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Bacterial interactions and successions during plaque development

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Studies from the 1960s indicated that increased microbial diversity and a succession in the predominant bacterial species in plaque correlate with the appearance of gingival inflammation and the development of periodontal disease. In the past few years, molecular characterization of the microflora found in various sites of the oral cavity of different subjects has detected around 700 bacterial species or phylotypes (1, 63, 93). Some of these species are considered commensal and a positive feature of our healthy microflora, while others are considered pathogenic. The colonization of pathogenic bacteria is probably dependent upon the interaction of pathogens and commensal organisms. The clinical relevance and periodontal microbial ecology of these bacteria have been presented in an outstanding, comprehensive review (101). The discussion in the present article centers on interactions among bacterial species and how these interactions contribute to the development of plaque and ultimately to the formation of periodontopathogenic communities.

Interactions among human oral bacteria are integral to the development of plaque. From the early stages of colonization to the formation of mature supragingival and subgingival plaque, a diverse array of bacterial species colonizes into densely populated communities. Interactions among different bacterial cell types are proposed to drive the maturation of plaque. These interactions occur at several levels, including physical contact, metabolic exchange, small-signal-molecule-mediated communication and exchange of genetic material.

A principal feature of human oral bacteria is their ability to interact by coaggregation with other oral bacteria (48). Coaggregation is defined as the specific cell-to-cell recognition that occurs between genetically distinct cell types. Each cell type bears on its surface one or more types of coaggregation mediator, which are called adhesins and receptors (described below). The adhesins and receptors confer a particular set of coaggregation properties. Coaggregation partnerships are central to the development of biodiversity in supragingival and subgingival plaque. Coaggregations effect changes in populations, from low diversity in initial communities of supragingival plaque to high diversity in subgingival plaque. This article will provide an overview of the physical and metabolic interactions that occur among the oral microflora in the context of plaque development.

The basics about coaggregation

Gibbons and Nygaard (31) discovered coaggregation among plaque bacteria when they conducted pairwise testing of 23 strains. Only five of the 253 pairs showed strong coaggregation, and these five were pairs composed of a streptococcus and an actinomyces or a coccobacillus. Gibbons and Nygaard called it interbacterial aggregation. The term coaggregation was coined to describe a clumping phenomenon that occurred when sucrose-grown streptococci were paired with actinomyces and was used to distinguish this intergeneric type of clumping from the dextran-mediated intraspecies aggregation of actinomyces (5).

More than 1,000 strains (cell types) of human oral bacteria have been tested for their ability to coaggregate. A convenient assay for coaggregation
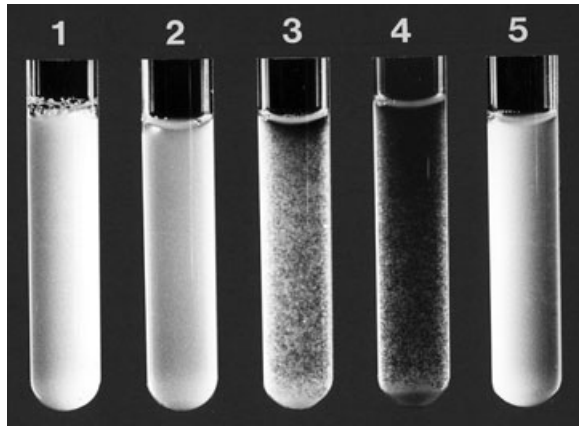
Fig. 1. The visual assay for coaggregation (48). Homogeneous suspensions of cell types A and B are shown before mixing (tubes 1 and 2, respectively) and immediately after mixing of equal volumes (tube 3). Within seconds, coaggregates settle to the bottom of the tube, leaving a clear supernatant (tube 4). Addition of sugar inhibitor reverses the interaction (tube 5).

is the mixing of a pair of cell types (Fig. 1). A dense suspension ($\approx 10^9$ cells/ml) of one cell type (Fig. 1, tube 1), for example a streptococcal strain, and a dense suspension of a second cell type (Fig. 1, tube 2), for example an actinomyces strain, are mixed together. If they are coaggregation partners, they form clumps (Fig. 1, tube 3), and if the coaggregation is extensive, the clumps fall immediately (within seconds) to the bottom of the tube (Fig. 1, tube 4). Some of these coaggregations are reversed by the addition of lactose or other sugars, which causes the coaggregates to disperse into an evenly turbid suspension (Fig. 1, tube 5). By using this simple method, thousands of strains were paired with other strains to yield the coaggregation profile of each strain.

Delineation of a coaggregation profile

Delineation of a small set of pairwise coaggregations for four strains shows the breadth of variety of coaggregation profiles (Table 1). Each strain coaggregates with a specific set of partner strains. *Actinomyces naeslundii* T14V (a gram-positive bacterium) coaggregates with several gram-positive strains (*Streptococcus* spp.) as well as gram-negative strains (*Prevotella* spp. and *Capnocytophaga* spp.). *Capnocytophaga ochracea* ATCC33596, however, only coaggregates with gram-positive partners. Some strains, such as *Porphyromonas gingivalis* PK1924, do not coaggregate with any of the partners in this table matrix, whereas others, such as *Fusobacterium nucleatum* PK1594, coaggregate with all of the partner strains as well as with *P. gingivalis* PK1924. This ability to coaggregate with a wide variety of partner strains is highly unusual and is restricted to the genus *Fusobacterium*. These pairwise coaggregations of most oral bacterial strains yield a set of partner strains, as seen with *A. naeslundii* T14V and *C. ochracea* ATCC33596. They coaggregate with some partners, but not with others, which indicates the specificity of the coaggregation profiles for each strain. Knowledge of coaggregation profiles is helpful in understanding the vital role of coaggregation in bacterial succession and colonization of hard tissue surfaces.

Table 1. Delineation of coaggregation profiles of four oral bacterial strains

| | Partner strain designation | | | | | | | | | | | | | | | | |
| | *Streptococcus* spp. | | | | | | *Prevotella* spp. | | | *Capnocytophaga* spp. | | | *Rothia* spp. | *Actinomyces* spp. | | | |
	DL1	H1	34	J22	509	488	1277	1295	1511	ATCC 33612	ATCC 33596	ATCC 33624	44	16	T14V	19	606
Actinomyces naeslundii T14V	+	+	+	+	−	−	+	−	+	+	+	+	−	−	−	−	−
Capnocytophaga ochracea ATCC33596	+	+	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+
Porphyromonas gingivalis PK1924	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fusobacterium nucleatum PK1594	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The streptococcus–actinomyces coaggregation groups

An example of the specificity of coaggregation is clearly demonstrated by *Streptococcus* spp. and *Actinomyces* spp., two of the initial colonizing genera on enamel surfaces. These streptococcus–actinomyces partnerships are highly specific and can be sorted into coaggregation groups. More than 300 isolates of these genera have been tested in pairwise intergeneric coaggregation, and more than 90% coaggregate (56). The defining characteristics of the pairwise interactions include the suite of sugars that inhibit coaggregation, the effect of heat (85°C/30 min) and protease treatment of cells before mixing the partners, and the simultaneous loss of multiple coaggregation partnerships by substituting a coaggregation-defective mutant for the wild type. Consider a matrix of 300 isolates, which lists the outcome of each isolate tested pairwise with each other isolate. Thousands of types of interactions could result if streptococcus–actinomyces coaggregations are random, but instead six coaggregation groups of streptococci (streptococcus coaggregation groups 1–6) and six coaggregation groups of actinomyces (actinomyces coaggregation groups A–F) are found (Fig. 2). Each coaggregation is mediated by one or more complementary sets of adhesin–receptor pairs. Although the number of coaggregation groups is small, large differences in coaggregation profiles are readily seen. For example, streptococcal coaggregation group 6 coaggregates only with actinomyces coaggregation group D. At the other end of the spectrum, actinomyces coaggregation group D is the most reactive group in that it coaggregates with all six streptococcal coaggregation groups. Some groups, such as actinomyces coaggregation group F, only participate in lactose-inhibitable coaggregations. Thus, specificity of coaggregation partnerships occurs, and defined coaggregation groups are delineated.

A striking feature of these streptococcus–actinomyces coaggregation groups is the clear relatedness of the groups to each other. Actinomyces coaggregation group F possesses a single type of adhesin for lactose-inhibitable coaggregation. Actinomyces coaggregation group B exhibits identical coaggregations to those exhibited by group F, plus this group bears a receptor for the complementary adhesin on streptococcal coaggregation group 2. Actinomyces coaggregation group C participates in all the coaggregations of groups B and F, as well as bearing receptors for lactose-inhibitable coaggregations with streptococcal

coaggregation groups 1 and 3. And, finally, actinomyces coaggregation group D exhibits all the coaggregations observed with groups B, C and F, plus additional types of coaggregations. Likewise, the relatedness of actinomyces coaggregation groups A and E is clear. Less obvious is the relatedness of streptococcal groups. However, streptococcal coaggregation groups 3, 4, and 5 bear receptors for lactose-inhibitable coaggregations as well as a few adhesins, whereas streptococcal coaggregation groups 1, 2, and 6 bear only adhesins. Although the coaggregation groups between streptococci and actinomyces (Fig. 2) have been studied in detail, coaggregations of other genera, such as *Fusobacterium* (59) and *Veillonella* (36), are also defined.

Coaggregation-defective mutants

The characteristic of 'simultaneous loss of multiple coaggregation partnerships by substituting a coaggregation-defective mutant for the wild type' determines the relatedness of coaggregation mediators (adhesins or receptors) on the partners. This characteristic is defined after selecting a coaggregation-defective mutant by its inability to exhibit coaggregation with one of the partner strains of the wild type (parent) (47). Coaggregation-defective mutants are obtained by mixing the parent strain with its partner; this can be visualized by examining tube 4 in Fig. 1, where the upper layer contains the coaggregation-defective mutants. Removal of an aliquot of the upper layer and mixing it with additional coaggregation partner cells gives another round of coaggregation. A gentle centrifugation pellets the coaggregates and leaves potential coaggregation-defective mutants in the supernatant. A few more rounds of addition of the coaggregation partner and gentle centrifugation, followed by plating the mixture, yield a desired coaggregation-defective mutant. Surprisingly, mutants selected for the inability to coaggregate with one partner are unable to coaggregate with certain other partners. For example, a mutant of actinomyces coaggregation group F, which fails to coaggregate with streptococcus coaggregation group 3 (bottom right of Fig. 2), simultaneously loses its ability to coaggregate with streptococcus coaggregation groups 4 and 5 (45, 46).

Sugar inhibition

McIntire et al. (73) reported the highly specific nature of certain coaggregations between streptococcus and actinomyces in that these coaggregations were
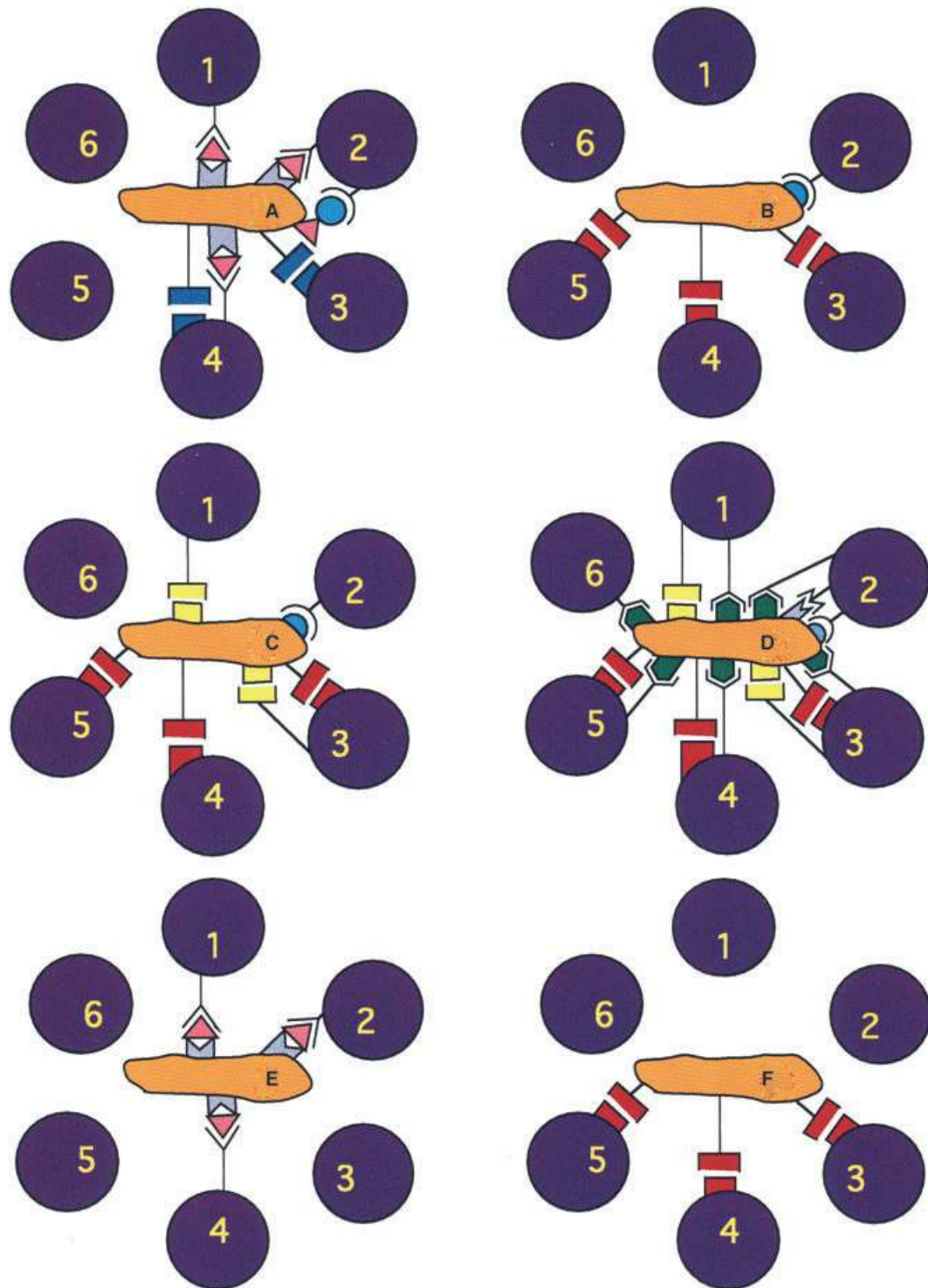
Fig. 2. Diagrammatic representation of the coaggregations between members of the six streptococcal coaggregation groups (numbered circles) and the six actinomyces coaggregation groups (lettered oblong shapes). Each interaction is depicted by one or more pairs of complementary symbols. Symbols with a stem represent inactivation by heat or protease treatment, and symbols without a stem represent resistance to these treatments. The rectangles (blue, red, yellow) and the symbol M (light blue) represent interactions inhibited by 60 mM lactose and sialic acid, respectively. Rectangular symbols of different colors represent components that are functionally similar (lactose inhibitable) but might not be structurally identical. The semicircle/circle (blue) without a stem on the surface of actinomyces coaggregation groups A, B, C, and D represents a complex polysaccharide-containing cell wall component isolated by Mizuno et al. (77). The triangle (pink) without a stem represents a dual-function surface molecule that acts as a receptor for bacteriophage BF307 (13) and as a receptor for streptococcus coaggregation groups 1, 2 and 4. The receptor represented by an obelisk (green) on actinomyces coaggregation group D is the only receptor recognized by streptococcus coaggregation group 6 (55).

inhibited by lactose, but not sucrose or many other sugars. McIntire advanced the idea that sugar-inhibitable coaggregations were mediated by lectin–carbohydrate interactions, because only specific sugars inhibit, while other sugars, which are structurally closely related, do not inhibit (11, 12, 74). One of the coaggregation partners bears the receptor polysaccharide (carbohydrate) and the other partner bears the complementary adhesin (lectin). The partner bearing the adhesin is inactivated from participating in coaggregation when it is heated (85°C for 30 min), but the partner bearing the complementary receptor polysaccharide is unaffected when it is heated. It is this specificity of coaggregations between oral bacteria that has allowed delineation of partnerships and promoted ideas of nonrandomness of coaggregation among oral bacteria.

The wide range of lactose-inhibitable coaggregations among oral bacteria is shown in Fig. 3. Each of the cellular shapes depicted represents a strain of bacteria tested pairwise with another. Each cell exhibits an adhesin or a receptor polysaccharide (rectangular symbols). Some cells exhibit a receptor polysaccharide that is complementary to an adhesin on a partner, and they exhibit an adhesin that is complementary to a distinct receptor polysaccharide on a different partner. These adhesins and receptor polysaccharides are represented as different-colored complementary symbols. Clearly evident in this two-dimensional drawing are the variety of lactose-inhibitable coaggregations, but what is not depicted is the sugar specificity of the lactose inhibitions. Some are inhibited better by L-rhamnose, others by D-fucose, and still others by *N*-acetylated aminosugars, yet all are also inhibited by lactose. Also not shown are the numerous other types of coaggregations found by pairwise testing between some of these partners that are not inhibited by sugars. Thus, while the figure shows extensive numbers of lactose-inhibitable coaggregations, it represents a simplified
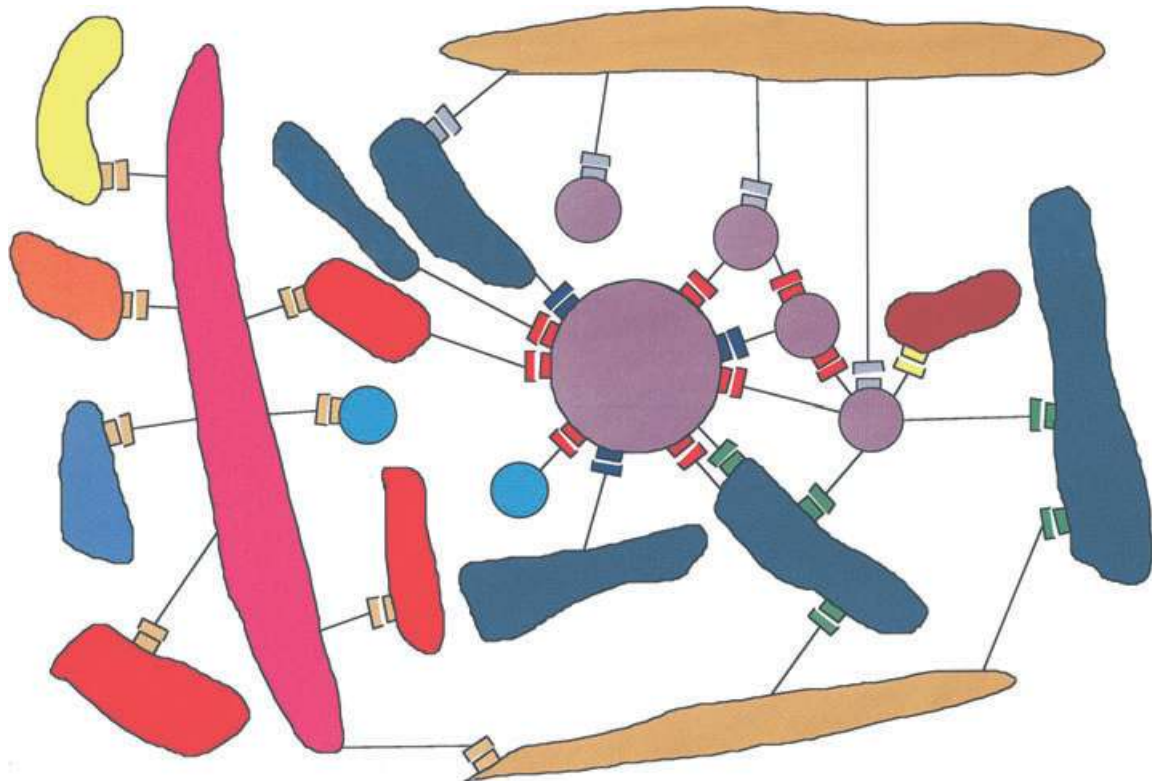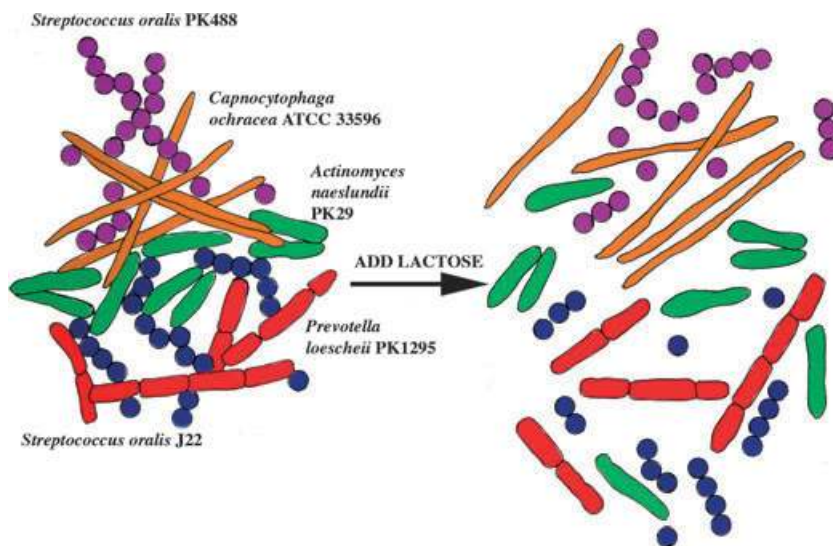

Fig. 3. Diagrammatic representation of lactose-inhibitable coaggregations known to occur between pairs of oral bacteria (56). The emphasis in this figure is on the variety of lactose-inhibitable coaggregations. The genus/species strain names have been removed to emphasize the subtle differences among these coaggregations. Cell shapes of the same color represent species strains of the same genus. The complementary pairs of adhesin–receptor are shown as the same color. Adhesins are depicted as symbols with a stem; adhesins are proteins and are inactivated by heating the cells that bear them or by treating the cells with protease. Receptor polysaccharides are depicted as the complementary rectangles that do not have a stem; the receptor polysaccharides are insensitive to heating or to protease treatment of the cells that bear them. The complementary symbols of the same color are thought to be functionally related and may be structurally related.

Fig. 4. Graphic representation of lactose-reversible multigeneric co-aggregates by visual examination of cell suspensions before (left) and after (right) the addition of lactose. Cell types of various shapes in large aggregates are completely dispersed to individual cells or small clusters by the addition of lactose. The table at the bottom indicates the results obtained when one strain (*Streptococcus oralis* J22, *Prevotella loescheii* PK1295, or *Actinomyces naeslundii* PK29) is radioactively labeled and mixed with the other four non-radioactive cell types to form coaggregates. The mixture is given a low-speed centrifugation ($100 \times g$ for 1 min) to pellet coaggregates. The percentage of input radioactivity in coaggregates before and after the addition of 100 mM lactose is indicated. For details, see Kolenbrander and Andersen (54).

LACTOSE-REVERSIBLE COAGGREGATIONS

Radioactive strain	% of Input CPM in coaggregated cells	
	No sugar additions	100 mM lactose
J22	40	0
PK1295	51	0
PK29	93	0

view of these coaggregations and does not show why adding lactose cannot disassociate all coaggregations of oral bacteria *in vivo*.

Independent nature of lactose-inhibitable and lactose-noninhibitable coaggregations

To test the nature of coaggregations involving large aggregates composed of several species, we chose certain partners known to participate pairwise only in lactose-inhibitable coaggregations. One of the partners was radioactively labeled and mixed with the others to form multigeneric coaggregations (Fig. 4). Addition of lactose has been previously shown to dissociate the coaggregates (54). Such observations pushed our thinking towards dissociating plaque *in vivo* with lactose rinses. However, the failure of lactose to dissociate coaggregations *in vivo* and disrupt dental plaque on enamel could be modeled *in vitro*. Lactose-inhibitable and lactose-noninhibitable coaggregations act independently. To show that

lactose-inhibitable and lactose-noninhibitable coaggregations act independently, we mixed two lactose-inhibitable pairs with several pairs that were not inhibited by lactose (Fig. 5). Adding lactose does, indeed, dissociate the cells that participate solely in lactose-inhibitable coaggregations from the multigeneric coaggregate. Cells that participate solely in lactose-noninhibitable coaggregations remain firmly bound in the coaggregates, indicating the independent nature of pairwise coaggregations in the presence of numerous other coaggregations. These observations suggest that *in vivo*, in the development of dental plaque, the sequential accretion of cell types occurs independently of the surrounding coaggregations.

Sequential coaggregating partnerships

Sequential arrangement of coaggregating cells is depicted in Fig. 6. Only the cells depicted as touching each other are coaggregation partners. The purple cells (*Streptococcus oralis* 34) coaggregate with green

INDEPENDENT NATURE OF LACTOSE-REVERSIBLE AND LACTOSE-NON REVERSIBLE COAGGREGATIONS

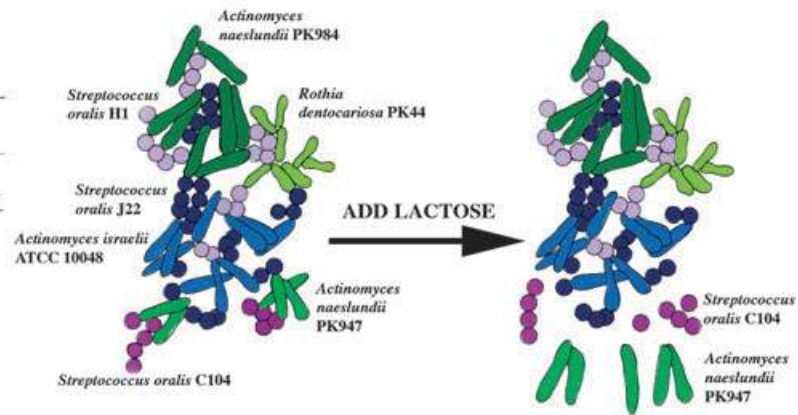| Radioactive strain	% of Input CPM in coaggregates	
	Before lactose	After lactose
PK44	95	96
J22	69	64
PK984	93	85
C104	42	5
PK947	86	6

Fig. 5. Diagrammatic representation of the independent nature of lactose-reversible and lactose-nonreversible coaggregations. Illustrated here is the selective release of cells that coaggregate only by lactose-reversible mechanisms and the maintenance of coaggregates consisting of lactose-nonreversible interactions following the addition of lactose to the multigeneric coaggregate. The table on the left indicates results obtained when one strain (*Rothia dentocariosa* PK44, *Streptococcus oralis* J22, *Actinomyces naeslundii* PK984, *S. oralis* C104, or *A. naeslundii* PK947) is radioactively labeled and mixed with the other six nonradioactive cell-types to form coaggregates. The mixture is given a low-speed centrifugation ($100 \times g$ for 1 min) to pellet coaggregates. The percentage of input radioactivity in coaggregates before and after the addition of 100 mM lactose is indicated. For details, see Kolenbrander and Andersen (54).
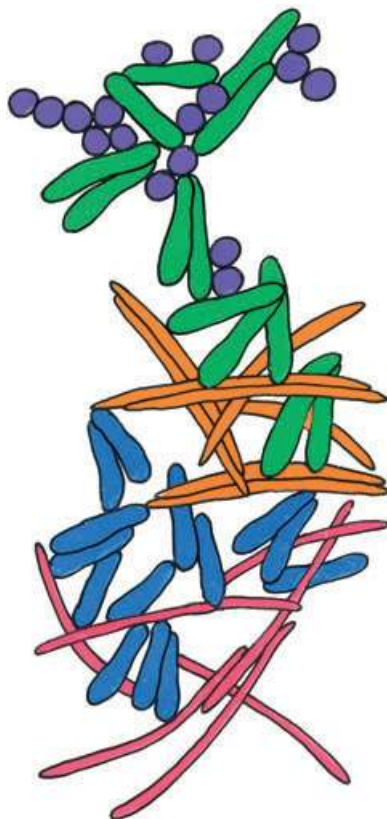

Fig. 6. This model depicts the sequential arrangement of coaggregating cells and the formation of multiple bridges in the accretion of cells as multigeneric coaggregates (54). Purple cells, *Streptococcus oralis* 34; green cells, *Actinomyces naeslundii* PK25; orange cells, *Capnocytophaga ochracea* ATCC33596; blue cells, *A. israelii* PK16; red cells, *C. gingivalis* DR2001.

cells (*Actinomyces naeslundii* PK25), which coaggregate with orange cells (*C. ochracea* ATCC33596), which coaggregate with blue cells (*A. israelii* PK16), and finally the red cells (*C. gingivalis* DR2001) accrete. Close examination of this type of sequential coaggregation reveals the extremely limited coaggregation profile of *C. gingivalis* DR2001. To be successful *in vivo*, an organism with equivalent limitations in accretion would require specific, already accreted, partners for it to attach to the plaque biofilm.

Coaggregation bridges

The basic coaggregation principle exhibited by sequential coaggregation is the principle of bridging (Fig. 7). A coaggregation bridge is formed when the common partner bears two or more types of coaggregation mediators. These mediators can be various types of receptor polysaccharides, or various types of adhesins, or a mixture of the two. Figure 7 illustrates an example where the common partner (red cell) exhibits an adhesin and an unrelated (noncomplementary) receptor polysaccharide. One of the cell types (purple cell) recognizes the adhesin, and the other cell type (blue cell) recognizes the receptor polysaccharide. In this way, the two cell types (blue and purple cells) do not compete with each other for binding to the common partner, which acts as a coaggregation bridge to connect the three
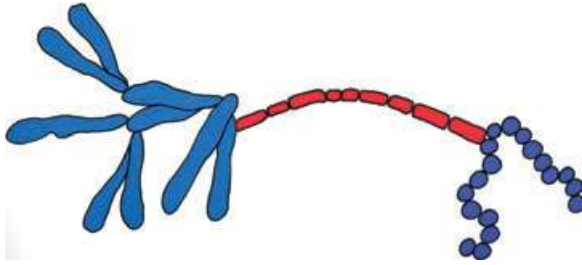
Fig. 7. Model depicting *Prevotella loescheii* PK1295 (red cells) acting as a coaggregation bridge between two non coaggregating cell types, *Actinomyces israelii* ATCC 10048 (blue cells) and *Streptococcus oralis* 34 (purple cells). Coaggregation between the bridge bacterium and *S. oralis* is lactose reversible, but coaggregation with *A. israelii* is not (58).

cell types. An example of bridging coaggregations is shown in Fig. 8. *S. oralis* C104 coaggregates with each partner; *Prevotella loescheii* PK1295 does not coaggregate with *F. nucleatum* PK1909. To illustrate setting up a coaggregation bridge, *S. oralis* C104 and *P. loescheii* PK1295 are mixed together (at a ratio of 3:1, respectively) to form coaggregates; two focal planes of the same field of view are shown (top, Fig. 8). Then, *F. nucleatum* PK1909 (center, Fig. 8) is added to complete the coaggregation bridge (bottom, Fig. 8). Bridging is usually considered to be a co-operative event that brings three or more cell types into close proximity and fosters symbiotic relationships. However, bridging can also be an antagonistic event which brings together organisms that compete with each other for nutrient or other needs.
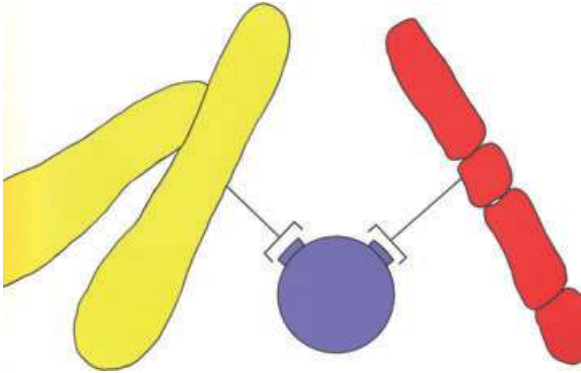
Coaggregation competition

The basic coaggregation principle of competition occurs when multiple cell types recognize the same

coaggregation mediator on the common coaggregation partner (Fig. 9). In this simple example, the streptococcus (purple) is recognized by two species, an actinomyces (yellow) and a prevotella (red). Both the actinomyces and the prevotella recognize the same receptor on the streptococcus and thus compete for binding. Competition is easily demonstrated by using a radioactivity-based assay. One of the competing cell types is radioactively labeled. Then,
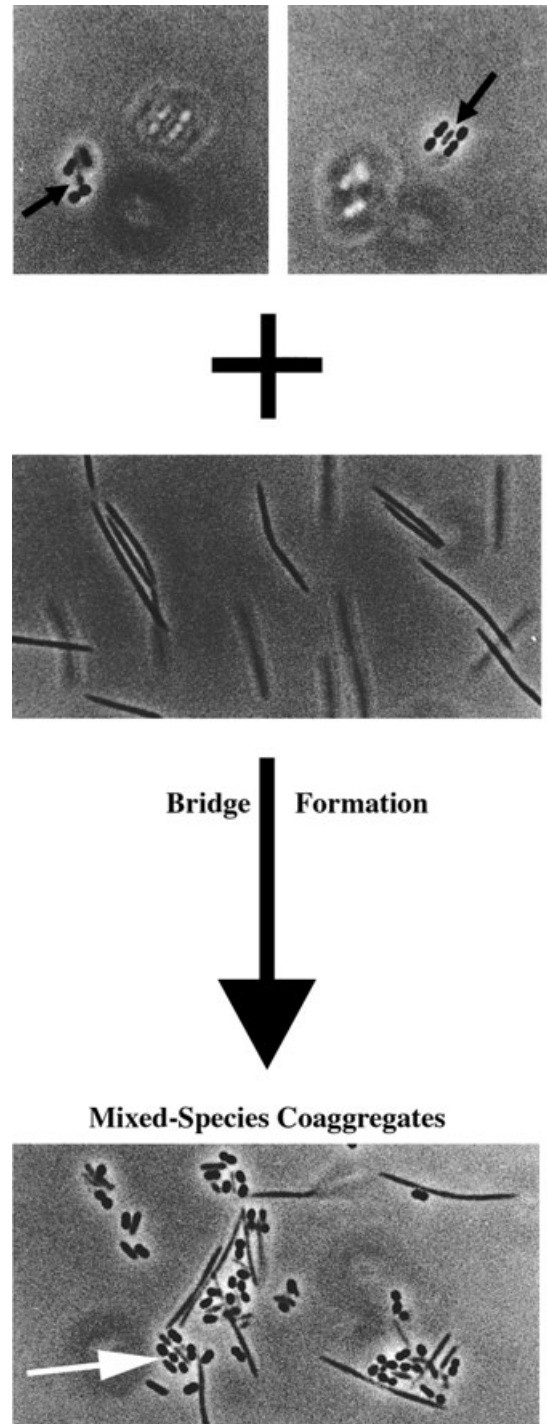
Fig. 8. Phase-contrast photomicrographs depicting coaggregation bridges mediating multigeneric coaggregations. Two views at different focal planes (top panels) of *Streptococcus oralis* C104 (dark cells) in three-fold excess over partner *Prevotella loescheii* PK1295 (grey short rods, black arrows); these coaggregates are found in the supernatant after centrifuging the coaggregation mixture at low speed (100 × *g* for 2 min). Long slender cells of *Fusobacterium nucleatum* PK1909 (middle panel) are mixed with the streptococcus–prevotella pairs in the supernatant to complete the coaggregation bridges and develop into mixed-species coaggregates (bottom panel). *P. loescheii* PK1295 does not coaggregate with *F. nucleatum* PK1909, but *S. oralis* C104 coaggregates with both and acts as a coaggregation bridge (the white arrow indicates one of several examples) between the fusobacteria and the prevotellae.

Fig. 9. Model depicting competition for binding sites (58) on *Streptococcus oralis* 34 (purple cell). These coaggregations are lactose reversible, and although the same symbols are used to represent interactions between different cell types, the identity of structures on the yellow and red cells is not intended. Rather, it is likely that these structures are just part of a larger network of functionally similar, lactose-reversible coaggregations among oral bacteria (see Fig. 3).

by keeping the number of radioactively labeled cells constant while adding increasing numbers of the other competitor, it can be shown that the radioactively labeled cell type is effectively competed from its binding to the common partner (58). Lactose-inhibitable coaggregations are examples that are susceptible to coaggregation competition. Numerous and broadly distributed among genera of oral bacteria (Fig. 3), they can confer competitive accretion properties on the cells.

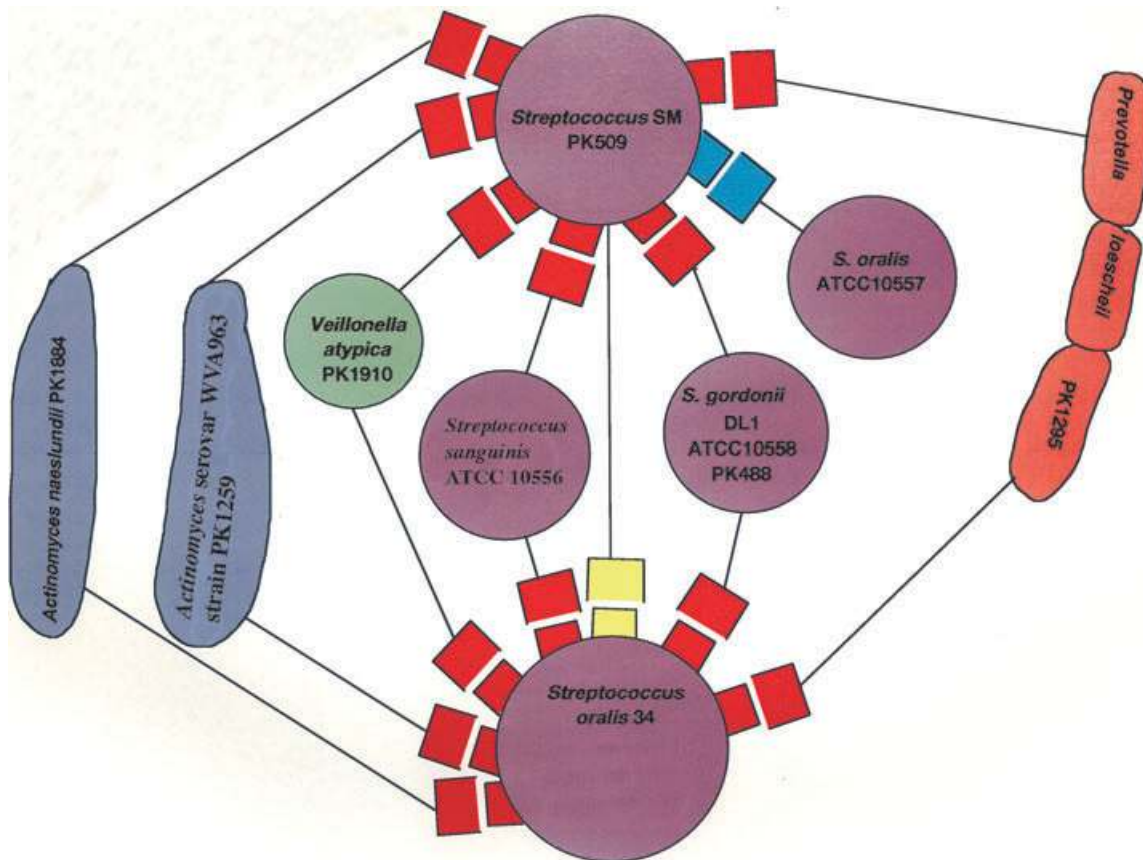## Functional similarity of coaggregation mediators

The principle of competition was illustrated by using just two competing cell types, but it can be expanded to include many cell types, which supports the hypothesis of functional similarity (Fig. 10). Strains from multiple species (two actinomyces, three streptococci, and one each veillonella and prevotella) can bind to a common partner by recognition of the identical receptor on the common partner [*S. oralis* (bottom) or *Streptococcus* SM (top)]. The functionally similar adhesins (surface structures with stems) on each species bind to the same receptor (red rectangles) on the common partner. Note that a coaggregation-defective mutant of the common partner (lacking red rectangle) could no longer coaggregate with *P. loescheii* PK1295, *Streptococcus gordonii* strains DL1, ATCC10558, and PK488, *S. sanguinis* ATCC10556, *Veillonella atypica* PK1910, *Actinomyces serovar* WVA963 strain PK1259, and *A. naeslundii* PK1884, but retains coaggregation with *Streptococcus*

SM PK509 (Fig. 10, yellow symbol) or *S. oralis* ATCC10557 (Fig. 10, blue symbol). Functional similarity of adhesins is documented by radioactively labeling one of the cell types (for example, *P. loescheii* PK1295) and then testing its ability to bind to the common partner (for example, *S. oralis* 34) in the presence of an excess of any one of the other unlabeled cell types (53). The radioactively labeled cell type is out-competed by the binding of cells bearing functionally similar adhesins. Two coaggregations are depicted with different colors (blue and yellow) to indicate that these coaggregations, while inhibited by lactose, are different from the others (red). They might be inhibited more efficiently by galactose or another related sugar. Thus, functional similarity appears to be widespread among oral bacteria, while at the same time, the sugar specificity of coaggregation inhibition modulates the competition inherent in functional similarity.

We propose that each of these basic principles of coaggregation (high specificity, bridging, competition and functional similarity) plays a role in the succession of bacterial colonization in dental plaque. The initial colonizers are primarily streptococci with minor proportions of other genera, such as *Actinomyces*, *Gemella*, *Neisseria*, *Rothia* and *Veillonella*. A wide variety of gram-negative genera coaggregate with the initial colonizers. Bacterial succession facilitates the maturation of plaque communities, and the establishment of periodontal pathogens is predicted to depend on the changes in bacterial diversity that occur as the biofilm develops. Socransky et al. (101, 104) reported clinical findings that indicate the colonization by fusobacteria and certain other bacteria of the orange complex as a prerequisite for the appearance of periodontal pathogens such as *Treponema denticola*, *P. gingivalis* and *Tannerella forsythia*. The temporal appearance of organisms is corroborated spatially by the immunohistological examinations of subgingival dental plaque by Ebisu et al. (82–85). A role for coaggregation in coordinating this temporal order is presented below. We also urge the readers to consult the extensive discussion of periodontal microbial ecology in Socransky and Haffajee (101) for a complete description of microbial diversity in the oral cavity.

Bacterial diversity: from initial communities to mature plaque

The resident microflora of the oral cavity reside in a convenient location for study. Since the 1960s,

Fig. 10. Diagrammatic representation of the functional similarity of adhesins borne on six species that recognize one receptor (red rectangle) on *Streptococcus oralis* 34 and another receptor (red rectangle) on *Streptococcus* SM PK509. A coaggregation-defective mutant of *S. oralis* 34 that fails to coaggregate with *Prevotella loescheii* PK1295 is also unable to coaggregate with *S. gordonii* strains DL1, ATCC10558 and PK488, *S. sanguinis* ATCC10556, *Veillonella atypica* PK1910, *Actinomyces* serovar WVA 963 strain PK1259 and *A. naeslundii* PK1884. The mutant retains coaggregation (yellow symbols) with *Streptococcus* SM PK509. Likewise, a coaggregation-defective mutant of *Streptococcus* SM PK509 fails to coaggregate with *P. loescheii* PK1295, *S. gordonii* strains DL1, ATCC10558 and PK488, *S. sanguinis* ATCC10556, *V. atypica* PK1910, *Actinomyces* serovar WVA 963 strain PK1259 and *A. naeslundii* PK1884, but retains coaggregation (blue symbols) with *S. oralis* ATCC10557.

various reports have established a comprehensive knowledge on how the composition of dental plaque changes as it matures over time. Ritz (98) described changes that occur in the microbial composition of supragingival plaque over an observation period of 9 days. Facultative and aerobic organisms belonging to the genera *Streptococcus* and *Neisseria* predominated on day 1 of plaque formation. After 9 days, a shift occurred and the proportions of these organisms decreased, while the proportions of *Veillonella, Corynebacterium* and *Fusobacterium* increased. The experimental gingivitis studies conducted by Löe and coworkers (69, 112), over a 28-day period, demonstrated that a shift occurred from plaque dominated by gram-positive bacteria, mainly cocci, to one composed largely of gram-negative morphotypes, including rods, filamentous organisms, vibrios and spiro-

chetes. These shifts in the microbial composition of plaque have great significance as they correlate with the appearance of gingivitis.

A later study by Listgarten (66) described the ultrastructural characteristics of mature plaque present on extracted teeth that were associated with healthy periodontal tissues and various degrees of periodontal disease. Intimate associations ('corn cob' and 'bristle brush' formations) between different bacterial morphotypes were commonly seen in subgingival plaque. 'Corn cob' formations were occasionally seen as a feature of plaque present on teeth associated with gingivitis, while 'bristle-brush' formations, composed of a central axis of a filamentous bacterium with perpendicularly associated short filaments, were commonly seen in the subgingival plaque of teeth associated with periodontitis. It is evident that the close proximity of different bacterial cell types

allows the formation of microenvironments in which cell–cell interactions easily occur. This study also revealed that the health-associated microbiota consisted of a thin layer of adherent bacterial cells with the characteristics of gram-positive cocci. In contrast, the samples from teeth with gingivitis contained a greater variety of morphotypes, including coccoid and filamentous forms, as well as gram-positive and gram-negative bacteria. Numerous examples of distinct morphotypes in close association (coaggregation) are seen at the periphery of developing plaque (67, 68, 66). Diversity further increased in the samples from teeth with chronic periodontitis, which contained a dense, predominantly filamentous, supragingival plaque and a subgingival component containing flagellated bacteria, spirochetes and small gram-negative bacteria. These early studies demonstrated that the maturation of plaque is accompanied by changes in the predominant bacterial species, close association of distinct bacterial morphotypes and an increase in bacterial diversity over time. These studies established a cause–effect relationship between these temporal changes in the microflora and the appearance of disease. The notion that plaque maturation was the trigger necessary for the appearance of the inflammatory processes leading to periodontitis made the microbiological studies of plaque formation an important part of understanding the etiology of periodontal diseases.

Bacterial diversity in initial communities

The cellular morphology of early colonizers in the first 4 h of biofilm formation, as determined by scanning electron microscopy, is consistent with that of gram-positive cocci (86). After 8 h, rod-shaped organisms are also seen, but the majority of the bacterial population continues to be largely coccoid. Within 24–48 h, thick deposits of cells with various morphologies are observed, including coccoid, cocco–baccillary, rod-shaped and filamentous bacteria. Consistent with these reports on the morphology of the early colonizing bacteria, several studies have identified streptococci as the predominant colonizers of early enamel biofilms (18, 65, 87). Nyvad and Kilian (87) characterized the culturable microflora colonizing enamel pieces carried in the oral cavity. Streptococci were shown to represent $\approx 63\%$ (mean value of samples from four individuals) of the bacteria isolated after 4 h of plaque formation and 86% of bacteria isolated after 8 h. A variety of other bacteria, such as veillonellae and *Actinomyces*, were also reported to be present. However, this study was

performed at a time when rapid polymerase chain reaction (PCR)-based taxonomic characterization of bacterial communities was not available. As a consequence, the microflora did not include uncultured organisms, and many of the rarer isolates were not assigned an identity, but rather were placed into broad groups such as gram-negative cocci.

The era of molecular biology has made it possible to characterize bacterial communities without selecting just for culturable microorganisms. Li et al. (65) used the checkerboard DNA–DNA hybridization technique, developed by Socransky (103) in 1994, to analyze the early supragingival plaque of 15 healthy individuals. They utilized DNA probes for 40 cultured bacterial species to investigate samples of supragingival plaque collected after 0, 2, 4 or 6 h of plaque formation. This study also identified *Streptococcus* spp., in particular *S. mitis* and *S. oralis*, as being predominant early colonizers, increasing in numbers especially after 4 h of biofilm formation. Other identifiable and moderately abundant species were *A. naeslundii*, *S. gordonii*, *Eikenella corrodens* and *Neisseria mucosa*. The finding of low levels of periodontal pathogens at this early stage of biofilm formation is significant: they report that *T. forsythia*, *P. gingivalis*, *T. denticola* and *Actinobacillus actinomycetemcomitans* all gave positive, but extremely low, reactions.

A recent study in our laboratory characterized the initial microflora by using 16S rRNA gene sequencing (18). The use of broad taxonomic identification tools, such as 16S rRNA gene sequencing, allows the study of bacterial communities regardless of whether the microorganisms are culturable. Our study utilized retrievable enamel chips (91, 92), which were placed in the oral cavity of volunteers for different time periods and then used to visualize undisrupted biofilm architecture (discussed below). 16S rRNA gene libraries were constructed for three subjects who were sampled at 4 and 8 h of colonization of the enamel chips. In accordance with previous studies (65, 87), we observed that the initial communities from all subjects were dominated by *Streptococcus* spp. belonging to the *S. oralis*/*Streptococcus mitis* group. We grouped sequences as phylotypes, which were defined as those sequences similar to each other by 98%. The most abundant phylotypes, apart from those classified as streptococci, belonged to the genera *Actinomyces*, *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia* and *Veillonella*, as well as uncultured species from the class *Clostridia*. The initial communities of some subjects contained gram-negative anaerobic bacteria such as *Prevotella*

spp. and *Porphyromonas* spp., confirming that anaerobic periodontopathogens can colonize early biofilms. Furthermore, the most intriguing finding of our study was that the libraries obtained from different subjects appeared to be statistically different from each other. We found that early dental plaque microflora varies on a subject-specific basis. More than two-thirds of a total of 97 phylotypes found in the three subjects were unique to a specific subject. Subject-specific variation is commonly overlooked as investigators usually pool samples from different subjects and report means or ranges (65, 71, 87, 93). Our finding of interindividual variation in dental plaque colonizers agrees with a recent extensive characterization of the human intestinal microflora that reported major differences in molecular community composition and diversity among three individuals (23). It is possible that interindividual variation in microflora of the digestive tract, including the oral cavity, could be attributed to differences in host factors that modulate colonization of an individual by a specific set of species. Perhaps members of a specific community have adapted to each other and to the host, thus creating inter-relationships among community participants that ensure spatiotemporal repeatability and stability of the microbial community composition.

We also found that of the total 97 phylotypes, 11 were common to all three subjects included in the study. These phylotypes found in all subjects were closely related to *S. mitis*/*S. oralis*, *Streptococcus sanguinis*, *Streptococcus vestibularis*/*Streptococcus salivarius*, *Neisseria pharynges* and *Gemella haemolysans*. Perhaps these microorganisms are highly adaptable to different hosts and constitute an integral part of the development of initial communities independently of other early colonizers.

It is also worth noting that desquamated epithelial cells might be a part of this initial biofilm and are not seen so often in mature plaque. This observation has been reported by various investigators (69, 113). We also observed epithelial cells at 4 and 8 h of biofilm development and, interestingly, streptococci predominate, as found in the initial colonization of enamel surfaces (Fig. 11). This observation suggests that epithelial cells could be a reservoir involved in the relocation of oral bacteria from one site to another.

Subgingival plaque is probably formed by the spread of supragingival plaque down into the gingival sulcus (111). The study of initial subgingival biofilms represents a greater challenge owing to the lack of models that mimic subgingival colonization or that
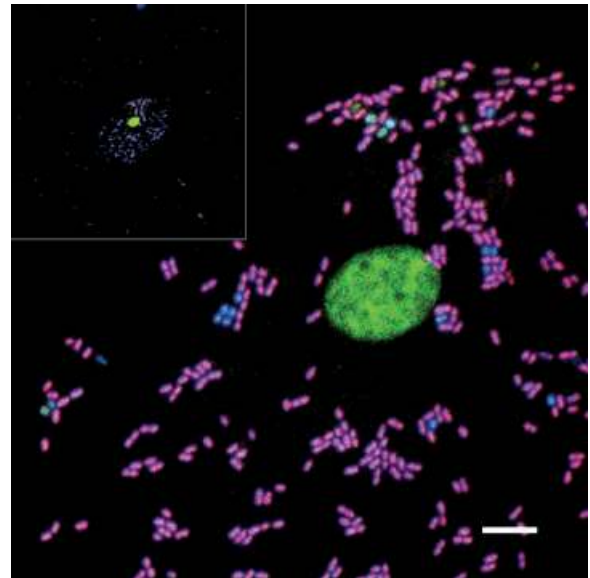

Fig. 11. An epithelial cell detected on the enamel surface is colonized with multi-species bacterial biofilm communities in 8 h supragingival dental plaque. Communities are documented with fluorescence *in situ* hybridization probes (eubacterial probe EUB338, blue; *Streptococcus* spp. probe STR405, red) in conjunction with general nucleic acid stain (acridine orange, green). The nucleus of the epithelial cell is stained with acridine orange (green). *Streptococcus* spp. cells (purple, colocalization of red + blue) are closely associated with non-*Streptococcus* spp. cells (blue) on the epithelial surface. (Bar, 5 μm; insert, the same region at lower magnification).

allow appropriate sampling. Quirynen et al. (94) tried to overcome this limitation by analyzing the subgingival microflora present in so-called pristine pockets, namely pockets created after insertion of transgingival abutments in previously submerged dental implants. The microbiologically 'pristine' pockets had depths of 2.5–6 mm, and were sampled after 1, 2, and 4 weeks of abutment connection. Each site sampled was analyzed for the presence of 40 bacterial species using DNA–DNA checkerboard hybridization. The higher counts detected after 1 week of subgingival biofilm development corresponded to *N. mucosa*, *A. naeslundii*, *Veillonella parvula* and *S. gordonii*, all of which are abundant in early enamel biofilms (101). Quirynen et al. (94) reported the presence of low counts of other organisms, including periodontopathogens, which also agrees with their presence on initial supragingival enamel communities (18, 65). Periodontopathogens were more commonly found when other teeth in the dentition of the individual sampled also harbored them. The authors suggested that the colonization of pristine pockets around implants occurs from the bacteria present in saliva (representing the microbial load in the remaining

dentition) that accumulates first supragingivally and then subgingivally around the newly placed implants. It seems then that the development of subgingival plaque is directly influenced by the supragingival environment. This statement is also supported by studies which show that control of the supragingival microbial load through scaling and oral hygiene decreases the proportion of gram-negative anaerobic bacteria in moderate periodontal pockets (32). Although it appears that the development of subgingival plaque might be influenced by supragingival plaque, the development of subgingival plaque might follow a distinct pattern of maturation because of different selective environmental pressures.

One major challenge in understanding bacterial interactions in subgingival biofilms is the acquisition of undisturbed samples in which spatial relationships between bacteria are maintained, and for which the orientation within the oral cavity is known. The best solution to this problem is removal of the entire tooth with as much surrounding gingival tissue as is practical. Such samples have been obtained (44, 82–85) and have been useful in pioneering studies that map subgingival plaque structure on a macro scale using immunohistochemical approaches. Electron microscopy has also been applied to these samples to examine the biofilm on a finer scale. Sectioning of samples prior to microscopy makes standard microscopy approaches cumbersome and time-consuming; the use of confocal microscopy would free investigators from the need to section their samples extensively prior to examination. A steady supply of extracted teeth is limited in most research situations, therefore a noninvasive human model system, similar to the retrievable enamel chip model discussed below, has been developed (114). In this model, a small rod surrounded by a plastic membrane is inserted into the sulcus of a human volunteer. After removal, the membrane is embedded and minimally sectioned prior to examination by confocal microscopy or electron microscopy. Fluorescence *in situ* hybridization has been used to stain these samples, but other approaches, such as immunofluorescence, are also possible. Knowledge of sample orientation allowed these investigators to conclude that spirochetes and gram-negative bacteria predominated in deeper regions of the pocket, whereas streptococci were abundant in the shallow regions. Biofilms within and around the tooth, in sites such as peri-apical lesions (107), are also amenable to fluorescence *in situ* hybridization staining and confocal analysis; these biofilms also show interbacterial interactions.

Bacterial diversity in mature plaque

Initial biofilm formation seems to be a subject-specific, repeatable process. Regardless of the specific strains carried by each subject, streptococci predominate in this early stage until approximately 2 days of undisrupted plaque accumulation (113). As plaque matures and bacterial accumulation affects the local environmental conditions, minor species of these initial communities will have the opportunity to flourish and become predominant species in mature plaque. Changes in the bacterial composition of plaque could be a result of the continuous accretion of new cell types. Changes in the types of predominant bacteria could be the result of increased numbers of those members of the community that find suitable environmental conditions for rapid cell division. After 7 days of undisrupted plaque accumulation, the bacterial population shifts to predominantly rods and filaments (113) with the appearance of spirochetes and vibrios (69, 112). Ramberg et al. (96) used DNA–DNA checkerboard hybridization to characterize interproximal supragingival biofilms formed on tooth surfaces after professional tooth cleaning. Although their results show unusually high proportions of *Actinomyces* on day 1 of plaque formation, it is interesting that on day 4 of plaque accumulation there is an increase in the numbers of bacteria from the orange complex (104), in particular *F. nucleatum*. As discussed above, the coaggregation properties of *F. nucleatum* would facilitate biofilm development. One coaggregation partnership of particular interest is that between *F. nucleatum* and *P. gingivalis*. Coaggregations with fusobacteria are noteworthy morphological arrangements: these corncob shapes were noted by Listgarten (66–68) and others (40) in dental plaque, as well as *in vitro* (20, 63, 81), indicating that these types of coaggregations are not uncommon in developing oral biofilms. Coaggregations can exhibit a variety of morphologies, which depend upon partner cell shapes and the ratio of the partner cell types (Fig. 12). When partners are in 10-fold excess, *F. nucleatum* forms corncob arrangements with porphyromonads (Fig. 12B), actinobacilli (Fig. 12C), veillonellae (Fig. 12D), selenomonads (Fig. 12F), and streptococci (Fig. 12H). Corncob configurations can be formed with other species; a chain of *S. oralis* C104 is surrounded by *P. loescheii* PK1295 when *P. loescheii* is in 10-fold excess (Fig. 12G). Significant changes in the appearance of coaggregates occurs with just a fivefold adjustment in the cell ratio (compare Fig. 12I with Fig. 12J). Owing to its long rod morphology, a single fusobacterial cell
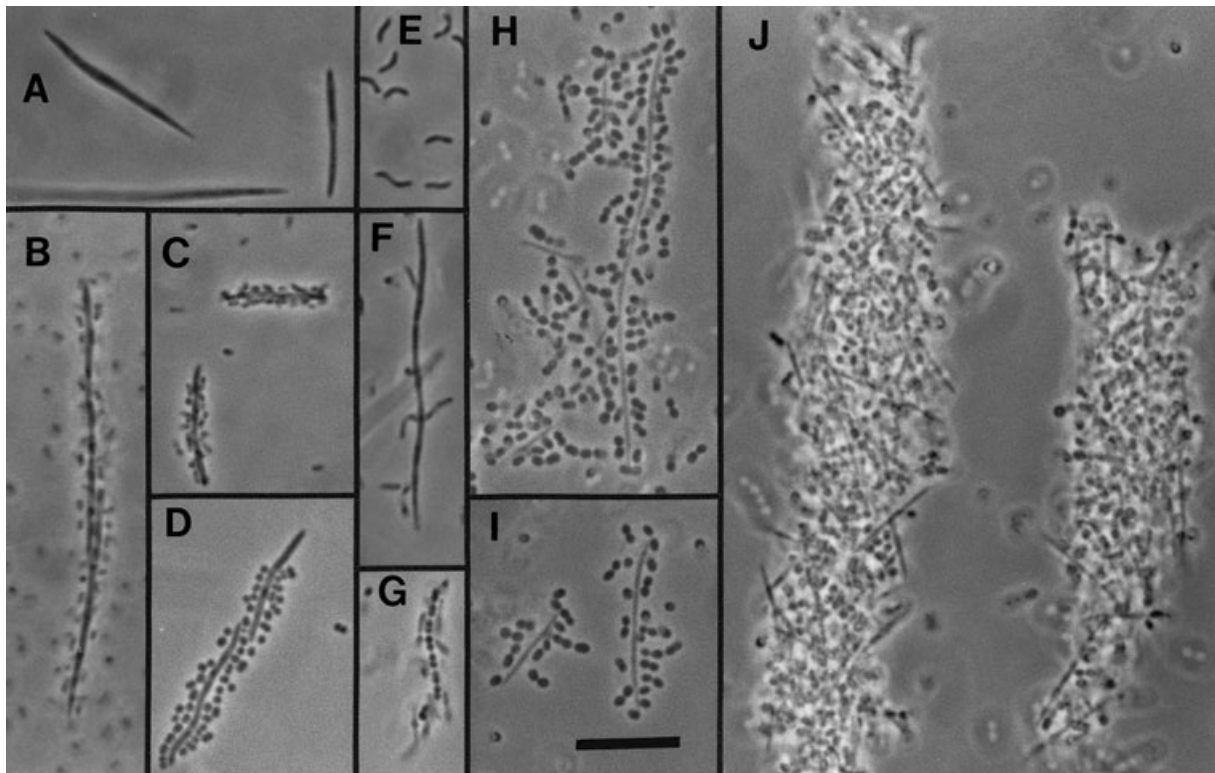
Fig. 12. Intergeneric coaggregations in the form of corncob configurations (49, 50, 59). *Fusobacterium nucleatum* PK1594 (A) forms corncob configurations with its partners *Porphyromonas gingivalis* PK1924 (B), *Actinobacillus actinomycetemcomitans* Y4 (C), *Veillonella atypica* PK1910 (D), *Selenomonas flueggei* PK1958 (F), and *Streptococcus oralis* C104 (H) when the partners are present at a 10-fold excess. Corncob configuration with a chain of *S. oralis* C104 surrounded by *Prevotella loescheii* PK1295 when *P. loescheii* is at a 10-fold excess (G). Changes in appearance of coaggregates with different ratios of partners; *S. oralis* C104 at a 10-fold excess over *F. nucleatum* PK1909 (I), and at only a two-fold excess (J). Suspension of *S. flueggei* PK1958 (E). Bar, 10 μm.

could harbor, on its surface, a variety of coaggregation partner cell types. We propose that fusobacteria are central structural components of plaque and essential for plaque maturation and an increase in plaque diversity.

The diversity of mature supragingival plaque has been studied by DNA–DNA checkerboard hybridization (119), but the focus of most of the extensive characterizations of mature plaque samples using broader methods, such as 16S rRNA gene sequencing, has been on subgingival plaque (63, 93). The checkerboard studies have shown that one of the most abundant gram-negative anaerobes in mature supragingival and subgingival plaque of healthy subjects, and those with periodontitis, is *F. nucleatum*. These studies confirm its ubiquity and its potential role as a core organism essential for plaque development. Ximenez-Fyvie et al. (118) also demonstrated that gram-negative periodontopathogenic bacteria from the red complex [*P. gingivalis*, *T. forsythia* and *T. denticola*; (104)] are present in the supragingival and subgingival plaque of healthy

subjects and subjects with periodontitis. However, red complex organisms were present in higher proportions in the supragingival plaque of subjects with periodontitis that harbored the organisms subgingivally. This result confirms the influence of supragingival plaque on the subgingival microbiota. It is also possible that once established in the subgingival environment, gram-negative anaerobes leave the periodontal pocket to colonize supragingival plaque and subgingival sites of other teeth. The process of bacterial detachment has been described in biofilms formed by extra-oral organisms such as *Pseudomonas aeruginosa* (38) and by oral bacteria such as *A. actinomycetemcomitans* (41). It is possible that most bacteria have the ability to detach from a surface as a mechanism involved in relocation and attachment to a surface at a different site. *A. actinomycetemcomitans* produces a soluble β-N-acetyl-glucosaminidase that hydrolyzes a cell-synthesized extracellular polysaccharide in which the biofilm cells are embedded. The glycoside hydrolase dispersin B facilitates detachment and dispersion of cells

(42, 95). Mechanisms of dispersal of attached cells are required for nonmotile oral bacteria. Once they are suspended in oral secretions, they are moved from place to place and into position for accretion at a new oral site, or they are swallowed. Imagining frequent intra-oral movement of detached bacteria, coupled with coaggregation, gives clear pictures of how microbial diversity on oral surfaces could occur.

Inflammatory diseases of the periodontium result from an imbalance in environmental conditions and/or host-associated factors that trigger changes in the microbiota, allowing an increase in the proportions of virulent bacteria. In subjects with periodontitis, the diversity of the subgingival resident organisms seems higher than in healthy subjects. Paster et al. (93) estimated that ≈ 415 species are present in subgingival plaque. The establishment of a climax community with high diversity increases the potential for metabolic co-operation and interactions among community members. However, not all the bacteria present in sites with periodontitis might necessarily be part of the transition from health to disease; virulent bacteria might not be able to colonize a site until the microbiota has shifted and the inflammatory process has initiated. The role of many microorganisms considered to be periodontopathogens might be to perpetuate the imbalance in the microflora and the inflammatory response induced by other bacteria. Tanner et al. (109) studied the bacterial species associated with the initial development of a periodontal lesion. This study is one of the few longitudinal studies carried out to identify the organisms associated with the shift from health to disease. Their data suggest that *T. forsythia*, *Campylobacter rectus* and *Selenomonas noxia* were the major species that characterized sites converting from health to disease. *P. gingivalis* and *A. actinomycetemcomitans* were detected infrequently in this population, which suggests that they might arrive later during the disease process. It is also evident that subject-specific factors make it very difficult to categorize a microorganism as being absolutely causative of disease. The same organism might not display the same virulence potential in all subjects because the interaction with other bacteria could condition its growth and gene expression. Similarly, immune host factors have been demonstrated to be essential for disease susceptibility (19, 62). Now that a large 16S rRNA database is available, the application of 16S rRNA-based molecular tools, such as microarrays, might facilitate future monitoring of changes in the microbiota associated with gingivitis and periodontitis.

Integration of specificity of coaggregation, taxonomic identification of species and temporal succession of species-colonizing enamel

In 1965 and 1966, Löe and colleagues (69, 112) established the succession of bacterial morphotypes with progression from periodontally healthy sites (predominated by gram-positive cells) to gingivitis (predominated by gram-negative cells). Taxonomic identification confirmed the shift from primarily streptococci and actinomyces in healthy sites to primarily fusobacteria in gingivitis (Fig. 13) (79, 80, 102). Kilian and colleagues showed that 63–86% of the initial colonizing bacteria were streptococci along with some actinomyces and veillonellae (87, 88). Recent studies, based on molecular techniques, have expanded the types of bacteria found in initial and mature communities but have not changed the overall understanding of change from gram-positive to gram-negative flora (18, 63, 65, 93, 101, 103, 104).

Considering these facts, and knowing the results of numerous pairwise coaggregation profiles of isolates from subgingival sites, led us to propose an integration of these observations (51, 57). The sequential nature of colonizing species and their coaggregation properties is depicted in Fig. 14. The acquired pellicle coating the enamel consists of a variety of receptor molecules that are recognized primarily by streptococci. This ability to bind to nonshedding surfaces gives streptococci a great advantage and is consistent with the observation that they constitute 60–90% of the initial bacterial flora. Streptococci are less sensitive to exposure to air than most oral bacteria, and they participate in modifying the environment to a more reduced state, a condition often considered to favor the shift towards gram-negative anaerobes. Moreover, another property that we had proposed to be of critical significance for initial streptococcal dominance is their ability to coaggregate with other streptococci (60). Although the coaggregation partners belong to the same genus, they are genetically distinct cell types, exemplified by the *S. oralis* and *S. gordonii* partnerships. While most of these coaggregations are lactose-inhibitable, all are more efficiently inhibited by *N*-acetyl-D-galactosamine, indicating the high specificity for intrageneric sugar inhibitions (60). These intrageneric coaggregations are a clear departure from the prevalent intergeneric coaggregations; *Streptococcus* spp. and a few members of *Actinomyces* were the only early colonizers to

Species:	Health	Rank in different periodontal health conditions:				
		Gingivitis	Moderate	Adult	Juvenile	Severe
A. naeslundii	1 (18)	2	1	1	2	4
F. nucleatum	2 (11)	1	2	2	3	1
S. sanguinis	3 (9)	14	32	3	23	41+
S. oralis	4 (7)	24	17	8	8	41
S. intermedius	5 (5)	19	14	11	36	9
P. micros	6 (2)	7	3	4	10	6
A. meyeri	7 (2)	43+	30	31	20	31
S. gordonii	8 (2)	40	35	47+	42+	41+
G. morbillorum	9 (2)	31	44+	27	37	41+
A. odontolyticus	10 (2)	18	22	12	18	41+

Species:	Gingivitis	Moderate	Adult	Juvenile	Severe	Health
F. nucleatum	1 (12)	2	2	3	1	2
A. naeslundii	2 (9)	1	1	2	4	1
O. uli	3 (4)	9	9	7	5	38+
C. concisus	4 (3)	44+	6	42+	41+	30
S. sputigena	5 (3)	15	10	39	23	36
C. gracilis	6 (3)	21	5	25	25	17
P. micros	7 (3)	3	4	10	6	6
P. anaerobius	8 (2)	20	23	16	29	38+
A. israelii	9 (2)	38	22	34	21	21
A. rimae	10 (2)	29	13	13	12	11
P. gingivalis	11 (2)	6	20	28	41+	38+

Fig. 13. Genera of oral bacteria in health and disease. The most numerous species are listed in order of percentage of isolates taken at random from subgingival crevices of subjects with different periodontal health conditions. The species rank is based on data obtained from Moore and Moore (78). The color bars identify members of the microbial complexes given in Socransky et al. (104). The number in parentheses is the percentage of isolates taken at random from subgingival samples. The gram-positive bacterial species are *Actinomyces israelii, A. meyeri, A. naeslundii, A. odontolyticus, Atopobium rimae, Gemella morbillorum,* *Olsenella uli, Peptostreptococcus anaerobius* ID, *P. micros, Streptococcus gordonii, S. intermedius, S. oralis* and *S. sanguinis.* The gram-negative bacterial species are *Campylobacter concisus, C. gracilis, Fusobacterium nucleatum, Porphyromonas gingivalis* and *Selenomonas sputigena.* The species rank in other periodontal health conditions is based on data obtained from Moore and Moore (78). Species rank with a number and a plus sign indicates a rank lower than that number. These rankings were obtained on the basis of culturability of isolates observed in samples taken from sites with different periodontal health conditions.

exhibit extensive intrageneric coaggregations (60). These three properties, and probably several others, contribute to the numerical dominance of streptococci in the early colonizers.

The analysis of the coaggregation profiles of hundreds of subgingival isolates has provided evidence that coaggregation might be important for subsequent plaque development. Certain streptococci (for example, *S. oralis*) (Fig. 14), which bear receptors (depicted as red rectangles in Fig. 14), are coaggregation partners of members of several genera (57, 61). Early colonizing partners of receptor-bearing streptococci include *S. gordonii, A. naeslundii, E. corrodens, V. atypica, P. loescheii* and *Haemophilus*

Fig. 14. Spatiotemporal model of oral bacterial colonization, showing recognition of salivary pellicle receptors by early colonizing bacteria, and coaggregations between early colonizers, fusobacteria and late colonizers of the tooth surface (61). Each coaggregation depicted is known to occur in a pairwise test. Collectively, these interactions are proposed to represent development of dental plaque. Starting at the bottom, primary colonizers bind via adhesins (round-tipped black line symbols) to complementary salivary receptors (blue–green vertical round-topped columns) in the acquired pellicle coating the tooth surface. Secondary colonizers bind to previously bound bacteria. Sequential binding results in the appearance of nascent surfaces that bridge with the next coaggregating partner cell. Several kinds of coaggregations are shown as complementary sets of symbols of different shapes. One set is depicted in the box at the top. Proposed adhesins (symbols with a stem) represent cell-surface components that are heat inactivated (cell suspension heated to 85°C for 30 min) and protease sensitive; their complementary receptors (symbols without a stem) are unaffected by heat or protease. Identical symbols represent components that are functionally similar but may not be structurally identical. Rectangular symbols represent lactose-inhibitable coaggregations. Other symbols represent components that have no known inhibitor. The bacterial species shown are *Actinobacillus actinomycetemcomitans, Actinomyces israelii, Actinomyces naeslundii, Capnocytophaga gingivalis, C. ochracea, C. sputigena, Eikenella corrodens, Eubacterium* spp., *Fusobacterium nucleatum, Haemophilus parainfluenzae, Porphyromonas gingivalis, Prevotella denticola, P. intermedia, P. loescheii, Propionibacterium acnes, Selenomonas flueggei, Streptococcus gordonii, S. mitis, S. oralis, S. sanguinis, Treponema* spp., and *Veillonella atypica.*

parainfluenzae, as well as *C. ochracea*. It is worth noting that these coaggregating partners of the initial colonizing *S. oralis*, *S. sanguinis* and *S. mitis* are nearly all gram-negative, which correlates with the 40-year-old reports of a temporal shift from gram-positive to gram-negative bacterial flora. We showed, by molecular phylogenetic characterization, that the dominant species in initial dental plaque was *S. oralis*

(which cannot be distinguished from *S. mitis* by the 16S ribosomal RNA gene sequence) (18). All *S. oralis* are receptor-bearing cells (35), indicating that receptor-bearing streptococci are an abundant surface readily available for recognition by gram-negative bacteria expressing complementary adhesins which recognize receptor polysaccharides. Possibly, receptor polysaccharides on the early colonizing
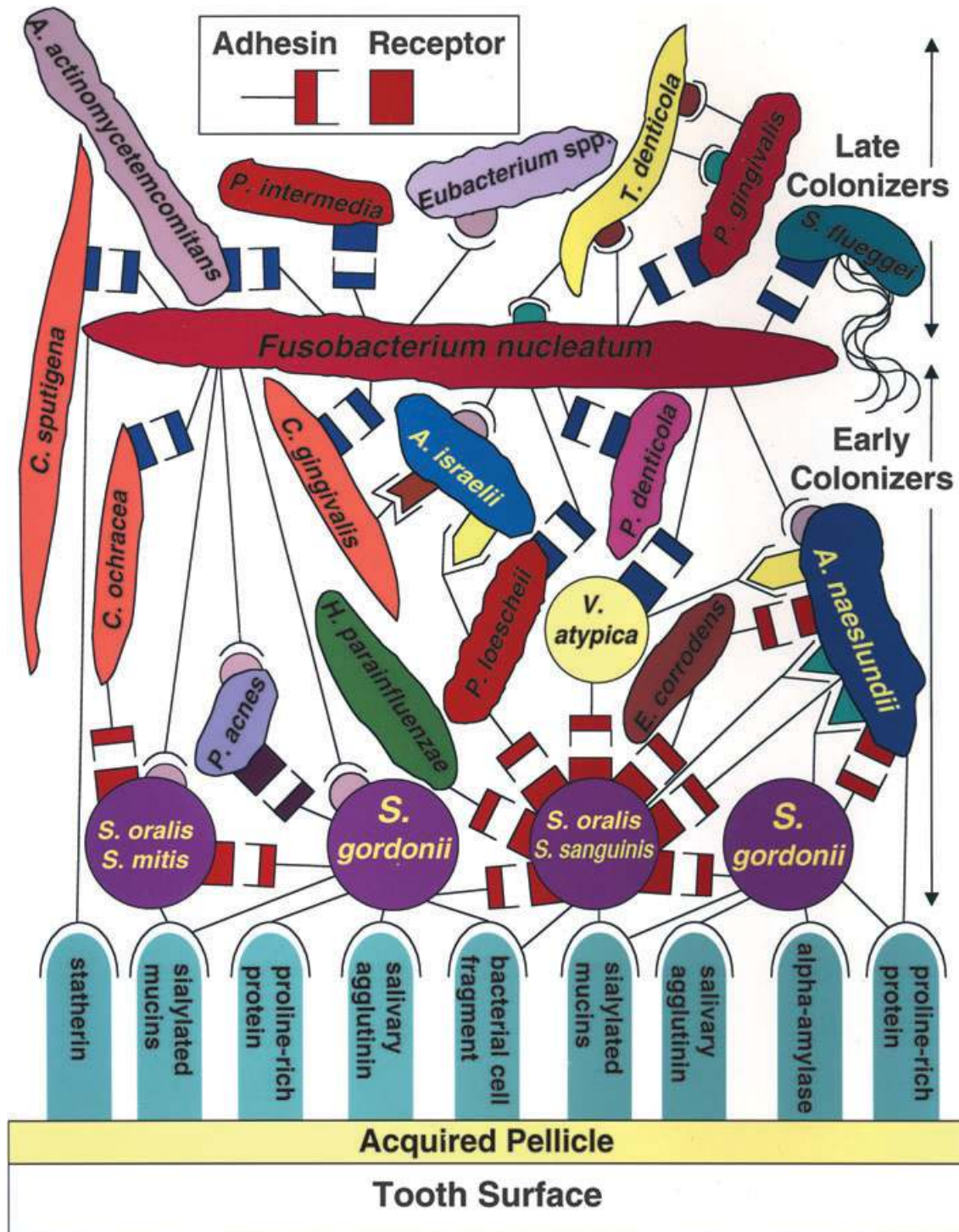
streptococci are a prerequisite for the shift from gram-positive to gram-negative flora accompanying the shift from health to gingivitis.

In addition, these coaggregations offer another illustration of the principle of functional similarity. Each of the species bears an adhesin that is functionally similar to the others, but is structurally distinct. The adhesins of the gram-negative species vary in molecular size, being 34 kDa in *H. parainfluenzae* (64), 45 kDa in *V. atypica* (37) and *E. corrodens* (22, 120) and 75 kDa in *P. loescheii* (115, 116). The adhesins of the gram-positive species are 95 kDa for *Actinomyces* serovar WVA 963 strain PK1259 (45, 46) and 215 kDa for SspA of *S. gordonii* (14, 24). Only a few of these species have been tested in competition experiments for binding to the common partner, *S. oralis* (53). Radioactively labeled *P. loescheii* was effectively competed by unlabeled *S. gordonii*, and unlabeled *A. naeslundii*. *V. atypica*, *H. parainfluenzae* and *E. corrodens* could also compete for binding to the common partner; however, they could equally act as coaggregation bridges for other bacterial colonizations. Both *P. loescheii* and *V. atypica*, for example, bridge between *S. oralis* and *F. nucleatum*.

Coaggregation bridges among early colonizers are common with other early colonizers as well as with *F. nucleatum*, which in turn coaggregates with numerous late colonizers. Examination of the coaggregation profiles of the late colonizers indicates that most do not coaggregate with early colonizers, most do not coaggregate with other late colonizers, but they all coaggregate with fusobacteria. This observation led us to propose that fusobacteria are a major turning point in colonization of periodontally healthy sites. Fusobacteria act as major coaggregation bridges between early and late colonizers. Fusobacteria also offer a second example of functional similarity of coaggregation mediators. Unlike the example of the streptococci, which bear receptors (Fig. 10), the fusobacteria bear the adhesin (Fig. 14; stemmed blue rectangle) that recognizes functionally similar receptors (Fig. 14; blue rectangles) on both early and late colonizers. All of the coaggregation partners that bear this functionally similar receptor are gram-negative cells. Note that several species (*V. atypica*, *P. loescheii* and *C. ochracea*) also bear the adhesin which recognizes the lactose-inhibitable receptor (red rectangular symbol) on the streptococci described above. Importantly, the receptor and adhesin borne on the same cell do not recognize each other, which is another example of the exquisite specificity of lactose-inhibitable coaggregations.

To investigate the occurrence of coaggregations *in vivo*, we used a retrievable enamel chip model system (91) (Fig. 15). A volunteer wears an acrylic appliance on each side of the mandible. Each appliance contains three $3 \times 3 \times 1$ mm enamel chips, as shown. Thus, six replicates are possible for a time point. However, usually two time points are chosen, for example 4 and 8 h of wear. The appliance is removed at the proper time and the enamel chips are retrieved and processed for microscopy without disturbance to spatial relationships within the native biofilm. To verify the occurrence of coaggregation-mediated interactions *in vivo*, we used antibodies directed against bacterial surface components (adhesins and receptors) that mediate coaggregation between streptococci and actinomyces. Antibodies against adhesin-bearing type 2 fimbriae of *A. naeslundii* T14V (anti-type 2), and antibodies against complementary receptor polysaccharides borne on streptococci, such as *S. oralis* 34, were conjugated to fluorescent dyes and used as direct immunofluorescent probes (Fig. 16). This image showed the first unambiguous evidence of a role of coaggregation-mediated cell–cell recognition in plaque development. The dark areas evident in the large coaggregate are likely to be occupied by other bacteria that do not react with either of the two antibodies used. A third antibody, against *S. gordonii* DL1 (anti-DL1), was used to investigate intra-generic coaggregations (Fig. 17). Although it is not possible to identify coaggregation as a mechanism in establishment of these latter interactions, it is clear that, even very early in plaque development, cells of different types are found juxtaposed in multi-species communities.

As all oral bacteria have coaggregation partners, clearly this kind of cell–cell recognition is key to

Fig. 15. Retrievable enamel chip model showing intra-oral acrylic appliances in place in a volunteer's mouth (91). Each mandible carries an appliance, which contains a $3.5 \times 10 \times 1.5$-mm groove to accommodate three $3 \times 3 \times 1$ enamel chips side-by-side.
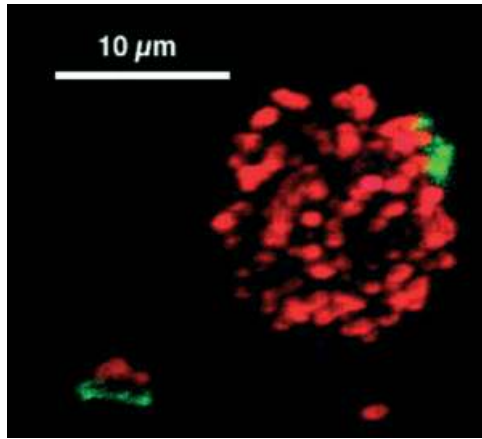
Fig. 16. Interaction between anti-receptor polysaccharide-reactive cells (red) and anti-type-2-reactive cells (green) in 8-h-old plaque. Streptococcal receptor polysaccharides and actinomyces type-2 fimbriae are mediators of coaggregation: juxtaposition of the mediators is strong evidence for coaggregation *in vivo* (92).

successful colonization of teeth. Oral bacteria must attach to a surface or they will be swallowed and removed from the oral cavity. Almost nothing is known in regard to how these bacteria communicate to make successful communities. In most people, these communities form and are removed twice a day with each oral hygiene procedure. Besides the physical interaction of cells, it is likely that these bacteria indicate their presence through other means of communication. This area of research is building momentum with the development of confocal scan-

ning laser microscopy and the discovery of small-molecule signals.

# Communication, signaling and partnership among oral bacterial species

This section will describe the different *in vitro* approaches we have used to characterize the outcomes of bacterial interactions. We propose that coaggregation and close proximity facilitate bacterial mutualism, which influences biofilm development. Metabolic interactions can occur at many levels and include nutritional co-operation, environmental modification through oxygen detoxification, and small-molecule signal-mediated gene regulation. A current review gives an ecological and evolutionary perspective to bacterial communication and provides a useful lexicon for describing communication and signaling (43).

The confocal scanning laser microscope has had a major impact on the types of experiments that are possible to carry out with multispecies communities, such as dental plaque. As a tool used in parallel with our studies on plaque formed *in vivo*, we used a flowcell with a glass coverslip (89), and we used unamended saliva as the sole nutritional source (52) (Fig. 18). Biofilms are formed on the surfaces, and those biofilms attached to the underside of the cov-
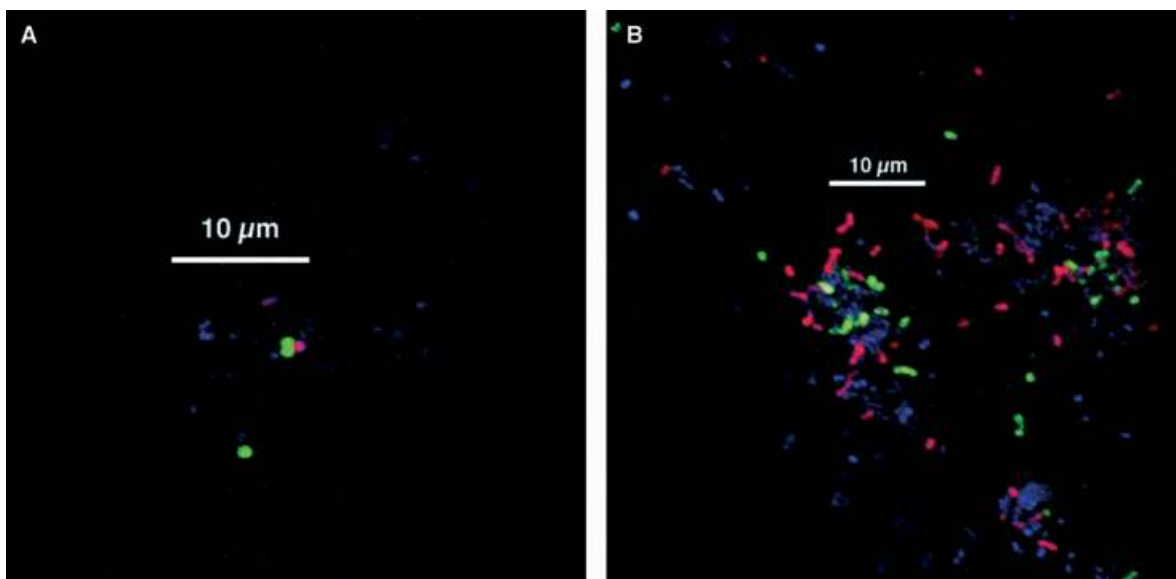
Fig. 17. Unambiguous interactions *in vivo* of at least two coccoid genotypes (92). Staining with anti-DL1 (green), anti-receptor polysaccharides (red), and SYTO 59 (blue). (A) Anti-DL1-reactive cells in association with an anti-receptor polysaccharide-reactive cell in 4-h-old plaque. (B) Interaction of anti-DL1-reactive cells, anti-receptor polysaccharide-reactive cells, and antibody-unreactive cells in 8-h-old plaque.
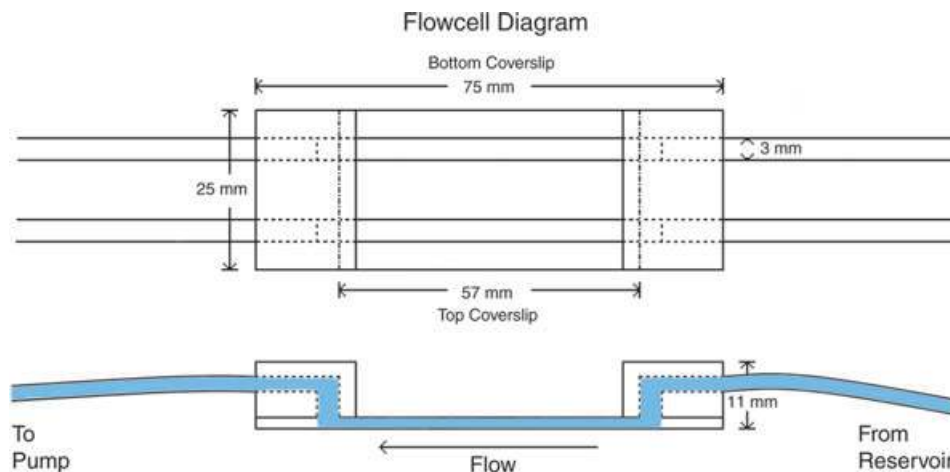
Flowcell Diagram

Fig. 18. The disposable flow cell design viewed from above (top diagram) and from the side (bottom diagram). Each flow cell contains two channels, and each channel is connected by tubing to a reservoir on the right and to a pump on the left. The biofilm formed on the bottom-side of the upper glass surface is viewed from above by using a confocal scanning laser microscope (52, 89).

erslip were imaged with the confocal scanning laser microscope. The inoculum can be undefined, as from a whole saliva sample, and a simple method used to stain the bacteria is to use Live/Dead Stain (Fig. 19). These communities are complex in structure and morphotypes, and it is not possible to distinguish species with a general staining procedure such as the Live/Dead Stain. Many initial attachments occurred by only a few cells (Fig. 19A, arrow), compared with the contiguous, more voluminous colony mass extending toward the lumen (Fig. 19B–F, arrows). This feature is characteristic of multispecies biofilm growth in laminar flow conditions, as used in the flowcell.

Mutualism in dual-species flowcell communities

It is possible to examine defined multispecies communities using fluorescent probes that specifically label each species. Such fluorescent probes can be prepared by conjugating a fluorophore with an antibody against specific bacteria, and the probes can be used to locate bacteria in a mixture of organisms. Other fluorescent probes include conjugating a fluorophore to an oligonucleotide fragment that targets the 16S rRNA of a particular species or by inserting a gene encoding green fluorescent protein into one of the species used to study multispecies biofilms. By using dual-species inocula of *A. naeslundii* with either *S. gordonii* or *S. oralis*, we showed that *S. gordonii* grows equally well with or without *A. naeslundii* (90). *A. naeslundii* is unable to grow by itself on saliva; it is retained in the dual-species biofilm, although it does not grow well. Likewise, *S. oralis* is unable to grow by itself on saliva or in dual species with *S. gordonii*, although it is retained while *S. gordonii* grows. Thus, *S. gordonii* grows on saliva independently of the other two species. It is significant that *S. oralis* and *A. naeslundii* are retained in biofilms with *S. gordonii*, even though they show little or no growth. Retention is a critical feature because it

Fig. 19. Human oral biofilm formed *in vitro* with a saliva inoculum and using sterile saliva as its sole source of nutrient (61). The 25-μm-thick biofilm was grown overnight suspended from the underside of the coverslip of a flowcell with saliva flowing through once at 0.2 ml/min. Bacterial juxtaposition and biofilm architecture were imaged by confocal scanning laser microscopy after staining the cells with Live/Dead stain (Molecular Probes, Eugene, OR). The color of the cells is from the red (propidium iodide: permeable cell membrane, low transmembrane potential) and green (SYTO 9; healthy cell) fluorescent stains. Colocalization of both fluorophores results in yellow staining. Confocal scanning laser microscopy acquires optical sections through the biofilm: each optical section is 0.5-μm thick. Progressive depths through the entire biofilm are shown in six images (A–F). Panel A is the 0.5-μm optical section at the substratum and shows the biofilm footprint. Panel F is the top 0.5 μm of the biofilm where it projects into the lumen of the flowcell. The other four projection images contain eight sections per projection and show the 4-μm-thick regions 4 to 8 μm from the substratum (B), 8–12 μm from the substratum (C), 12–16 μm from the substratum (D), and 16–20 μm from the substratum (E). Regions indicated by arrows are described in the text. Bar, 10 μm.

allows the organism to be available for more favorable conditions. This may be different if the pH is changed or under other environmental changes, or in

the presence of a more favorable coaggregation partner. Thus, although neither *S. oralis* nor *A. naeslundii* grow in monoculture on saliva, together they
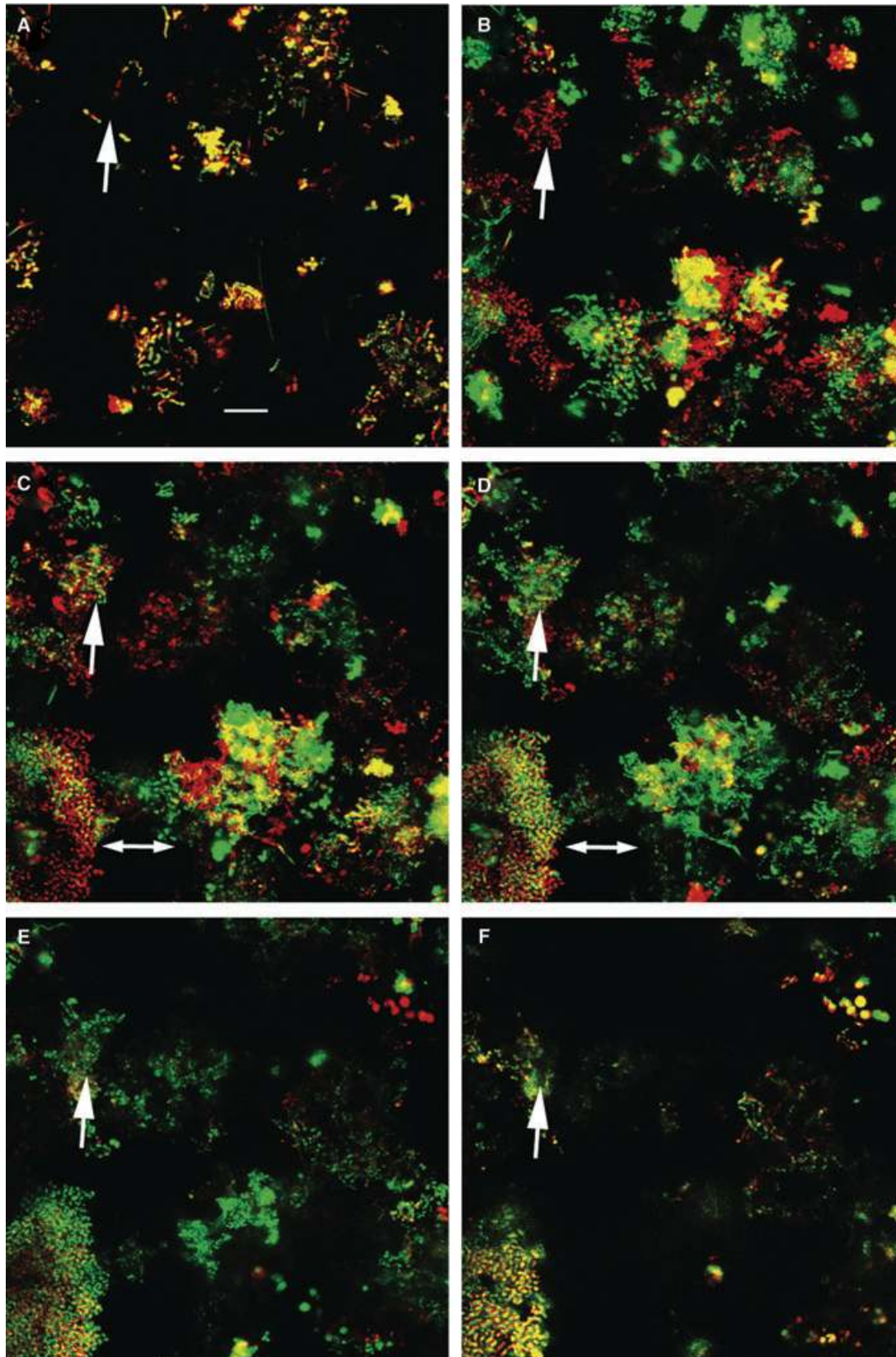
grow abundantly (Fig. 20) and to a significantly higher biomass than observed with *S. gordonii* growing alone or with coaggregation partners. These results emphasize the possibility of mutualistic interactions *in vivo* and point out the differences in abilities of species to grow alone vs. in communities. These results are consistent with the observations of *S. oralis* and *A. naeslundii* in early dental plaque communities and suggest that mutualism is a key element in successful colonization.

## Symbiotic relationship in coaggregated four-species flowcell communities

We used the flowcell, with saliva as the sole nutritional source, to investigate multispecies community building with *A. naeslundii*, *F. nucleatum*, *S. gordonii* and *V. atypica* (28). Fluorescence *in situ* hybridization

was used to distinguish cell types (Fig. 21). Of interest to colonization and growth was the influence of adding the four species sequentially to the flowcell vs. adding all four as a coaggregate. The fluorescence was quantified and converted to biovolumes. Sequentially inoculated flowcells gave higher overall biovolumes than coaggregate-inoculated flowcells. However, coaggregate-inoculated flowcells contained significantly higher numbers of *A. naeslundii* and *V. atypica* than sequentially inoculated flowcells, indicating the influence of pre-formed coaggregates on the composition of multispecies biofilm communities. Preformed coaggregations appear to favor a symbiotic relationship between *A. naeslundii* and *V. atypica* and may be essential to initiate other symbiotic interactions. In all experiments, whether sequentially added first or last or as part of the four-species preformed coaggregates, *S. gordonii*
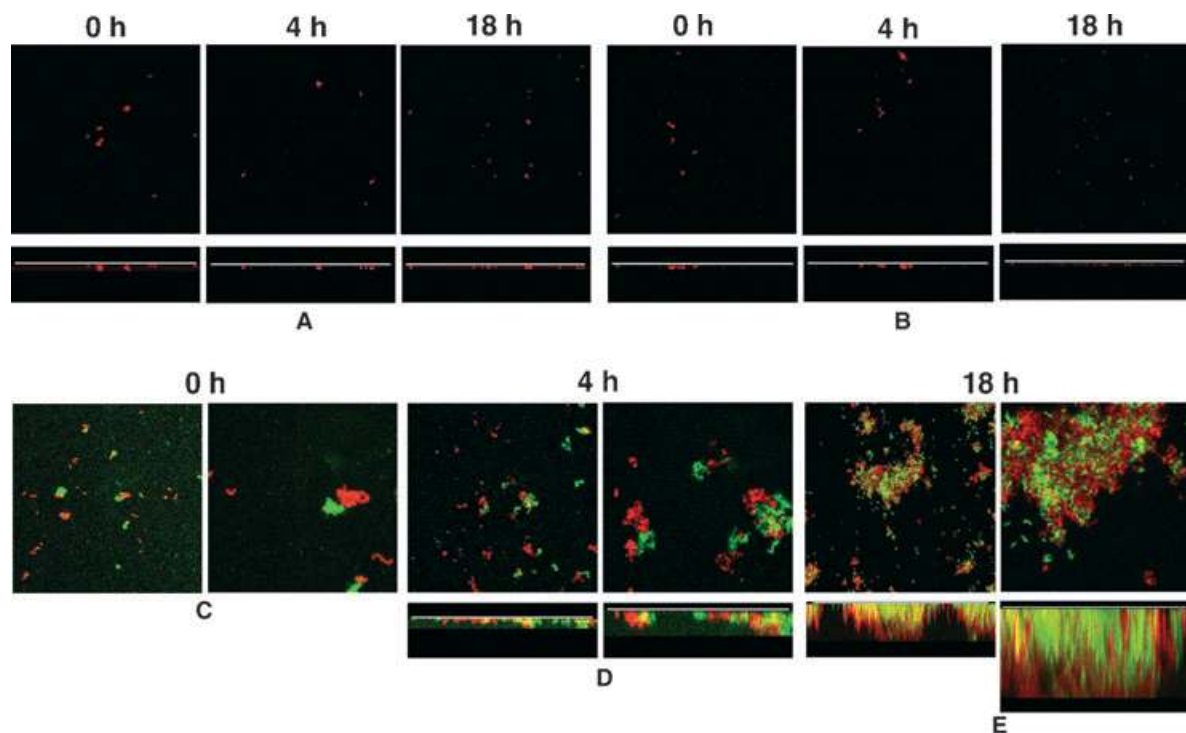
Fig. 20. Time course of biofilm development in coculture of *Actinomyces naeslundii* T14V and *Streptococcus oralis* 34 (90). (A) *A. naeslundii* monoculture control (Syto 59 staining). (B) *S. oralis* monoculture control (Syto 59 staining). (C) Coculture biofilm at 0 h. Inoculation was with *A. naeslundii* (secondary immunofluorescence, green) followed by *S. oralis* (primary immunofluorescence, red). *S. oralis* cells are frequently located in direct proximity to *A. naeslundii* cells. (D) Coculture biofilm after 4 h of saliva flow. Growth of both strains is apparent, especially in mixed-species colonies. Note increased interdigitation of the two cell types within the colonies. (E) Coculture biofilm after 18 h of saliva flow. Marked growth of both strains has occurred. Mixed-species colonies dominate the biomass. In all six subpanels of panels A and B and in the left-hand subpanels of panels C, D, and E, one representative maximum projection image from the set of three randomly selected *x–y* stacks (square panels) and rotation of the maximum projection to display *x–z* perspective (rectangular panels) are shown. Dimensions of the regions displayed are 250 × 250 μm (*x–y* perspectives; square panels) and 250 × 73 μm (*x–z* perspectives; rectangular panels) in panels A and B. The image pairs presented in panels C through E are 250 × 250 μm (*x–y* perspectives; left panel) and 83 × 83 μm (*x–y* perspectives; right panel); the right panel is a 3× zoom of the center portion of the left panel. For the *x–z* perspectives, the dimensions are 250 × 73 μm (left panel) and 83 × 24 μm (right panel) (i.e. the right panel is a 3× zoom of the left panel). One exception is the right *x–z* perspective in panel E, which is 83 × 48 μm.
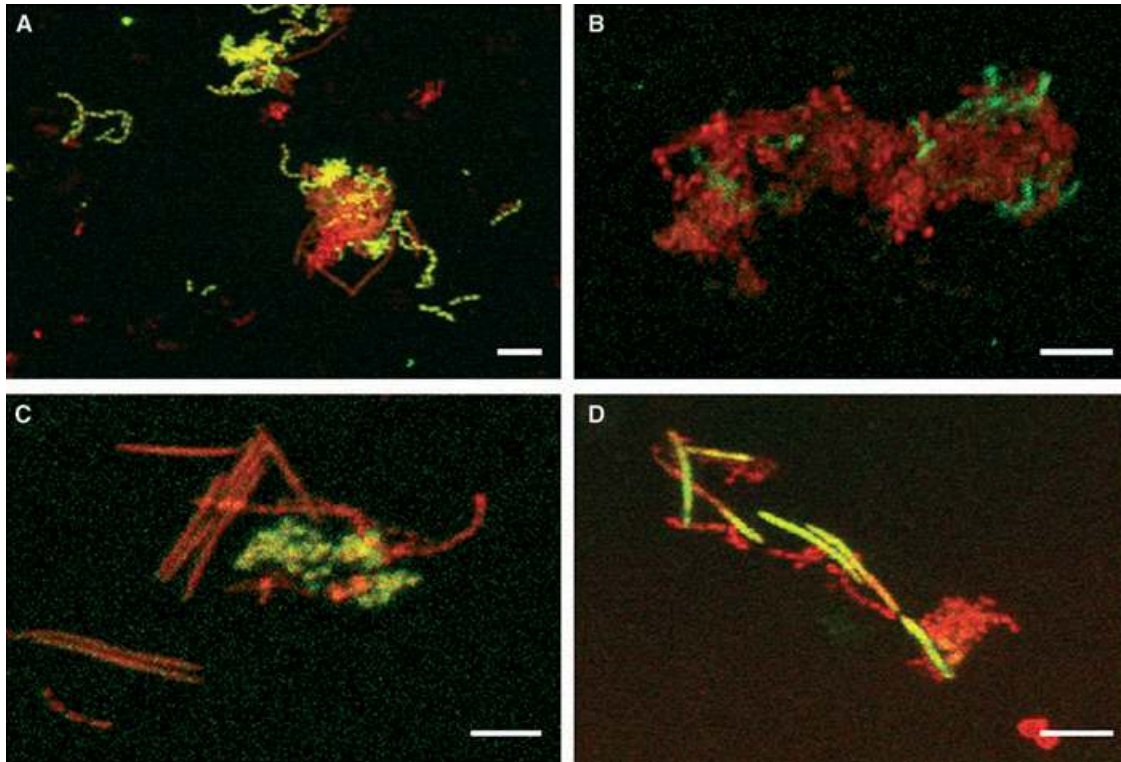
Fig. 21. Confocal micrographs of planktonic cultures containing coaggregates of *Streptococcus gordonii*, *Actinomyces naeslundii*, *Veillonella atypica* and *Fusobacterium nucleatum* processed for fluorescence *in situ* hybridization with fluorescein isothiocyanate-labeled probes (green) and counterstained with general nucleic acid stain Syto 59 (red) (28). Colocalization of both fluorescent markers appears yellow to yellow–green. (A) *Streptococcus*-specific probe targeting *S. gordonii*. (B) Species-specific probe labeling short rod-shaped *A. naeslundii* cells. (C) Clustered *V. atypica* cells hybridized with a *Veillonella*-specific probe. (D) Long slender rod-shaped *F. nucleatum* cells labeled with a *F. nucleatum*-specific probe. Bar, 5 μm.

dominated the other three species. As *in vivo*, the streptococci *in vitro* dominate the initial multispecies biofilm communities. These results support the usefulness of parallel *in vitro* flowcells to study *in vivo* plaque development.

Signaling in dual-species flowcell communities

We found that inter-species signaling in flowcells requires the interacting cell types to be in cell–cell contact in the biofilm (25). *S. gordonii* ferments carbohydrates to form lactic acid. *V. atypica* uses lactic acid as a preferred fermentation substrate, thus completing a metabolic coupling of the two species. When the two species are spotted one on the other on agar containing starch, a zone of hydrolysis was evident that was absent around monoculture spots. The increase in starch hydrolysis was caused by increased expression of the *S. gordonii* alpha-amylase-encoding gene, *amyB*. To monitor signaling between the species, we constructed a transcriptional fusion (*PamyB*–'gfp) of promoterless

gfp [encodes green fluorescent protein (GFP)] under control of the promoter from *amyB*. A plasmid containing *PamyB*–'gfp was transformed into *S. gordonii*, which was cocultured with *V. atypica* in flowcells with saliva as the sole source of nutrient. We used confocal scanning laser microscopy to obtain single-cell resolution and found that only those streptococci in juxtaposition with *V. atypica* expressed GFP: nearby colonies composed solely of *S. gordonii* were not green, indicating that the *amyB* promoter was not activated (Fig. 22). These results suggest that coaggregation is required for signaling events, but when the two species were placed in a closed vessel with one species inside a dialysis bag and physically separated from the other, *S. gordonii* containing the *PamyB*–'gfp reporter plasmid exhibited 20-fold higher fluorescence levels than *S. gordonii* incubated alone. Thus, cell–cell contact is not required in a closed vessel where the signal concentration can build, but it is required in a flowing environment, where the signal is washed out and which is the natural environment in the oral cavity.

Autoinducer-2 as a universal signal mediating mutualism among oral bacteria

In 2001, Schauder et al. (99) proposed that a small molecule called autoinducer-2 was a universal signal mediating messages among the species in mixed-species communities. This idea is distinct from the regulation of gene expression mediated by autoinducer-1, a family of acyl homoserine lactones (3, 30), which regulate gene expression in genetically identical cells. We and others (29, 117) have found no
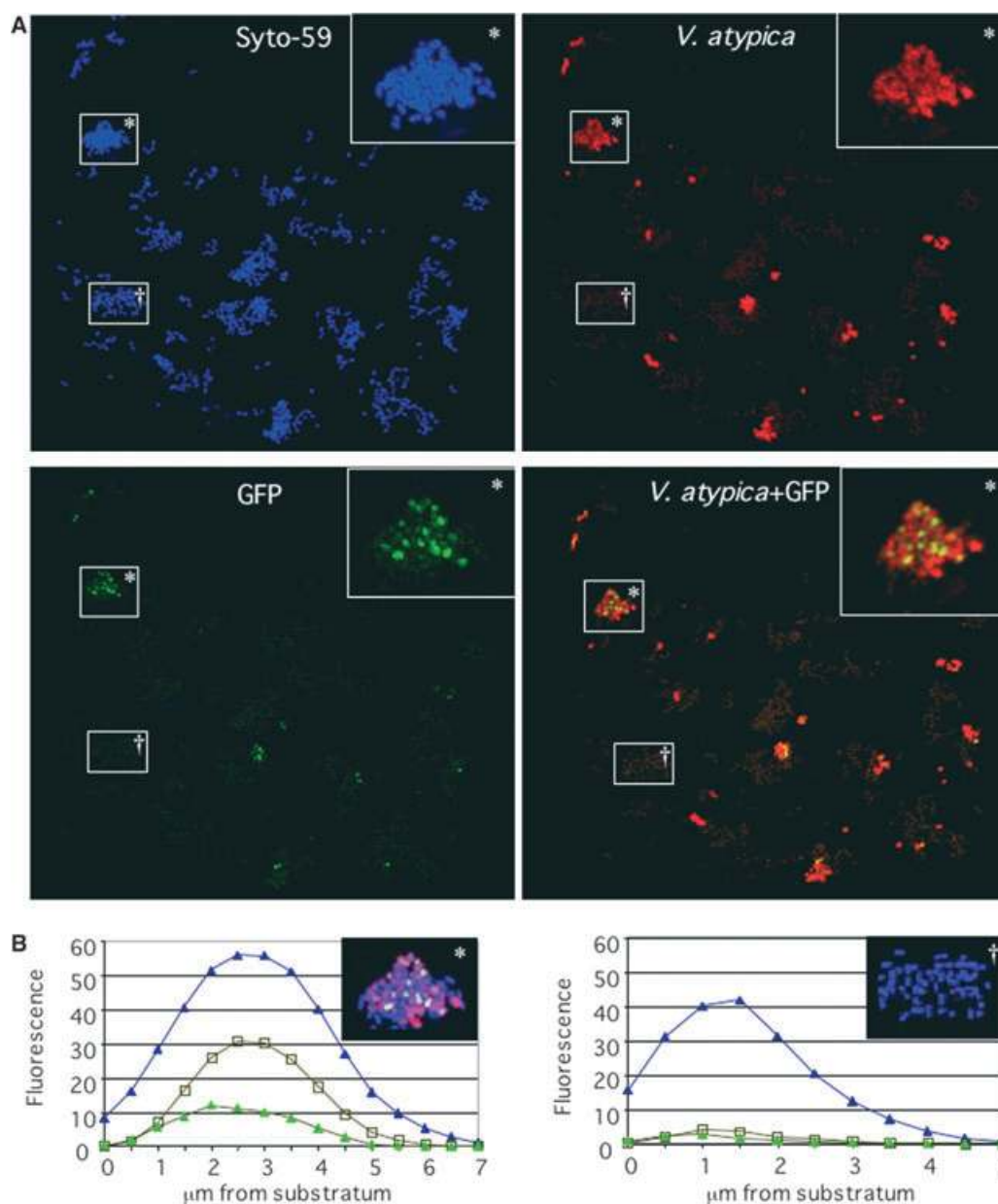
Fig. 22. Confocal scanning laser microscope analysis of dual-species biofilms (25). (A) Maximum projections (all confocal sections in a single field of view) of a single confocal stack showing fluorescence from Syto-59 (blue; all cells; upper left panel), Alexa Fluor 546-conjugated anti-*Veillonella atypica* immunoglobulin (red; upper right panel), and green fluorescent protein (green; *Streptococcus gordonii* expressing *amyB*; lower left panel). The lower right panel shows an overlay of green fluorescent protein with anti-*V. atypica*. (Inset) An enlargement of the boxed microcolony labeled with an asterisk. (B) Graphs of fluorescence intensity vs. depth in a dual-species micro-colony (left) and a monospecies microcolony (right) depicted in the upper right corner of each graph. The dual-species microcolony is the same colony marked with an asterisk in (A) and is shown as an overlay of all three colors. The monospecies microcolony is labeled with a dagger in (A). Microcolonies are shown as maximum projection images. Fluorescence of Syto-59 (blue triangles), Alexa Fluor 546-conjugated anti-*V. atypica* immunoglobulin (open squares), and green fluorescent protein (green triangles) are shown at each 0.5-μm-spaced optical slice of the confocal stack.

evidence of autoinducer-1 in oral bacteria. However, autoinducer-2 has been detected in the cell-free culture supernatants of several oral bacteria (4, 8, 10, 26, 27, 29, 75), suggesting that, indeed, autoinducer-2 might be a signal exchanged in mixed-species communities.

Autoinducer-2 is an umbrella designation that covers a collection of molecules formed from the spontaneous rearrangement of 4,5-dihydroxy-2,3-pentanedione (DPD) (21, 100), which is the product of LuxS. Autoinducer-2 produced by one oral species can be spontaneously rearranged to another form, which is recognized by a cognate receptor on a different species. This has been shown to occur; crystallized receptor–autoinducer-2 complexes from *Salmonella typhimurium* and from *Vibrio harveyi* are interconvertible; they can be released from one receptor, enter the autoinducer-2 equilibrium and bind to the other receptor (76). Given this inter-convertibility of autoinducer-2 and that potentially all oral bacteria produce autoinducer-2, a response to autoinducer-2 signaling by a particular member in mixed-species communities must be highly co-ordinated with the environment, such as pH, atmosphere, and autoinducer-2 concentration. Some isolates may respond to lower concentrations of autoinducer-2 than others. We found that *S. oralis* and *A. naeslundii* responded to picomolar concentrations of autoinducer-2 (97), which are 100-fold lower than the concentration detectable in the bioluminescence assay with *V. harveyi* (100, 108). Frias et al. (29) reported high luminescence induction with cell-free supernatant fluids from *F. nucleatum*, *P. intermedia*, and *P. gingivalis*, but they found low levels of luminescence induction with many commensal species, such as *S. oralis* and *A. naeslundii*. These results support a hypothesis that commensal oral bacteria respond to low levels of autoinducer-2, whereas periodontopathogenic bacteria respond to higher levels of autoinducer-2. A model depicting this hypothesis is presented in Fig. 23. Commensal bacteria send and receive autoinducer-2 signals at picomolar levels. They have optimal levels to which they respond with mutualistic interdigitated growth. Higher levels of auto-inducer-2 reduce commensal bacterial growth. As the diversity of commensal bacteria increases, the availability of coaggregation bridging increases: the 'orange complex' bacteria (104) act as coaggregation bridges for accreting 'red complex' bacteria. In this hypothesis we propose that the 'orange and red complex' bacteria send and receive autoinducer-2 signals at much higher concentrations and grow

rapidly. Importantly, the commensal bacteria are less able to flourish at the higher autoinducer-2 concentrations. Signaling occurs under conditions of salivary flow and crevicular fluid flow, which wash out the signal. Removal of signal keeps the signaling distance restricted and, thus, pathogens form microcommunities instead of globally infecting the site. Continued growth of pathogens maintains high signal levels. Brushing, flossing, and professional debridement remove pathogens, auto-inducer-2 is reduced to picomolar concentrations, and the commensals retake their dominant position in initial dental plaque communities. Thus, commensal life could proceed with autoinducer-2 signaling 'under the radar' of needed high levels of autoinducer-2 for signaling among pathogens. While this is an attractive hypothesis, much work will be required to test it.
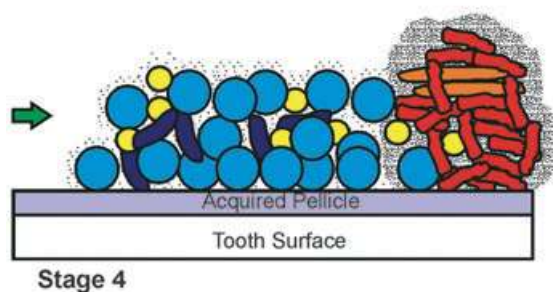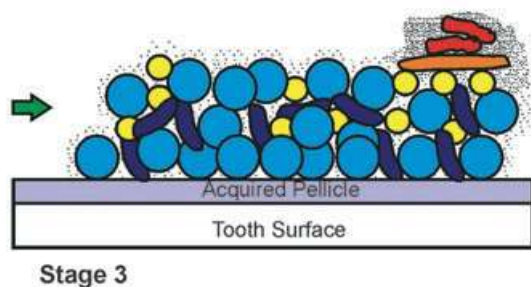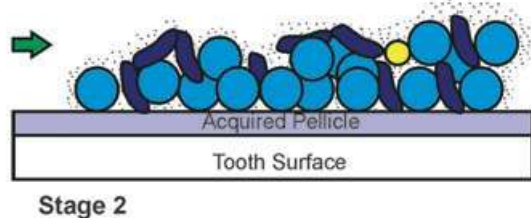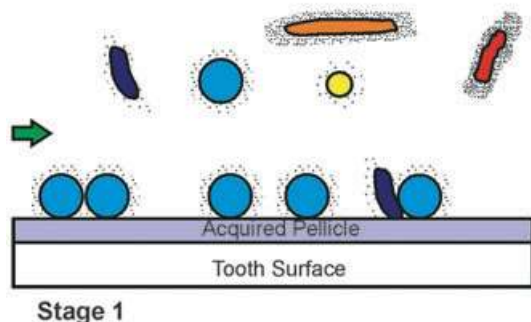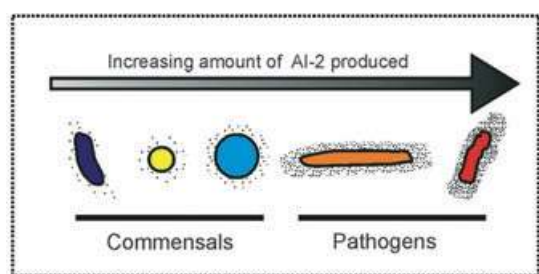
Role of aerotolerance and oxidative stress in plaque development

We propose that the spatial organization seen in plaque is a result of plaque maturation determined by the physical interactions with other cell types and the metabolic requirements of each microorganism. Evidence exists also for the nonrandom organization of bacteria in periodontal pockets. Kigure et al. (44) found that *T. denticola* inhabits the surface layers of subgingival plaque in moderately deep pockets, while *P. gingivalis* is predominantly seen beneath them. In deeper pockets, both bacterial species co-existed. *T. denticola* might be more aerotolerant than *P. gingivalis* and able to proliferate in the outside layer of moderate pockets, while *P. gingivalis* is restricted to the inner layer. In deeper pockets, where the oxidation–reduction potential is lower, both bacteria can flourish in all layers. Although this is just a hypothetical explanation, the aerotolerance of different bacterial species is likely to play a major role in plaque development. The late colonizing gram-negative species that have been associated with the development of disease are all anaerobes. It is therefore important to understand how these microorganisms survive unfavorable environmental conditions and establish in mature plaque.

Environmental modification by bacterial consortia

Initially, plaque is dominated by species (such as neisseriae, haemophilae and streptococci) that are

capable of rapid cell division under high oxidation–reduction potentials. Organisms with anaerobic requirements are also present, but are less numerous.

The ability of anaerobes to survive oxidative stress is referred to as aerotolerance, although it does not imply that cell replication can occur under aerated conditions. Some anaerobes, such as *P. gingivalis*, are able to survive under ambient atmosphere for several hours until the environment again becomes anaerobic and cell division and growth are re-initiated (17). The survival of anaerobes in dental plaque is thought to be the result of interactions with other members of the dental plaque community. *In vitro* studies demonstrate that the growth of obligate anaerobic periodontopathogens in a mixed population containing facultative anaerobes may allow the anaerobes to survive in environments containing oxygen (6, 7). During initial biofilm formation, it is likely that close cell contact with organisms able to metabolize oxygen facilitates the survival of anaerobes. We have observed close association of the anaerobic *Prevotella*

Fig. 23. A diagrammatic representation of biofilm formation on the tooth surface and the potential roles of coaggregation, bridging, and autoinducer-2 in the transition from health to disease. Stages show the colonization and growth of commensal organisms (Stages 1 and 2) and the integration and invasion of pathogenic species (Stages 3 and 4). Different colors represent different species. Yellow, light blue and dark blue cells are commensal species. Orange and red cells are pathogenic species. Relative amounts of autoinducer-2 are shown as sparsely placed dots (low level of autoinducer-2 produced) around commensals vs. densely packed dots (high level of autoinducer-2 produced) around pathogens (see the box at the top). A clean tooth surface, with the associated acquired pellicle, is colonized by commensal species (Stage 1). The small green arrow indicates that these oral communities form and flourish under conditions of flow of saliva or crevicular fluid. Coaggregation interactions contribute to colonization. The commensal cells produce picomolar amounts of autoinducer-2, and this fosters mutualistic interdigitated growth. The interdigitated commensal community expands rapidly through autoinducer-2-mediated mutualistic growth (Stage 2). As commensal cell numbers and diversity increase, the likelihood of pathogens integrating into the biofilm increases; integration can be through coaggregation interactions with commensals (orange cells with yellow cells) or through a 'bridging' species (red cells coaggregating with orange cells that coaggregate with yellow cells) (Stage 3). The pathogens produce higher concentrations of autoinducer-2 than the commensals, and this high concentration retards mutualistic growth between the commensal species. With growth being impaired by high autoinducer-2 concentrations (Stage 4), the commensal species are unable to compete with the invading, rapidly multiplying, pathogens. The community composition shifts from that of predominantly commensals to include a community of mostly pathogens.
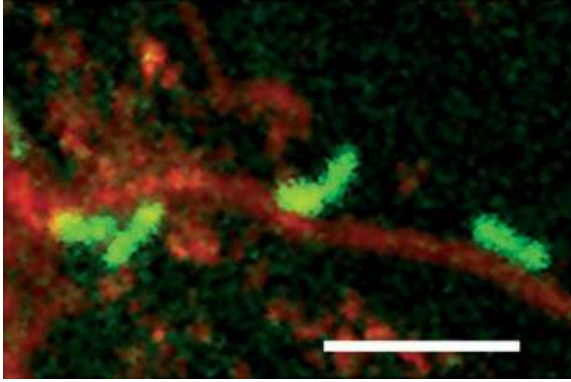
Fig. 24. Fluorescence *in situ* hybridization of cells of *Prevotella* spp. in close association with other bacteria found on enamel chips at 8 h of plaque development. Confocal micrograph of cells simultaneously labeled with the *Prevotella*-specific PRV392 probe (green) and eubacterial specific EUB338 probe (red). *Prevotella* cells are adherent to the surface of unidentified bacteria with rod-like morphology. Scale bar, 5 μm.

spp. with other bacterial cell types in 8-h biofilms (Fig. 24). This close proximity might allow the establishment of a reduced microcommunity in which species, such as neisseriae, remove oxygen. It is also likely that the aerotolerance of anaerobes varies from species to species, a characteristic that is proposed to impact bacterial succession. As plaque matures, the increased cell density lowers the oxidation reduction potential and some of the more aerotolerant anaerobic species increase in number in the biofilm. One of these organisms is *F. nucleatum*, which has been demonstrated to be more aerotolerant than *P. gingivalis* (16), and hence one of the reasons for its predominance in moderately aged supra-gingival plaque. In later stages of plaque development, and near the gingival margins, the environmental conditions might allow less aerotolerant anaerobic organisms, such as *P. gingivalis* and *T. denticola*, to increase in number.

Anti-oxidant defenses of anaerobes

Partnership with other species might be part of the process that facilitates the survival of anaerobes in plaque. However, it is likely that there are many situations in which microorganisms are not protected by the overall oxygen metabolism of the microbial community; for example, passage through oral fluids or mechanical disruption of dental plaque. In such situations, the survival of anaerobes will depend more upon defenses against oxidative stress, and these defenses are specific for each species. Oxidative stress response mechanisms could also be important

during the invasion of host tissues, as a defense against oxidative-mediated killing of neutrophils and macrophages and for survival in blood during bacteremias. In any of these roles, anti-oxidant defense systems are likely to contribute to the virulence of anaerobic microorganisms. The oxidative stress defenses of anaerobes have not been widely studied. In contrast to the vast knowledge on anti-oxidant mechanisms operating in aerobes or facultative species, the mechanisms by which anaerobes tolerate oxygen are poorly understood. It seems that anaerobes have common anti-oxidant defenses with aerobes but these defenses are not expressed at high levels in anaerobes or they are not all present simultaneously in the same species. Moreover, the metabolism of anaerobes relies on metabolic schemes built around enzymes that react easily with oxygen. For example, the dependence upon low-potential flavoproteins for anaerobic respiration probably causes substantial superoxide and hydrogen peroxide to be produced when anaerobes are exposed to air (39). The exposure of enzymes from the central metabolism of anaerobes to oxidants, and their subsequent inactivation, seems to be the common element of anaerobiosis. Organisms like *T. denticola* and *F. nucleatum* have been shown to possess enzymes, such as NADH oxidase, which are involved in oxygen detoxification and in maintaining the redox balance inside cells (9, 15). NADH oxidases are common in a wide range of microorganisms and are thought of as an adaptation by which microorganisms lacking a cytochrome-mediated reduction of oxygen are able to contend with, or take advantage of, oxygen in their environments (105). However, if we compare the NADH oxidase levels present in *S. mutans*, a facultative organism (33), with those present in *F. nucleatum* (15), cell extracts of *S. mutans* have 1000-fold more enzymatic activity. Similar comparisons are valid for other anti-oxidant enzymes, such as superoxide dismutase (SOD). The levels of SOD activity in facultative organisms such as *H. influenza* or *E. corrodens* are three- to seven-fold higher than the levels of SOD enzymatic activity found in *P. gingivalis* (2). Nevertheless, these 'low' levels of SOD are important for the relative aerotolerance of *P. gingivalis* (70).

We have recently demonstrated that *P. gingivalis* also possesses an OxyR protein (18), homologous to that present in many facultative and aerobic microorganisms (105). OxyR acts as a regulator of many oxidative stress-related genes, in response to hydrogen peroxide, in organisms such as *Escherichia coli* (120). In *P. gingivalis*, OxyR does not seem to respond

to hydrogen peroxide and its role in anti-oxidant defense requires further clarification. We identified, however, some anti-oxidant-relevant genes whose transcription depends on OxyR. These genes included the genes for SOD, Dps (a nonspecific, protective, DNA-binding protein), alkylhydroperoxide reductase subunits F and C, PG0421 (a hypothetical protein) and ferritin (17). A report by Sztukowska et al. (109) also identified rubrerythrin as an important mechanism for the H_2O_2 resistance of *P. gingivalis*. It seems then that although anaerobes, such as *P. gingivalis*, cannot survive highly oxygenated conditions, their own array of anti-oxidant defenses, plus the interaction with neighboring facultative or aerobic bacterial species, allows them to adapt to oral biofilms.

Microarrays as tools to detect communication among mixed species

Among the 308 bacterial genomes sequenced in the last decade, 15 genomes, representing 13 distinct species, are from human oral microorganisms. This surge in sequence data has led to the development of microarrays to monitor gene expression on a whole-genome scale. Five microarrays, containing complete gene sets of *P. gingivalis*, *S. mutans*, *A. actinomycetemcomitans*, *T. denticola* and *F. nucleatum*, are currently available by request from the NIDCR Oral Microbial Microarray Initiative (NOMMI; http://www.nidcr.nih.gov/Research/Extramural/NIDCR_TIGR_Facility.htm).

Microarrays are useful for investigating bacterial cell interactions with host proteins and host cells. The attachment of *P. gingivalis* to HEp-2 epithelial cells resulted in increased expression of over 30 bacterial genes that are primarily involved in protection against oxidative stress and protein stabilization (34). These responses suggest that *P. gingivalis* cells experience oxidative stress when bound to epithelial cells and respond accordingly. Microarray studies have great potential for identifying key genes that are switched on and off during the different stages of bacterial interactions, such as coaggregation, attachment to surfaces, colonization of the surface, and the establishment of multi-species commensal or pathogenic communities. Genes of interest can then be further analyzed by classical genetic approaches such as mutagenesis, functional complementation and promoter reporter constructs,

to determine their roles in the development of mixed-species oral microbial communities.

The incentive to use microarrays to study changes in gene expression elicited by inter-species interactions is evident. For example, *A. actinomycetemcomitans* autoinducer 2, the product of the *luxS* gene, complemented a *luxS*-deficient strain of *P. gingivalis* to modulate the expression of *uvrB* and *hasF* (27). Moreover, signaling between two early colonizers of the oral cavity, *S. gordonii* and *V. atypica*, occurs when cells are juxtaposed in oral biofilms and results in the up-regulation of *S. gordonii* amylase expression (25). It is possible to use microarrays to analyze gene expression in bacteria in co-cultures, provided that the partners in the interaction differ sufficiently in their DNA sequences so that extensive cross-hybridization is avoided. The exact amount of DNA sequence divergence that is acceptable for such studies will depend on the design of the microarray chip, as well as on the hybridization temperature. Cross-hybridization can be further reduced by using cell lysis protocols that are specific for one partner in the interaction (72). We have recently employed microarrays to compare gene expression in *S. gordonii* monocultures with cells in coaggregation with *A. naeslundii* using an *S. gordonii* microarray constructed by M.M. Vickerman and S.R. Gill (University at Buffalo, New York, NY). In control experiments, *A. naeslundii* RNA did not hybridize to the *S. gordonii* microarray chips. Eight *S. gordonii* genes were up-regulated in coaggregates, and thirteen genes were significantly down-regulated, including nine genes involved in arginine biosynthesis (N.S. Jakubovics and P.E. Kolenbrander, unpublished data). We hypothesize that *S. gordonii* can circumvent the energy input required for arginine biosynthesis by acquiring arginine from *A. naeslundii* cells, and thus obtain a competitive advantage. At present, microarray studies using coaggregates are in their infancy. However, with the recent release of several oral bacterial microarrays, we anticipate a dramatic increase in such investigations in the coming years. These experiments will undoubtedly shed new light on the microbial factors that control interbacterial interactions and establishment of complex biofilm communities on oral surfaces.

Summary

Oral populations shift from the low diversity found in initial communities of supragingival plaque to the

high diversity present in mature communities in supragingival and subgingival plaque. Physical interactions among bacterial species, attachment to surfaces, and successive colonizations to form communities, are processes facilitated by coaggregation. Metabolic interactions among different bacterial cell types are likely to play a decisive role in the changes in community composition during plaque maturation. Co-operation among community members might facilitate nutrient availability and oxygen removal, favoring the proliferation of specific bacterial species. Diffusible small-molecule signals mediate communication among members of the same microcommunity: we propose that the concentration of these signals in the microcommunity is critical for the gene-regulation response by commensal and pathogenic organisms. Bacterial communities co-evolve with their human host and thus are host specific. Each human host has developed an interrelationship with a specific set of bacterial species. The understanding of the factors that determine plaque maturation in that host is important, because the population shifts from predominantly gram-positive bacteria to obligately anaerobic gram-negative species. It remains unsolved as to how these shifts correlate with the appearance of gingivitis and, in the susceptible host, with the development of a periodontal lesion. Additional studies, involving natural mixed-species communities, confocal microscopy, signaling molecules, metabolic co-operation and antagonism, and molecular phylogenic analyses of culturable and yet-to-be cultured bacteria, are increasing our understanding of the biological and clinical processes involved in the development of plaque.

Acknowledgments

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