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Human Oral Cavity as a Model for the Study of Genome-Genome Interactions

JAMIE S. FOSTER, ROBERT J. PALMER, JR., AND PAUL E. KOLENBRANDER*

Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Building 30, Room 310, 30 Convent Drive MSC 4350, Bethesda, Maryland 20892-4350

The enormous diversity of culturable bacteria within the oral microbial community coupled with experimental accessibility renders the human oral cavity a valuable model to investigate genome-genome interactions. The complex interactions of oral bacteria result in the formation of biofilms on the surfaces of the oral cavity. One mechanism thought to be important in biofilm formation is the coaggregation of bacterial partners. In this paper, we examine the role of coaggregation in oral biofilms and develop protocols to elucidate the spatial organization of bacterial species retained within oral biofilms. To explore these issues, we have employed two experimental systems: the saliva-coated flowcell and the retrievable enamel chip. From flowcell studies, we have determined that coaggregation can greatly influence the ability of an oral bacterial species to grow and be retained within the developing biofilm. To examine the spatial architecture of oral biofilms, fluorescent in situ *hybridization protocols were developed that successfully target specific members of the oral microbial community. Together, these approaches provide insight into the development of oral biofilms and expand our understanding of genome-genome interactions.*

The human oral cavity contains more than 500 species of bacteria that interact among themselves and with their host tissues (Kroes *et al.,* 1999; Paster *et al.,* 2001). These complex interspecies associations result in the formation of microbial biofilms on the hard and soft tissues of the oral cavity (Gibbons and Hay, 1988; Hallberg *et al.,* 1998). Within these oral biofilms, numerous molecular and biochemical exchanges result in communication between distinct genomes (here called genome-genome interactions). To explore the nature of genome-genome interactions in the oral cavity, it is necessary to understand the composition of the microbial community, the mechanisms by which oral bacteria associate, and the spatial arrangement of the community. Until recently, description of plaque community composition relied on culture-dependent techniques (Moore and Moore, 1994; Socransky and Haffajee, 1994). Culture-independent methods have identified previously unknown and uncultured community members. These uncultured oral bacteria constitute a low percentage of the total bacterial numbers compared to the high percentage of uncultured bacteria in other natural environments (Kroes *et al.,* 1999; Paster *et al.,* 2001). Although these studies provide information on species composition, they do not address the spatial organization of the oral community. The juxtaposition of different bacteria in three-dimensional oral biofilms such as dental plaque probably contributes to and may direct metabolic cross-feeding symbioses and transcriptional signal exchange between organisms. Examining the precise architecture of oral biofilms may provide a clearer understanding of the role each organism plays in the overall community structure and in genome-genome interactions.

One mechanism through which oral bacteria may communicate and facilitate genome-genome interactions is coaggregation. In coaggregation, oral bacterial cells bind to specific bacterial partners (Cisar *et al.,* 1979). To date, all oral bacteria tested coaggregate with at least one other bacterial species (Whittaker *et al.,* 1996; Andersen *et al.,* 1998; Kolenbrander *et al.,* 2002). Coaggregation occurs

* To whom correspondence should be addressed. E-mail: pkolenbrander@dir.nidcr.nih.gov

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between genetically distinct bacteria and is mediated by protein adhesins on one cell type that recognize complementary carbohydrate receptors on the partner cell type. This phenomenon has been hypothesized to be essential to the formation of oral biofilms (Kolenbrander *et al.,* 2002), although little direct work has been performed on the consequences of coaggregation interactions. Several outcomes of these pairwise interactions are conceivable: (a) only one organism benefits, (b) one organism is detrimentally influenced, (c) both organisms benefit, (d) both organisms suffer, and (e) neither organism is influenced by the presence of the other. Although these scenarios are an oversimplification of what can occur in multispecies environments such as the oral cavity, they serve as starting points for assessing the consequences of coaggregation *in vivo.* Furthermore, it is becoming clear that coaggregation interactions exist outside the oral cavity in freshwater biofilms (Rickard *et al.,* 2000, 2002). Therefore, the significance of contact-based cell-cell interactions in the bacterial world has probably been underestimated, and the outcomes of these genome-genome interactions are likely to be a universal driving force in biofilm development.

Several experimental designs have been developed to examine the formation of oral biofilms (Wilson, 1999; Wimpenny, 2000). These model systems often rely on the flow of nutrients over a surface on which bacteria are able to attach and grow. In our laboratory we use two experimental models, a saliva-coated flowcell (Kolenbrander *et al.,* 1999) and a retrievable enamel chip (Palmer *et al.,* 2001a). Each method has its own advantages for the examination of oral microbial communities. The flowcell enables biofilms to form under defined conditions of species and nutrients. The basic design of a flowcell is a microscope slide and coverslip separated by a two-channel molded silicone gasket (Kolenbrander *et al.,* 1999). Inlet and outlet ports enable saliva, the sole nutrient source, to coat the glass surfaces with a salivary conditioning film of host proteins. After the salivary conditioning film is established, bacterial strains are injected into the flowcell chamber. As the biofilm forms within the flowcell, colonization and growth can be examined noninvasively by confocal laser microscopy (CLM). In comparison, the enamel chip facilitates the understanding of natural biofilms that form within the human oral cavity. Enamel chips cut from human third molars are placed into two acrylic appliances worn intraorally by volunteers. The chips are then recovered and examined using microscopy (Palmer *et al.,* 2001a).

Initial studies on the outcomes of coaggregation interactions have been conducted in the flowcell model with three primary colonizers of the tooth surface: *Streptococcus gordonii* DL1, *Actinomyces naeslundii* T14V, and *Streptococcus oralis* 34 (Palmer *et al.,* 2001b). Each bacterium can coaggregate with the other two. The behavior of the strains as monocultures was assessed by examining their abilities to grow planktonically (as liquid cultures) in saliva, and to grow as biofilms in saliva. In planktonic culture, *S. gordonii* reproducibly reached a cell density of 10^7 cells per milliliter of saliva and was transferable (*i.e.,* growth was maintained over three transfers). *A. naeslundii* numbers consistently tapered off within 18 h after the initial transfer to saliva. *S. oralis* behaved inconsistently: growth occurred, but the maximum cell density varied between 10^5 and 10^6 cells per milliliter of saliva, and cultures were not always transferable. These behaviors were duplicated in the flowcell system: monoculture biofilms of *S. gordonii* grew reproducibly, those of *A. naeslundii* never grew, and those of *S. oralis* grew only once in six experiments.

Once behavior as monocultures was assessed, the outcome of pairwise interactions between the strains in biofilms was investigated (Palmer *et al.,* 2001b). The first strain was inoculated into the flowcell and allowed to adhere for 20 min; nonadherent cells were then washed out and the second strain was introduced and allowed to adhere for 20 min. After the subsequent washout of nonadherent cells, the coculture biofilm was examined immediately (time 0), after 4 h, and after overnight growth with flowing saliva. When the initial strain was *S. gordonii,* combination with either of the other two strains produced identical results: *S. gordonii* grew as it did in monoculture, and the partner strain (*A. naeslundii* or *S. oralis*) failed to grow (Fig. 1A). Cells of the partner strain were retained within the *S. gordonii* biofilm, but biomass of either partner strain was clearly reduced over the course of the experiment. Thus, the growth of *S. gordonii* was apparently unaffected by the presence of *A. naeslundii* or *S. oralis.* In marked contrast to these interactions, when *S. oralis* and *A. naeslundii* were combined in a biofilm, both bacteria grew luxuriantly (Fig. 1B). Growth as a coculture biofilm of these two organisms (neither of which could grow reproducibly as a monoculture biofilm) was much greater than that of *S. gordonii,* which grew independently under identical culture conditions. This is a clear example of a mutualism in which both organisms benefit from the interaction. Such interactions may be important in establishment of regional heterogeneity in oral biofilms *in vivo,* and we are currently using the retrievable enamel chip system to relate the results of our *in vitro* investigations to the situation *in vivo.*

The study of bacterial symbiotic interactions described above was conducted by using antibodies to identify the organisms and give spatial information on the oral community. To complement these studies, we have begun to employ fluorescence *in situ* hybridization (FISH) coupled with the flowcell and enamel chip models. The advantages of using FISH are that uncultured bacteria can be detected and that development of the probes is more rapid than production and characterization of antibodies. Fluorescently labeled oligonucleotide probes designed to the 16S rRNA sequence of different oral bacteria were hybridized *in situ*

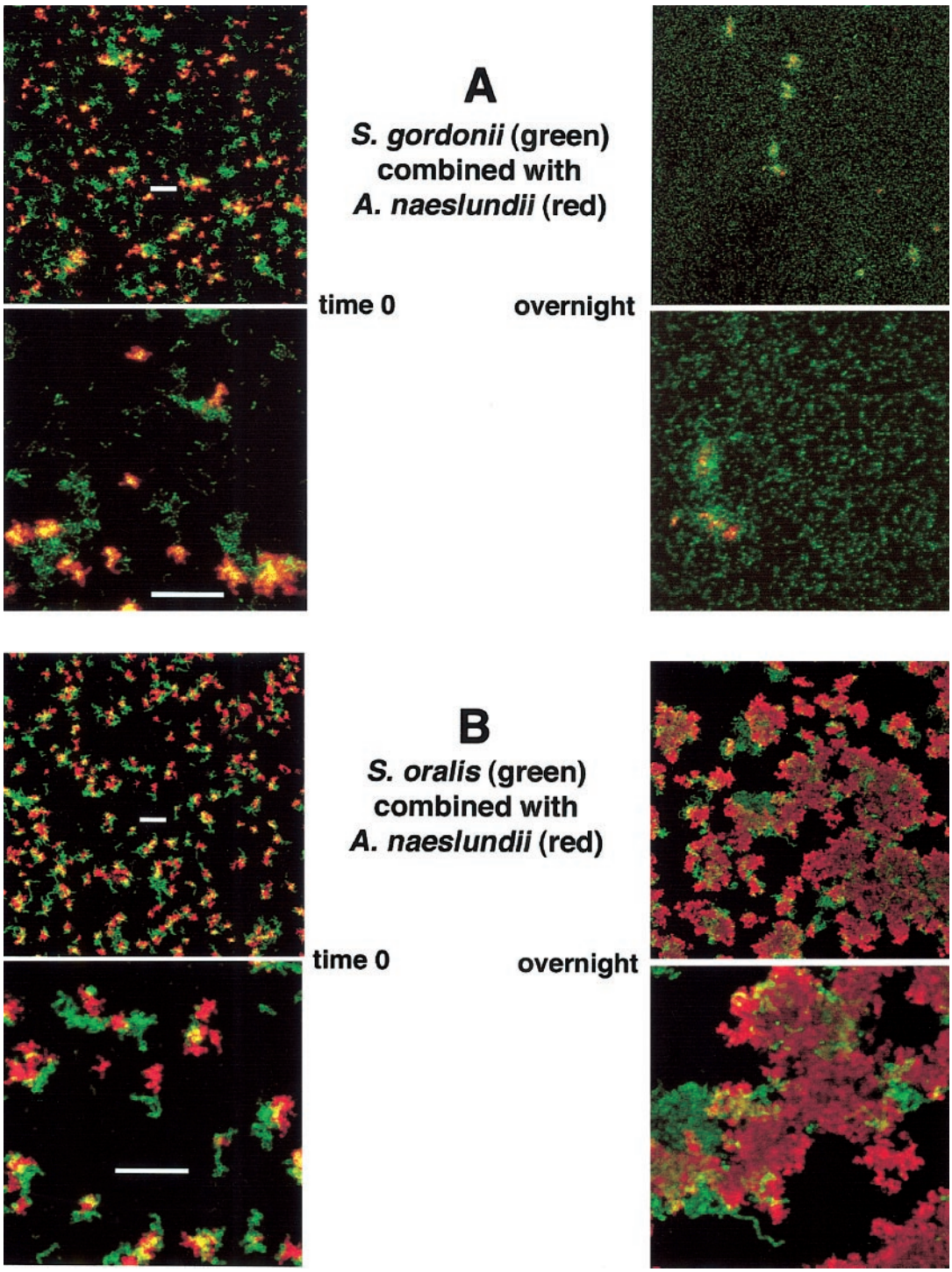

Figure 1. Coculture biofilms showing multiple outcomes of coaggregation. (A) *Streptococcus gordonii* interaction with *Actinomyces naeslundii*. *S. gordonii* was introduced first, followed by *A. naeslundii*. Biofilms were examined with confocal microscopy at time 0 (immediately after washout of nonadherent cells, left panels) and after overnight growth on saliva (right panels). Lower panels of each vertical pair are 3× zooms of the center of the corresponding upper panels. *S. gordonii* (green) was detected by constitutive green fluorescent protein fluorescence; *A. naeslundii* (red) was detected by secondary immunofluorescence. (B) *Streptococcus oralis* interaction with *A. naeslundii*. Details as above, except that *S. oralis* (green) was detected by primary immunofluorescence. All scales, 25 μm.

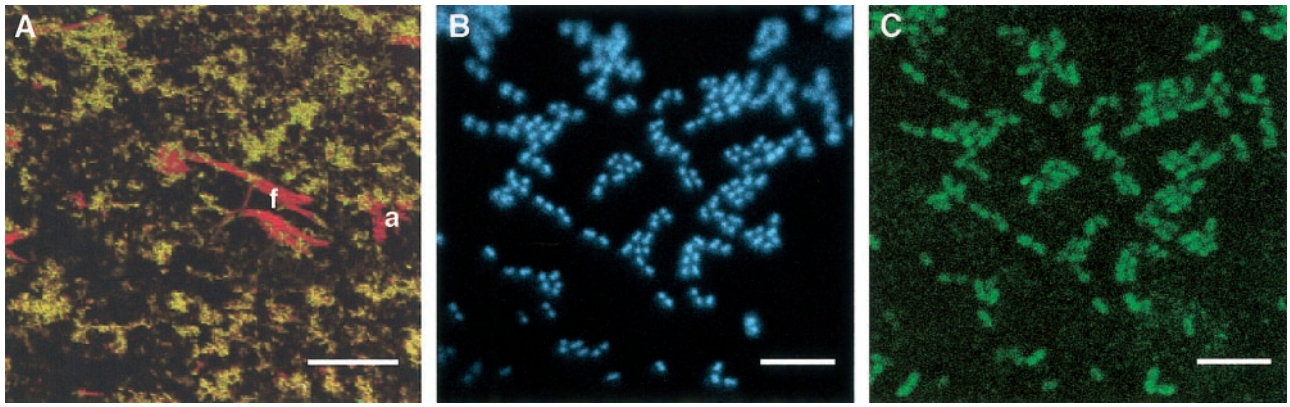

Figure 2. Confocal micrographs of oral biofilms examined with fluorescence *in situ* hybridization. (A) Mixed-species biofilm containing *Streptococcus gordonii* DL1, *Actinomyces naeslundii* PK19(a), and *Fusobacterium nucleatum* PK1594(f) grown on saliva in a flowcell for 4 h. The biofilm was stained with an FITC-labeled oligonucleotide probe (green) targeted to *S. gordonii* and counterstained with the nucleic acid stain Syto 59 (red). Colocalized stains are yellow. Scale, 25 μm. (B, C) Monospecies biofilms inoculated with *S. gordonii* DL1 and grown on enamel chips for 4 h. Biofilms were stained with an oligonucleotide probe specific for *S. gordonii* and the nucleic acid stain DAPI. Scale, 10 μm. (B) High-magnification image of enamel chip showing total number of cells visualized by DAPI stain. (C) Same location on enamel chip as in B, but only detecting cells labeled with oligonucleotide probe.

with the growing biofilm, thus enabling bacterial species to be located without biofilm disruption (Fig. 2). Flowcells were consecutively inoculated with cultures of *S. gordonii* DL1, *A. naeslundii* PK19 (each an early colonizer), and *Fusobacterium nucleatum* PK1594 (late colonizer). After 4 h of saliva flow, biofilms were probed with a fluorescently labeled oligonucleotide designed to target streptococci (5′-GCTGCCTCCCGTAGGAGT-3′; JF20) as well as with a general nucleic acid stain to detect all cells (Fig. 2A). Based on distinctive morphologies of *S. gordonii* (coccus shaped), *A. naeslundii* (rod shaped), and *F. nucleatum* (slender rods with tapered ends), all cell types could be visualized within the biofilm by staining with the nucleic acid marker Syto 59 (Molecular Probes, Eugene, OR). However, labeling by the fluorescent oligonucleotide probe was visible only in *S. gordonii* cells; the other two organisms did not bind the streptococcal probe (JF20) (Fig. 2A). RNA levels *in situ* are frequently very low, and therefore detection can be problematic. To test the detection efficiency of FISH probes compared with general nucleic acid stains, monospecies biofilms containing *S. gordonii* were grown in saliva *in vitro* on enamel chip surfaces and exposed to a streptococcal-specific probe (Fig. 2B, C). The biofilms were also stained with the nucleic acid stain diamidino-2-phenylindole dihydrochloride (DAPI) to fluorescently mark all cells within the biofilm. All of the *S. gordonii* cells could be stained with the oligonucleotide probe without high background fluorescence (Fig. 2C). Taken together, these results suggest that our FISH protocols can be employed in conjunction with the flowcell and enamel chip systems.

This work represents just a few of the approaches used to study oral bacterial interactions. Despite the complexity and diversity of organisms present within the human oral cavity, experimental model systems such as these can be used to explore the consequences of the interactions between distinct genomes and to elucidate common underlying mechanisms of communication within microbial biofilms.

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