INFECTION AND IMMUNITY, June 1983, p. 1178–1185
0019-9567/83/061178-08$02.00/0

Vol. 40, No. 3

Coaggregation of Human Oral *Cytophaga* Species and *Actinomyces israelii*

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Received 21 December 1982/Accepted 11 March 1983

A total of 19 strains of oral *Cytophaga* sp. obtained from subgingival plaque deposits were tested for their ability to coaggregate with strains of *Actinomyces israelii, A. viscosus, A. naeslundii, Streptococcus sanguis, S. mutans, S. salivarius*, and *S. mitis*. Coaggregation was observed only with *A. israelii*. Based on their coaggregation patterns with eight *A. israelii* strains, the *Cytophaga* strains were distributed among three distinct groups: those that coaggregated with *A. israelii* PK16 but not with *A. israelii* W1011 (ATCC 29322), those that coaggregated with *A. israelii* ATCC 29322 but not with *A. israelii* PK16, and those that coaggregated with none of the eight *A. israelii* strains. In each of the coaggregations, prior heat treatment (85°C, 30 min) of the *Cytophaga* cells prevented coaggregation, whereas identical treatment of the *A. israelii* cells had no effect. The ability of *A. israelii* PK16 to form adherent plaque on a tooth surface previously coated with *Cytophaga* plaque was tested with one of the coaggregating *Cytophaga* strains. White patches of *A. israelii* plaque were found covering both the amber-colored *Cytophaga* plaque on the cementum surface as well as the enamel surface to which *Cytophaga* strains do not adhere. Electron micrographs of thin-sectioned mixed-plaque material revealed both cell types in close proximity. In addition, electron micrographs of negatively stained coaggregated cells showed interbacterial adherence between surface fimbrae on *A. israelii* and outer membrane blebs on the gram-negative *Cytophaga* sp. The kinetics of binding of *A. israelii* to spheroidal hydroxyapatite and to root powder were indicative of a high-affinity binding system with comparatively large numbers of available binding sites on both substrata. These results indicate the highly specific nature of *Cytophaga* sp.—*A. israelii* recognition. The contribution of such recognition toward the mechanisms that are responsible for the indigenous nature of these oral bacteria is discussed.

The property of coaggregation between cells of different genera is widespread among oral bacteria. In a recent review, cell-to-cell interactions were reported between members of different gram-positive genera (e.g., *Streptococcus* and *Actinomyces*), gram-positive and gram-negative genera (e.g., *Actinomyces* and *Capnocytophaga*), and different gram-negative genera (e.g., *Bacteroides* and *Fusobacterium*) (3). Although many bacteria participate in coaggregation, a great deal of specificity is apparent in the choice of coaggregating cell types. For example, human strains of *Actinomyces viscosus* and *A. naeslundii* exhibit lactose-inhibitable coaggregation with *Streptococcus sanguis* but show no interaction with *S. salivarius* or *S. mutans* (4, 9); *Propionibacterium acnes* coaggregates with members of a single coaggregation group of *S. sanguis* but not with members of other *S. sanguis* coaggregation groups (9), *A. viscosus*, or *A. naeslundii* (P. E. Kolenbrander, unpublished observations).

Bacterial cell surface structures involved in the coaggregation process potentially may serve as ecological determinants in the formation of plaque on tooth surfaces. Electron micrographs of the multilayered consortia at the plaque-tooth interface (11) reveal cell arrangements consistent with the interpretation that microbial plaque results from growth of an initial microcolony, attachment, by cell-to-cell recognition, of other bacteria to those in adherent microcolonies, and growth of newly attached cell types. Of course, other possibilities exist (e.g., microbial entrapment and columnar growth of adjacent microcolonies); therefore, further studies of cell-to-cell recognition are essential to understanding plaque formation in vivo.

A new group of filamentous gram-negative gliding bacteria that preferentially adhere to the

cementum surfaces of teeth has recently been described (2). Tentative characterization of this group places its members in the genus *Cytophaga* (1, 12). These bacteria exhibit a shift from fermentative to respirational metabolism when switched from anaerobic to aerobic growth (12). However, they do not require a carbon dioxide-supplemented atmosphere during anaerobic growth, a property of *Capnocytophaga* species (10, 16). Since the oral *Cytophaga* strains exhibited the ability to form micro- and macrocolonies on cementum surfaces, it was of interest to determine if they were also capable of cell-to-cell interaction with other bacteria found in human plaque samples. Information obtained from these studies may provide new insights into the understanding of the ecological implications of preferential adherence to cementum.

MATERIALS AND METHODS

Bacterial strains. The 19 human oral *Cytophaga* strains used in this study were isolated from subgingival plaque by procedures described previously (2). All *A. israelii* strains were of human origin. B. Williams (University of Washington, Seattle) kindly supplied strains CROB2030, CROB2051, CROB2052, ATCC 23860, and ATCC 12103. Strain X522 (ATCC 10048) was from S. L. Bragg (Centers for Disease Control, Atlanta, Ga.), and strains W1011 (ATCC 29322) and WVU307 were from M. A. Gerencser (West Virginia University Medical Center, Morgantown). Strain PK16 was derived from WVU307 by adaptation to facultatively anaerobic growth through repeated transfer and static culture incubation in an aerobic atmosphere. PK16 retained the *A. israelii* serotype 2 expression of WVU307. All other bacterial strains of *A. viscosus*, *A. naeslundii*, *S. sanguis*, *S. mitis*, *S. mutans*, and *S. salivarius* have been identified previously (4, 9).

Culture conditions. All *Cytophaga* strains were grown in Schaedler broth (BBL Microbiology Systems, Cockeysville, Md.). All other strains were grown in Trypticase soy broth (BBL Microbiology Systems)-based medium containing 0.2% glucose and 0.05% Tween 80 (13). All bacterial cultures were incubated at 37°C in an atmosphere of H_2 and CO_2 (GasPak; BBL Microbiology Systems) for maintenance. Cells used for coaggregation experiments were grown as static cultures in screw-capped bottles and were harvested in the late exponential phase of growth. Cells were washed three times with coaggregation buffer (4) between centrifugations at 10,000 × *g* for 10 min.

Coaggregation assay. Cell suspensions were adjusted to a density of about 5×10^9 cells per ml (260 Klett units with a red filter at 660 nm). Equal volumes of potentially coaggregating cell types were mixed and checked visually for coaggfegation as previously described (4). The degree of coaggregation was assigned a score of from 0 to 4. A score of 0 was given when the mixture had the appearance of a homogeneous cell suspension, and a score of 4 was given to a mixture with clear supernatant fluid and large coaggregates which settled immediately to the bottom of the tube (i.e., maximum coaggregation).

Electron microscopy. Negative staining was accomplished by mixing equal volumes of 2% sodium phosphotungstate (pH 6.5) and coaggregated cell mixtures. The resulting mixtures were layered on collodion film-coated, carbon-stabilized, 300-mesh copper grids and immediately examined with a Phillips 200 electron microscope (accelerating voltage, 60 kV).

Plaques containing two different cell types were developed in vitro on human teeth by the method of McCabe et al. (14). Clean, autoclave-sterilized, human teeth were first suspended by wire supports in a freshly inoculated Schaedler broth culture of *Cytophaga* sp. strain DR2001 (1% glucose was substituted for sucrose) and incubated at 37°C for 6 days. After the first 3 days of incubation, spent culture medium was replaced with fresh broth, and incubation was continued for an additional 3 days. Upon completion of the initial 6-day incubation period, the DR2001-plaque-bearing teeth were removed from the broth culture tubes and immediately suspended in actively growing Schaedler broth cultures of *A. israelii* PK16. Again, the suspended teeth were incubated for 6 days at 37°C, with one fresh broth substitution after 3 days.

After the last incubation period, the teeth were carefully removed from the broth culture tubes and gently rinsed in 0.02 M phosphate buffer, pH 7.2. Small, macroscopically visible zones in plaque (regions of *A. israelii* colonization appeared as white-colored zones over the underlying amber-colored *Cytophaga*-containing plaque) were cut away from the tooth surface with sharpened scalpel blades and placed in Ryter-Kellenberger (8) buffered 3% glutaraldehyde, pH 6.1. Each sample was postfixed in osmium tetroxide, dehydrated through an increasing ethanol concentration series, and embedded in Spurr. The sections obtained with glass knives were stained with uranyl acetate and lead citrate and examined with the Phillips 200 electron microscope.

RESULTS

Coaggregation of *Cytophaga* strains with other oral bacteria. When the 19 *Cytophaga* strains were tested for their ability to coaggregate with 8 *A. israelii* strains, three coaggregation patterns emerged (Table 1). Nearly 50%, 9 of 19, coaggregated strongly with *A. israelii* PK16, weakly with ATCC 23860 and CROB2051, and not at all with W1011; they were designated group 1. Those strains given the group 2 assignation coaggregated strongly with W1011 and poorly or not at all with the other *A. israelii* strains. Members of group 3 exhibited little or no coaggregation with any of the *A. israelii* strains. No coaggregation was observed between *Cytophaga* strains and *A. viscosus* (13 strains), *A. naeslundii* (12 strains), *S. sanguis* (14 strains), *S. mitis* (2 strains), *S. mutans* (7 strains), or *S. salivarius* (4 strains).

Unlike coaggregations between *S. sanguis* and *A. viscosus* or *A. naeslundii*, which are inhibited by 1.0 mM EDTA (4), none of these coaggregations were affected by 10 times that

TABLE 1. Coaggregation between oral *Cytophaga* strains and *A. israelii* strains

Oral *Cytophaga* strains	Degree of coaggregation[a] with indicated *A. israelii* strain:							
	W1011	X522	PK16	CROB 2030	ATCC 12103	CROB 2052	ATCC 23860	CROB 2051
Group 1								
DR2001	0	0	3	0	1	0	1	2
DR2004	0	0	3	0	0	0	1	1
DR2007	0	2	3	0	0	0	1	0
DR2008	0	0	2	0	0	0	1	1
DR2013	0	1	3	0	0	0	1	2
DR2015	0	0	3	0	0	0	2	3
DR2016	0	0	3	0	1	0	1	1
DR2018	0	0	3	0	0	0	1	1
DR2020	0	0	3	0	0	0	1	1
Group 2								
DR2005	3	0	0	0	0	0	1	0
DR2006	3	0	0	0	0	0	0	0
DR2012	3	0	0	0	0	0	0	0
DR2022	3	0	0	0	1	0	0	0
Group 3								
DR2002	0	0	0	0	0	0	0	0
DR2003	0	0	0	0	0	0	0	0
DR2011	0	0	0	0	1	0	0	0
DR2014	0	0	0	0	0	0	0	0
DR2017	0	0	0	0	0	0	1	0
DR2021	0	0	0	0	0	0	1	0

[a] See text for description of scores.

concentration. Likewise, none were altered by the addition of 100 mM lactose, which totally inhibits many of the coaggregations involving *S. sanguis* (4). All coaggregations were prevented by simple heat treatment (85°C, 30 min) of *Cytophaga* strains, but none were affected by the same treatment of *A. israelii* strains.

The 19 *Cytophaga* strains were obtained from 15 patients with destructive periodontitis (2). The distribution of the 19 strains was as follows: the 9 strains of group 1 were obtained from eight patients, the 4 strains of group 2 were obtained from 3 patients, and the 6 strains of group 3 were obtained from six patients. Although a single *Cytophaga* isolate was obtained from most patients, two were isolated from each of four patients. In two cases, both isolates were members of either group 1 or group 2; in the other two cases, one isolate belonged to group 3 and the other isolate belonged to either group 1 or group 2. Thus, isolates from the same patient do not necessarily exhibit the same coaggregation pattern.

Electron microscopy of coaggregates. Negatively stained preparations of coaggregates composed of *Cytophaga* sp. strain DR2001 and *A. israelii* PK16 were viewed with an electron microscope (Fig. 1A). Close association of the two cell types forming a compact coaggregate is clearly visible. Only a portion of the coaggregate is shown, in that other clusters of cells extend to the right, bottom, and upper-left corner. Surface fimbriae on the *A. israelii* cell and extensive membranous blebs along the surface of the *Cytophaga* cell are evident. Interactions are apparent between the fimbriae along the *A. israelii* cell (oriented from the center to the upper-right corner) and membranous blebs and profuse membrane excrescenses at the pole of the *Cytophaga* cell (mostly hidden by the cluster of *A. israelii* cells in the upper-left corner). A higher magnification of these surface structures more clearly shows their sites of interaction (Fig. 1B).

Electron microscopy of plaque formed in vitro. Results from previous studies indicated that *Cytophaga* sp. selectively forms a heavy coating of amber-colored plaque on the cementum surface but not on the enamel surface of a tooth submerged in a bacterial culture (2). In contrast, recent experiments with *A. israelii* PK16 revealed the presence of occasional white patches of bacterial plaque on both the enamel and cementum surfaces. The ability of *A. israelii* to recognize the surface of *Cytophaga* sp. and form coaggregates prompted us to look for a possible interaction between these cells on a tooth surface. A presterilized tooth was initially suspended in a growing culture of *Cytophaga* sp. strain DR2001. When the tooth was subsequently suspended in a culture of *A. israelii* PK16, white

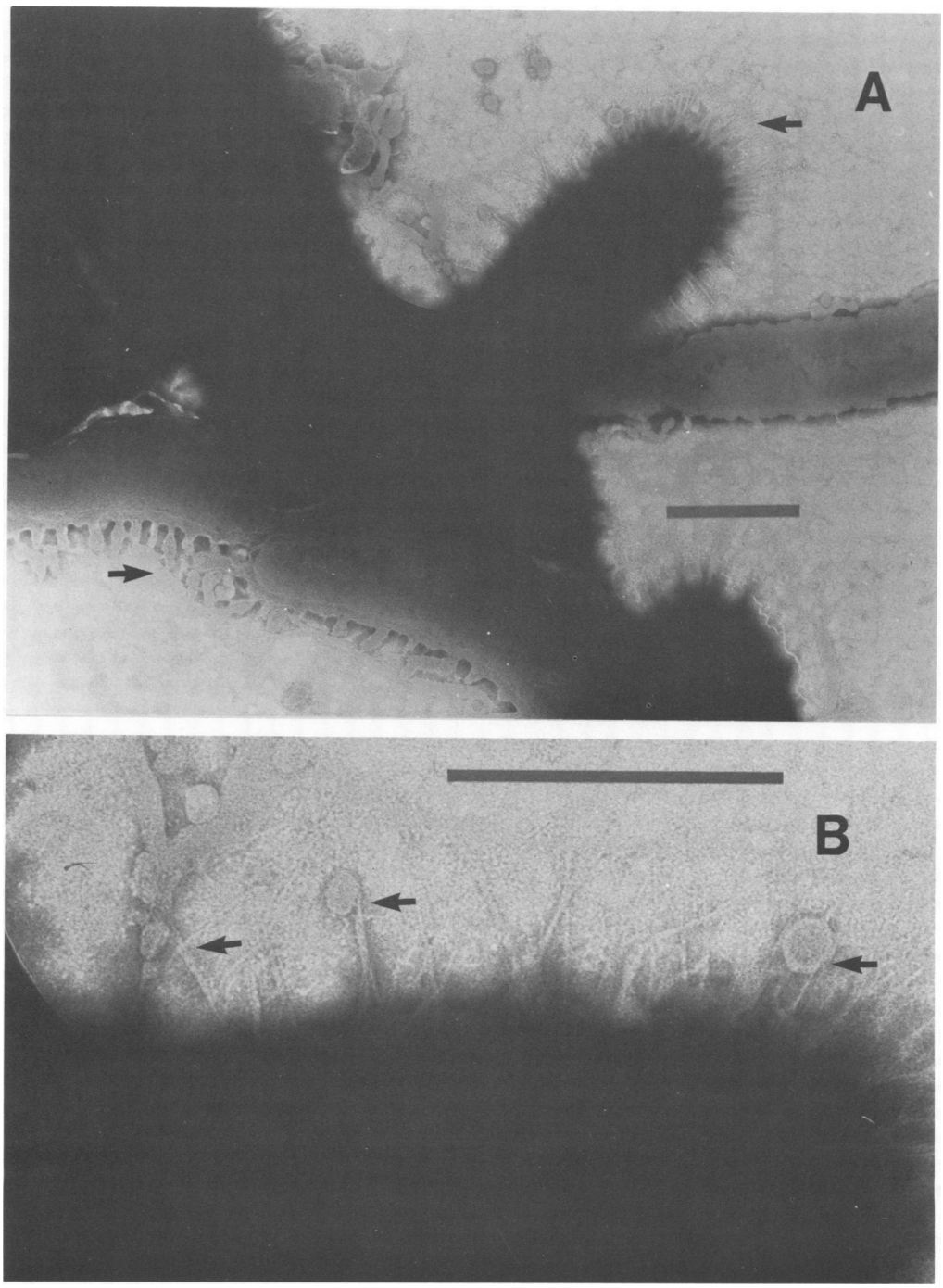

FIG. 1. Coaggregate of *Cytophaga* sp. strain DR2001 and *A. israelii* PK16 stained with sodium phosphotungstate. (A) *A. israelii* cells (heavily stained cells) show surface fimbriae (arrow), and the *Cytophaga* cells exhibit membranous excrescences (arrow). (B) A higher magnification of the region along the surface of the *A. israelii* cell in the upper central portion of (A) reveals several points of contact (arrows) between fimbriae and membranous blebs. Bars, 0.5 μm.

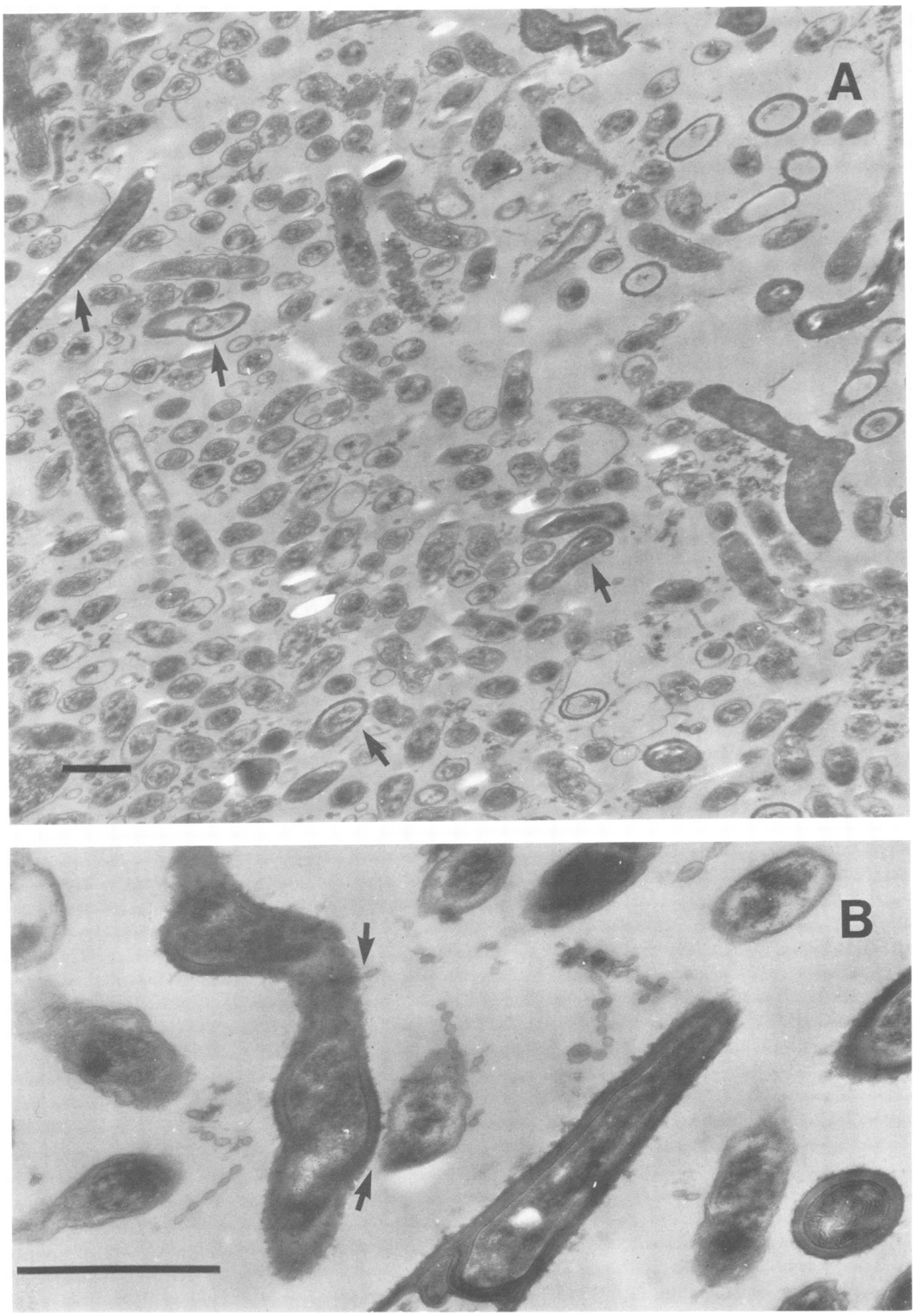

FIG. 2. Thin section of accumulated mixed plaque formed by successive incubation of a presterilized tooth in cultures of *Cytophaga* sp. strain DR2001 and *A. israelii* PK16. (A) *A. israelii* cells (arrows) surrounded by *Cytophaga* cells. The cementum surface is towards the lower-left corner, where there are only very tightly packed *Cytophaga* cells. The region beyond the upper-right corner is composed of loosely packed *A. israelii* cells. (B) Higher magnification of the mixed-plaque zone. Sites of interaction between the gram-positive *A. israelii* cells and both the gram-negative *Cytophaga* cells and one of the surface blebs are indicated by arrows. Bars, 1 μm.

patches of *A. israelii* plaque were found on both the enamel surface and the *Cytophaga* plaque-coated cementum surface. Examination of the mixed-plaque area by electron microscopy revealed tightly packed cells (Fig. 2A). Most of the cells shown are *Cytophaga* cells, with an occasional *A. israelii* cell surrounded by the gram-negative *Cytophaga* cells. The cell surfaces of these two cell types are quite distinct, as shown in a higher magnification of another field in the mixed-plaque zone (Fig. 2B). The *Cytophaga* cells are more lightly stained and exhibit a wavy cell envelope. The organization of the outer membrane excrescences into a string of bleb-like spheres is evident on the center cell. Other blebs in various states of organization are seen throughout the field. In contrast, the surfaces of the thick actinomycete murein layer display smooth topography. Direct cell-to-cell contact between the two cell types is evident, both between whole cells and between a bleb-like sphere and the *A. israelii* cell (arrows).

Adsorption of *A. israelii* PK16 to solid substrata. Quantitation of the adsorption parameters of *A. israelii* PK16 to root powder and spheroidal hydroxyapatite, model substrata for cementum and enamel surfaces, respectively, indicated high-affinity binding and large numbers of adsorption sites on both substrata (Table 2). For comparison, the binding characteristics of *Cytophaga* sp. strain DR2001 (1) and two other *A. israelii* strains (6) are included. The affinity constants for *A. israelii* PK16 and *Cytophaga* sp. strain DR2001 were nearly identical, whereas those for the other two *A. israelii* strains were about 10-fold higher. The maximum number of adsorption sites for *A. israelii* PK16 was considerably higher than that reported for the other two *A. israelii* strains (6) and 5- to 10-fold higher than that for *Cytophaga* sp. strain DR2001. Overall, the binding characteristics of *A. israelii* PK16 compared quite favorably with those for the strains compared here, indicating that *A. israelii* PK16 is capable of tenacious binding to both root powder and spheroidal hydroxyapatite.

DISCUSSION

One important aspect of studying the ecology of oral bacteria is an understanding of interactions between bacteria that inhabit the same econiche. In this study we present information that bears directly on cell-to-cell interactions between cells belonging to a relatively new group of gram-negative, flexuous bacteria tentatively placed in the genus *Cytophaga* and cells of a gram-positive bacterium, *A. israelii*. Both are known to inhabit subgingival sites and are readily isolated from samples obtained from patients with destructive periodontitis (2, 18). Of course, the observation that certain kinds of bacteria are isolated from the same sample does not prove that they occupy adjacent sites in a microeconiche. It also does not prove that cell-surface interactions occur between different kinds of cells. Indeed, the fact that different types of cells are coaggregating partners in vitro does not prove that they recognize each other in vivo. However, coaggregation does not appear to be a random event. Only certain pairs of cell types coaggregate. This was observed in the first report of interbacterial aggregation (7) and has been consistently found in several recent surveys of coaggregation between oral bacteria (4, 9, 17). The clear specificity of *Cytophaga* sp.-*A. israelii* coaggregations described here (and dis-

TABLE 2. Adsorption parameters for *A. israelii* PK16 interacting with root powder (RP) and spheroidal hydroxyapatite (SHA)[a]

Organism	Substratum	Maximum no. of adsorption sites (N)[b]	Affinity constant (K_a)[c]	Correlation coefficient	Reference
A. israelii PK16	RP	7.7×10^{8}[d]	1.3×10^{-9}	0.93	This work
A. israelii PK16	SHA	1.6×10^{9}[d]	1.8×10^{-9}	0.99	This work
A. israelii UFO512	SHA	2.1×10^{7}[e]	1.8×10^{-8}	0.71	6
A. israelii UFO243	SHA	9.0×10^{6}[e]	2.3×10^{-8}	0.79	6
Cytophaga sp. strain DR2001	SHA	2.7×10^{8}[d]	1.7×10^{-9}	0.98	1
Cytophaga sp. strain DR2001	RP	6.1×10^{7}[d]	2.5×10^{-9}	0.99	1

[a] The assay methods used are described in detail in a previous report (1); the procedures of Clark et al. (5) were followed.

[b] Derived from experimental curves consisting of at least five different cell concentrations (triplicate samples per cell concentration).

[c] Units are milliliters per cell.

[d] Units are cells per 20 mg of substrate.

[e] Units are cells per 40 mg of substrate.

cussed below) can be added to the growing list of specific cell-to-cell interactions between oral bacteria. We suggest that it is no coincidence that both cell types are found in subgingival samples but rather that it is likely that cell-to-cell recognition plays an important role in the establishment of these two cell types in the same econiche. Support for this proposal comes from the following observations: *A. israelii* PK16 formed plaque deposits on a tooth precoated with *Cytophaga* sp. strain DR2001, the region of overlapping plaque material consisted of an intermixture of the two cell types (Fig. 2), and negatively stained preparations of coaggregated cells showed surface fimbriae of *A. israelii* PK16 in direct contact with outer membrane blebs of *Cytophaga* sp. strain DR2001 (Fig. 1).

Although little is known about the mechanism of the coaggregations reported here, it is evident that *Cytophaga* strains show a great deal of specificity in their choice of a coaggregating partner cell. They do not coaggregate with *A. viscosus*, *A. naeslundii*, *S. sanguis*, *S. mitis*, *S. mutans*, or *S. salivarius*. Further, they do not coaggregate with all *A. israelii* strains. Members of group 1 recognize the largest number of *A. israelii* strains, and yet there is consistently strong coaggregation only with *A. israelii* PK16. Members of group 2, on the other hand, coaggregate specifically with *A. israelii* W1011. Although group 3 representatives do not coaggregate with any of the test *A. israelii* strains, this does not imply that they are unable to coaggregate with other strains not included in this study. In fact, a new coaggregation group of freshly isolated *S. sanguis* strains that failed to coaggregate with the routinely used test actinomycetes was recently discovered only after testing with *A. naeslundii* strains that were isolated from the same patient (P. E. Kolenbrander, Y. Inouye, and L. V. Holdeman, submitted for publication). A similar analysis of coaggregations between *Cytophaga* strains and *A. israelii* strains isolated from the same patient was not possible in the current study, since only *Cytophaga* strains were originally isolated for an independent investigation (2) and no selection for *A. israelii* strains was attempted. It is proposed that if such a study were conducted, *Cytophaga* strains unable to coaggregate with these test *A. israelii* strains would likely exhibit coaggregation with *A. israelii* strains isolated from the same patient.

The properties of coaggregation between *Cytophaga* strains and *A. israelii* strains are quite different from those found with *S. sanguis* and *A. viscosus* or *A. naeslundii*; the latter coaggregations appear to be mediated by lectin-carbohydrate complementary paris (4, 9, 15). Most of the latter coaggregations are inhibited by lactose (10 mM), and all are inhibited by EDTA (0.6 mM) and require divalent calcium ions (0.1 mM). One property shared by the *Cytophaga-A. israelii* coaggregations and the *S. sanguis-A. viscosus* (or *A. naeslundii*) coaggregations is that many are unimodal (heat treatment at 85°C for 30 min of one cell type but not the other will prevent coaggregation). In this case the *Cytophaga* cells are inactivated, but the *A. israelii* cells are resistant to heat treatment.

Although the *Cytophaga* isolates are morphologically similar to members of the recently designated genus, *Capnocytophaga*, they are metabolically distinct (2, 16). When their ability to coaggregate with *A. israelii* strains was compared, *Capnocytophaga sputigena* and *C. gingivalis* strains appeared similar to members of group 1, whereas *C. ochracea* exhibited lactose-inhibitable coaggregation with certain actinomycetes and *S. sanguis* strains (P. E. Kolenbrander and S. Hurst-Calderone, J. Dent. Res. **60A**:333, 1981). It is interesting to note that when *Cytophaga* sp. strains DR2001 and DR2002 were tested for DNA-DNA homology with the three *Capnocytophaga* species, 82 and 69% homology, respectively, were found with *C. gingivalis*, and little or no homology was observed with the other two species (John Johnson, personal communication). Thus, it appears that these *Cytophaga* strains may be similar to *C. gingivalis* but are quite distinct from *C. ochracea*.

ACKNOWLEDGMENTS

We thank John Johnson for providing the data on DNA-DNA homology of strains of *Cytophaga* and *Capnocytophaga*, Jack London and Angelika Kagermeier for critical reading of this manuscript, and Susan Hust-Calderone and Margaret Phillipson for excellent technical assistance.

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