/she interacts. When moving from the individual to the oral cavity, there are additional factors that influence microbial colonization. These include the nature of the receptors on both the hard and soft tissues for initial attachment of species, the nature of the species already colonizing available surfaces, the composition and nature of the major bulk fluid that sustains these organisms and once more the efficacy of self-performed oral hygiene measures. Data presented earlier indicate that each of these factors may impact on the nature of colonizing organisms and that species that colonize one area of the mouth have the potential to move to and reside in other areas, albeit in different numbers and proportions and occupying different niches (roles).

The teeth provide a unique habitat for the colonization of organisms. A critical factor is that the teeth (or dentures) are nonshedding surfaces and, as



such, allow development of very complex biofilms with a dense glycocalyx. The composition of the biofilms on the tooth is affected by the nature of the surface provided, the other organisms in the biofilm, and the nature of the bulk fluid. The receptors on the tooth are most likely to be salivary proteins deposited during the process of pellicle formation and these proteins will differ from person to person based on his/her unique genetic makeup. These proteins will select organisms with specific adhesins and they may attach, multiply, and provide the 'pioneer' species that set the stage for other colonizing bacteria. The first species may provide points of attachment (coaggregation) for other species or they may provide the environment for growth of species by altering physicochemical factors, providing nutrients or degrading complex macromolecules to forms that might be used by other species. Thus, the nature of the species that enter into biofilm development very much influences the nature of other species that might inhabit the same site. We were impressed by the speed at which this initial colonizing phase can take place. Certain species may reach peak levels within hours and many species will have attained maximum growth in 1–4 days. However, this rapid formation is analogous to the construction of a house. The frame may go up quite rapidly (within days), but the finished structure will take weeks to months to complete. The abundance and composition of the bulk fluid bathing this area also will impact on the total numbers and proportions of species on tooth surfaces. The data from the Sjogren's subjects suggest that decreased salivary flow limits the growth of bacterial species on both tooth and soft tissue surfaces and alters microbial composition. Finally, supragingival plaque and soft tissue biofilms harbor anaerobic species, including periodontal pathogens, although the numbers may be small.

The teeth together with the gingival tissues provide a unique domain for bacterial colonization, the subgingival environment. In this area, both hard and soft tissue surfaces are available for colonization providing a situation in which two types of biofilms may form. The first would initially be a subgingival extension of the tooth-associated supragingival biofilm, and the second, the biofilm that develops on the epithelial surface provided by the gingival sulcus or periodontal pocket wall. Once more we have the controlling themes of the nature of the surface(s) and the nature of the bulk fluid that sustains colonization. The soft tissue surface will differ from person to person based on genetic background in terms of the nature of the receptors provided for attachment of

oral bacteria. In addition, the colonizing species can alter the epithelial surfaces, leading to different colonizing species (21). A third zone may also be detected between the tooth and tissue-associated biofilms. This zone is most evident in deeper periodontal pockets and consists of loosely adherent bacteria that can exist in deeper pockets because the physical confinement of such pockets does not demand the tenacious surface attachment needed for the survival of most biofilms. The bulk fluid nourishing the subgingival biofilms is the gingival crevice fluid. This fluid is markedly different in composition from saliva and its composition and volume are dramatically influenced by the inflammatory status of the gingival tissues. Low gingival crevice fluid volume may limit the growth of the subgingival biofilms, whereas increased gingival crevice fluid volume containing breakdown products from the adjacent tissues may enhance bacterial growth, fostering the growth of specific species like *P. gingivalis* and *T. denticola* that require breakdown products such as proteins or peptides.

### Climax community

A second key concept is the stability of the 'climax' community, i.e. the complex mixture of bacterial species that occurs in mature biofilms. Eventually, after the interplay of all the local habitat, host and microbial determinants, a stable climax community is established. The microorganisms in this community have achieved an equilibrium with each other and with the habitat provided by the host. This equilibrium is dynamic in that minor perturbations are continuously occurring with adjustments made by both the host and the colonizing species. Once established, it is very difficult to make major changes to this climax community. Short-term changes in the host such as an upper respiratory infection, a brief change in diet, a lapse in oral hygiene procedures will modify the microbial community somewhat, but the community will return to its climax state once these factors are removed. Even periodontal therapy may only have a temporary modifying effect on the climax community, although therapies that provide major alterations to the microbial community or the habitat may lead to a new climax community that is hopefully beneficial to the host. As we examine the history of periodontal therapy, it is clear that a number of empiric procedures were employed to alter the subgingival microbiota with the hope that these procedures would benefit the host. For the most part, we had no idea as to the effects that these

procedures would have on the subgingival ecosystem. In recent years, a number of studies reviewed in Haffajee & Socransky (65) have attempted to define the shifts that occur as a result of individual or combined therapies. The next stage in this development will be to provide the optimal periodontal therapy to each subject in order to develop a climax community that is stable and provides long-term periodontal stability.

## What's next?

There is an awakened interest in the biomedical community in the study of biofilms since biofilm communities are responsible for the majority of bacterial infectious diseases of the human. This is a unique opportunity to meld *in vitro*, *in vivo*, and translational research, but it is critical that these three approaches be carried out in parallel with collaborative interactions. There is a great fear on the part of the authors that the research community and research administrators have become enraptured with 'metagenomics', 'proteomics', and *in vitro* systems. Studies of this nature, in isolation, may have little or unrecognized relevance to the *in vivo* ecosystem of interest. The challenge for the future will be to balance *in vitro*, *in vivo*, and translational research approaches so that major advances can be made in our understanding of biofilm ecosystems.

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