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Inhibition of Coaggregation between *Fusobacterium nucleatum* and *Porphyromonas* (*Bacteroides*) *gingivalis* by Lactose and Related Sugars

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The coaggregation of *Fusobacterium nucleatum* PK1594 and *Porphyromonas* (*Bacteroides*) *gingivalis* PK1924 was inhibited equally well by lactose, *N*-acetyl-D-galactosamine, and D-galactose, which caused 50% inhibition of coaggregation at 2 mM sugar concentration. Other sugars such as D-galactosamine, D-fucose (6-deoxy-D-galactose), and α-methyl- and β-methyl-D-galactosides also inhibited coaggregation. Sugar specificity was apparent, since neither L-fucose, L-rhamnose, *N*-acetyl-D-glucosamine, nor *N*-acetylneuraminic acid was an inhibitor. Protease treatment of the fusobacterium completely abolished coaggregation, whereas it had no effect on the coaggregating activity of the porphyromonad. Although numerous lactose-inhibitable coaggregating pairs are known to occur among gram-positive bacteria, this report and the accompanying survey (P. E. Kolenbrander, R. N. Andersen, and L. V. H. Moore, Infect. Immun. 57:3194–3203, 1989) are the first studies demonstrating the extensive nature of this type of interaction between gram-negative human oral bacteria. The significance of galactoside-inhibitable coaggregations between these two potential periodontal pathogens is discussed.

Human oral *Fusobacterium nucleatum* strains occur in high numbers in samples taken from healthy sites, and their numbers increase in periodontally diseased subgingival sites, but *Porphyromonas* (*Bacteroides*) *gingivalis* (33) strains appear in significant numbers only in the latter condition (5, 29, 38). It is generally accepted that both species are associated with periodontal disease. Both organisms are part of a mixed infection of subgingival microflora in a patient with refractory periodontitis (36), and they also coexist in vitro in a consortium of subgingival microflora growing on human serum in enrichment cultures (34). A survey of a large number of coaggregation partners for these two groups of gram-negative bacteria revealed numerous partners for *F. nucleatum* (18), but none except the fusobacteria was observed for *P. gingivalis* (17, 18).

Direct cell-to-cell contact between fusobacteria and porphyromonads is shown here to be inhibited specifically by galactosides. Galactoside sensitivity of cellular interactions involving fusobacteria and other cell types was first reported in 1979 by Mongiello and Falkler (28) in their studies of hemagglutination of human and sheep erythrocytes. The hemagglutination activity of hemagglutination-active *F. nucleatum* fragments was reduced by preadsorbing the fusobacterial preparations with cells of other oral bacteria, including *P. gingivalis*, and the adsorption was inhibited by D-galactose and N-acetyl-D-galactosamine but not by many other sugars (7). Recently, a galactose-binding lectin was reported on *F. nucleatum*, which binds to asialofetuin-Sepharose 6MB (32). The fusobacterial lectin may be involved in agglutination of human parotid saliva and hemagglutination of neuraminidase-treated rabbit erythrocytes, both of which are inhibited by galactose and its structural analogs.

Although none of its coaggregations with gram-positive bacteria was completely inhibited by 60 mM lactose, nearly all of the coaggregations between *F. nucleatum* PK1594 and members of five different genera of gram-negative bacteria were inhibited by lactose (18). Here, we present the properties of the galactoside-inhibitable coaggregation of *F. nucleatum* PK1594 with *P. gingivalis* PK1924.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *F. nucleatum* PK1594 (VPI E2S-11A) and *P. gingivalis* PK1924 (VPI 14020) were of human origin and were obtained from subgingival sites as already described (30, 31). The strains were kindly provided by L. V. H. Moore (the designations within parentheses are the strain numbers given at the time of isolation at The Anaerobe Laboratory at the Virginia Polytechnic Institute and State University, Blacksburg). *F. nucleatum* was grown in modified Schaedler broth (2), and *P. gingivalis* was grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.). Cells were grown in either screw-capped tubes (16 by 125 mm) or bottles (100- to 500-ml capacity) incubated at 37°C under an anaerobic atmosphere containing H_2, CO_2, and N_2 (10:10:80) with GasPaks (BBL). The cells were harvested in the late-exponential to early-stationary phase of growth and washed three times with coaggregation buffer, which consisted of the following (dissolved in 0.001 M Tris, adjusted to pH 8.0): $CaCl_2$ (10^{-4} M), $MgCl_2$ (10^{-4} M), NaN_3 (0.02%), and NaCl (0.15 M). Centrifugation was at 10,000 × *g* for 10 min; after the cells were washed, they were stored in coaggregation buffer at 4°C until used.

Protease treatment of cells. Cell suspensions of fusobacteria and porphyromonads were adjusted to 260 Klett units (660 nm [red filter]; Klett-Summerson, Inc., New York, N.Y.) with coaggregation buffer and contained 1×10^9 and 5×10^9 cells, respectively. Pronase (Calbiochem, San Diego, Calif.) was added to give a concentration of 0.5 mg/ml, and the suspension was incubated at 37 or 50°C for 60 min. The cells were washed three times with coaggregation buffer and suspended to the original volume with coaggregation buffer.

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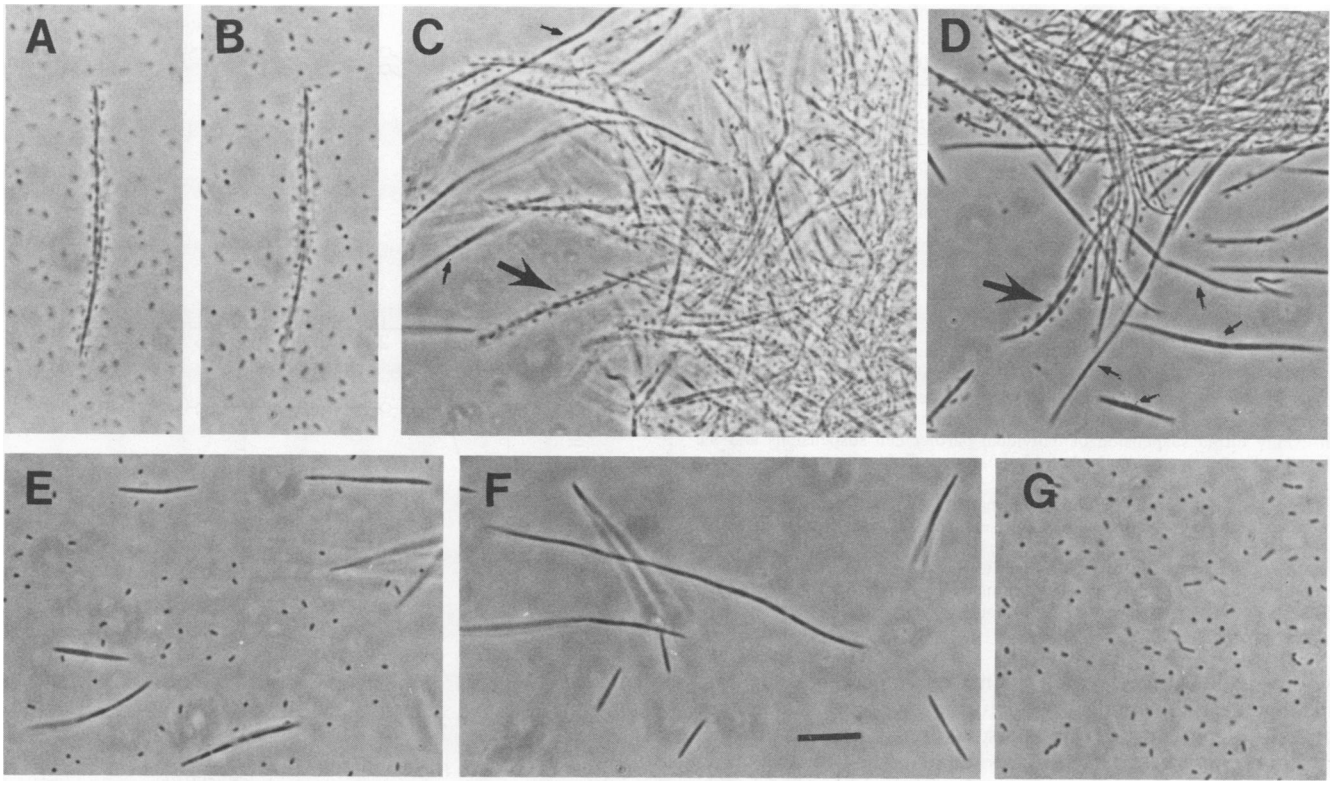

FIG. 1. Phase-contrast photomicrographs of coaggregation between *F. nucleatum* PK1594 and *P. gingivalis* PK1924. (A and B) Different focal planes of two fusobacteria surrounded by porphyromonads and apparently bridged by porphyromonads at a 1:80 proportion of fusobacteria to porphyromonads. The porphyromonads are aligned along the length of the fusobacteria, which gives a corncob appearance to the coaggregate. The corncob morphology is evident throughout large coaggregates as well and extending from these coaggregates (C and D, large arrows). (C) Coaggregate extending to the right of the frame showing that most but not all fusobacteria (small arrows) are coated with porphyromonads at a proportion of 1 to 10. (D) Many of the fusobacteria in coaggregates formed with a proportion of 1 to 4 contain fewer porphyromonads and often none (small arrows). Addition of lactose (60 mM, final concentration) to coaggregates completely dissociated them into individual cells in a mixed cell suspension (E). Suspension of fusobacteria showing the range of lengths of cells (F) and suspension of porphyromonads (G). Bar, 10 μm.

Radioactive labeling of bacteria. The two strains were labeled in their respective growth media; *F. nucleatum* was labeled with [^{14}C]uracil (58 mCi/mmol; ICN Radiochemicals Inc., Irvine, Calif.), and *P. gingivalis* was labeled with [^{3}H]thymidine (2.0 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) at 1.0 and 10 μCi, respectively, per ml of broth. After four to six cell doublings, the cells were harvested and washed as above by centrifugation, suspended in coaggregation buffer, and stored at 4°C (specific radioactivity, about 10^3 bacteria per cpm).

Radioactivity assay for coaggregation. The conditions of radioactivity measurements and this assay procedure were completely detailed previously (15). The errors in measurement in this assay are plus or minus 5% of the values reported. To determine the ratio of partner cells required to maximally coaggregate with the radioactively labeled cell type, increasing numbers of unlabeled partner cells were added to a constant number of labeled cells. After gentle rocking (setting 5 for 5 min with a DSG Titertek shaker; Flow Laboratories, Inc., McLean, Va.) of the tubes (either treated with Tween 80 as previously described [16] or siliconized tubes, from PGC Scientifics, Gaithersburg, Md., without further treatment) followed by centrifugation (1,000 rpm for 1 min in a Microfuge 12; Beckman Instruments, Palo Alto, Calif.), the percentages of input radioactivity distributed in the supernatant (free, uncoaggregated cells) and the pellet (coaggregated cells) were determined. Sugar inhibition of coaggregation was measured by using a ratio of cell types where the maximum number of radioactively labeled cells was in coaggregates (i.e., saturating unlabeled partner).

Chemicals. All sugars were purchased from Sigma Chemical Co., St. Louis, Mo. Solutions of amino sugars and N-acetylated amino sugars were adjusted to a pH of 7.4 with 1 N NaOH.

RESULTS

Coaggregation properties of the interaction between *F. nucleatum* PK1594 and *P. gingivalis* PK1924. After suspensions of the two cell types were mixed, the strongest coaggregation, by visual inspection of the resultant suspension, was found when equal numbers of the partners or an excess of fusobacteria was used. Mixed cell suspensions of various proportions of the partners were examined by phase-contrast microscopy (Fig. 1). At a proportion of 1 fusobacterium to 80 porphyromonads, the fusobacteria were coated with porphyromonads, and the background contained numerous unattached porphyromonads (Fig. 1A and B). The porphyromonads seem to line up along the length of the fusobacterial cell, giving the appearance of corncobs, similar to those reported with streptococcal partners and fusobacteria (19, 20). Although at this proportion very little coaggregation was detectable by visual examination of the mixed cell suspension in the test tube, microscopic viewing of the

TABLE 1. Effect of heat or protease on the ability of *F. nucleatum* PK1594 and *P. gingivalis* PK1924 to coaggregate[a]

P. gingivalis treatment	Coaggregation score with *F. nucleatum* treated with:			
	None	Heat (85°C for 30 min)	Protease	
			37°C for 60 min	50°C for 60 min
None	4^0	0	4^0	0
Heat (85°C for 30 min)	4^2	0	4^0	0
Protease (37°C for 60 min)	4^0	0	4^0	ND
Protease (50°C for 60 min)	4^0	0	ND	0

[a] The coaggregation score was determined by visual assay (4) after mixing equal volumes (0.1 ml) of each cell suspension (cell density of about 10^9 cells per ml). A score of 0 is given for no coaggregation as evidenced by no change in evenly turbid suspension of individual partner cell suspensions and the mixed-cell suspension. A score of 4 indicates maximal coaggregation, where immediate clumping occurs after mixing followed by immediate settling of coaggregates and leaving a clear supernatant. An intermediate score of 2 represents definite coaggregates easily seen but suspension remains turbid without immediate settling of coaggregates. Coaggregation scores are given in two parts: the first score is that given after mixing the two strains together, and the superscript is the score after adding lactose (final concentration equals 60 mM) to the coaggregates. ND, Not done.

same suspension revealed many examples like the two fusobacteria shown here, which appear to be attached by cross-bridging porphyromonads. When a proportion of 1 fusobacterium to 10 porphyromonads was mixed, larger coaggregates comprising a network of interacting fusobacteria and porphyromonads were formed (Fig. 1C). The corncob appearance of the individual paired cell types within the coaggregate is obvious, and one extending from the coaggregate is denoted by large arrows (Fig. 1C and D). Surprisingly, even in the presence of this large excess of porphyromonads, a few fusobacteria appear to be devoid of porphyromonads (small arrows). The coaggregates observed when the proportion was 1 to 4 showed fusobacteria with fewer attached porphyromonads per cell and several cells without any porphyromonads (small arrows) (Fig. 1D). The addition of lactose to coaggregates formed at this latter proportion completely dissociated the coaggregates to individual cells (Fig. 1E). Suspensions of the partners are shown for comparison (Fig. 1F and G).

Although heating the fusobacteria at 85°C for 30 min totally abolished their ability to coaggregate, heating the porphyromonads changed the coaggregation from fully lactose reversible to only partially reversible (18). Protease treatment of the two cell types at 37 and 50°C had different effects on the ability of treated cells to coaggregate (Table 1). The milder treatment at 37°C of the fusobacterium did not change the lactose-reversible nature of the coaggregation, whereas digestion at 50°C completely prevented coaggregation. In contrast, protease treatment of the porphyromonads at either temperature caused no visible change in the coaggregation with untreated fusobacteria. Digestions for 120 min did not increase the effects observed after 60 min of treatment (data not shown). Heating either cell type at 50°C for 60 or 120 min without protease had no effect on coaggregation. Thus, either heating (85°C for 30 min) or protease treatment (0.5 mg/ml at 50°C for 60 min) of fusobacteria completely abrogated coaggregation, suggesting that a proteinaceous surface component on the fusobacterium was altered by these treatments.

Inhibition of coaggregation by sugars. To determine the cell-type ratio appropriate to measure sugar inhibition of

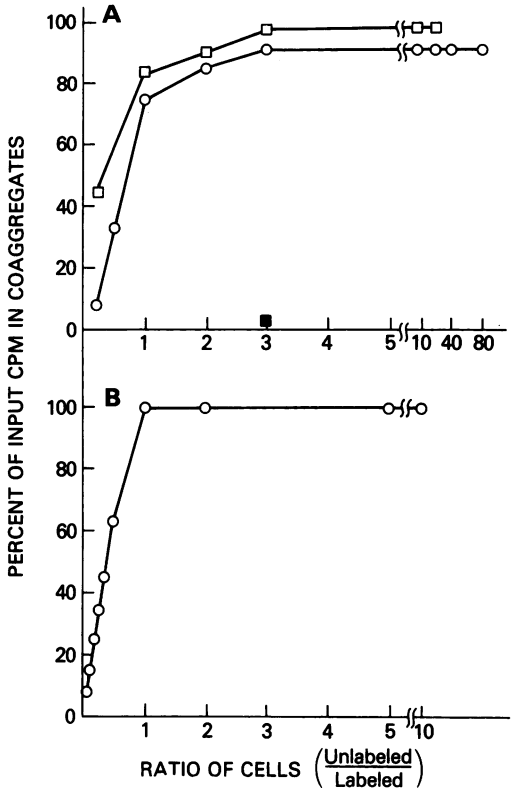

FIG. 2. Determination of unlabeled partner cells/radioactively labeled cells ratio at which maximum labeled cells participate in coaggregation. (A) The addition of increasing numbers of unlabeled *P. gingivalis* PK1924 to a constant number of radioactively labeled *F. nucleatum* PK1594 (○) showing maximum label in coaggregates remains constant (saturation) between ratios of 3 and 80, addition of increasing numbers of unlabeled and protease-treated *P. gingivalis* to a constant number of labeled *F. nucleatum* (□), and the percentage of input label in coaggregates when protease-treated, unlabeled *P. gingivalis* was mixed with labeled *F. nucleatum* (ratio of 3) in the presence of 60 mM lactose (■). (B) The addition of increasing numbers of unlabeled *F. nucleatum* to a constant number of labeled *P. gingivalis* saturated the porphyromonads, resulting in 100% of the porphyromonads found in coaggregates. Maximum labeled cells participated in coaggregation at an unlabeled/labeled cell ratio of >1.

coaggregation, each cell type was labeled and mixed with increasing numbers of unlabeled partner (Fig. 2). In the presence of excess labeled cells (unlabeled/labeled cell ratios of less than 0.5), the percentage of input label found in coaggregates was 40% or less (Fig. 2). This indicates a large fraction of uncoaggregated, labeled cells, which is consistent with the observations in the visual coaggregation assay and by phase-contrast microscopy (Fig. 1). As the cell ratios approached unity, most (Fig. 2A) or all (Fig. 2B) of the label was found in coaggregates. When the ratio was 3, a maximal amount of label was in coaggregates even when labeled fusobacteria were mixed with porphyromonad cells that had previously been digested with protease (Fig. 2A). Lactose inhibition of coaggregates formed by labeled fusobacteria and protease-treated, unlabeled porphyromonads at this cell ratio was nearly complete (3% of the label remained in coaggregates; Fig. 2A). The same or total inhibition was observed when both cells were untreated (see below).

With an unlabeled/labeled cell ratio of 3, the ability of various sugars to inhibit coaggregation was examined. Both

TABLE 2. Inhibition of coaggregation between *F. nucleatum* and *P. gingivalis* by different sugars

Sugar[a]	Concn (mM)	% Inhibition of coaggregation	
		Labeled *P. gingivalis*[b]	Labeled *F. nucleatum*[c]
Lactose	100	98	100
	10	93	98
GalNAc	100	93	94
	10	66	94
GalNH	100	91	97
	10	26	5
D-Fucose	100	47	47
	10	5	1
L-Rhamnose	100	0	0
	10	4	0
D-Galactose	100	89	98
	10	81	93
L-Fucose	100	0	1
	10	0	0
α-Methyl Gal	100	92	94
	10	75	70
β-Methyl Gal	100	93	94
	10	70	87
GlcNAc	100	0	2
	10	0	2
NeuAc	100	7	0
	10	0	0

[a] Abbreviations: GalNAc, *N*-acetyl-D-galactosamine; GalNH, D-galactosamine; α-Methyl Gal, methyl-α-D-galactoside; β-Methyl Gal, methyl-β-D-galactoside; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid.

[b] Values obtained with an unlabeled *F. nucleatum*/radioactively labeled *P. gingivalis* cell ratio of 3.

[c] Values obtained with an unlabeled *P. gingivalis*/radioactively labeled *F. nucleatum* cell ratio of 3.

combinations of labeled and unlabeled pairs were tested, with nearly identical results (Table 2). Each sugar was tested at 10 and 100 mM. More than 90% inhibition was found with 100 mM lactose, *N*-acetyl-D-galactosamine, D-galactosamine, D-galactose, α-methylgalactoside, and β-methylgalactoside. Except for D-galactosamine, the other sugars inhibited nearly as well at 10 mM. D-Fucose was a weak inhibitor and inhibited 47% at 100 mM. The other sugars tested, L-rhamnose, L-fucose, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid inhibited very little, if at all, at either concentration.

Further testing of the kinetics of sugar inhibition revealed that lactose, D-galactose, and *N*-acetyl-D-galactosamine were equally effective inhibitors; each of them caused 50% inhibition of coaggregation at 2 mM (Fig. 3). Inhibition was independent of the labeled cell type, and the only apparent difference in the kinetics of inhibition was that 100% inhibition was observed with the three sugars when the fusobacteria were labeled (Fig. 3A), but only 80% inhibition was seen with the latter two sugars at 10 mM, the highest concentration tested in this experiment, when the porphyromonads were labeled (Fig. 3B). These lower values of inhibition are consistent with the results given in Table 2 for inhibition at 10 mM sugar concentration. Two less potent inhibitors, D-galactosamine (Fig. 3A) and D-fucose (Fig. 3B), were tested and showed 50% inhibition at 55 and 85 mM, respectively.

DISCUSSION

Although previous studies have shown that lactose inhibits coaggregation between gram-positive bacteria (27) or between gram-positive and gram-negative bacteria (14; for a review, see reference 13), our current studies document widespread lactose-inhibitable coaggregations between different gram-negative cell types (18). Here, the *F. nucleatum* PK1594–*P. gingivalis* PK1924 pair was inhibited equally well by lactose, D-galactose, and *N*-acetyl-D-galactosamine; a 2 mM sugar concentration inhibited coaggregation by 50% (Fig. 3).

Coaggregations that are equally sensitive to all three sugars, however, have not been reported with other well-studied lactose-sensitive coaggregations. Some coaggregating pairs (e.g., *Streptococcus sanguis* 34 and *Actinomyces viscosus* T14V) are 5- to 10-fold more sensitive to lactose (about 50% inhibition with 2 mM lactose) than to the other sugars (24, 25, 27). Other coaggregating pairs (e.g., *S. sanguis* DL1 and *Propionibacterium acnes* PK93; *S. sanguis* 34 and *Eikenella corrodens* 1073 or *B. loeschei* PK1295) are more sensitive to *N*-acetyl-D-galactosamine (3, 6; P. Kolenbrander and R. Andersen, unpublished results). A third kind (*S. sanguis* H1 and *Capnocytophaga ochracea* ATCC33596) is inhibited 16-fold more effectively by L-rhamnose or D-fucose than by lactose and is only poorly inhibited by

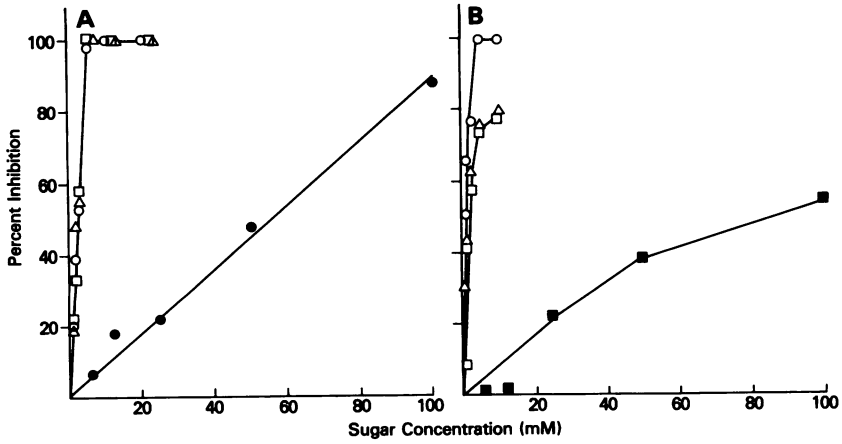

FIG. 3. Inhibition of coaggregation between *F. nucleatum* PK1594 and *P. gingivalis* PK1924 by lactose (○), D-galactose (□), *N*-acetyl-D-galactosamine (△), D-galactosamine (●), and D-fucose (■) with radioactively labeled *F. nucleatum* and unlabeled *P. gingivalis* (A) or labeled *P. gingivalis* and unlabeled *F. nucleatum* (B). All assays were performed at an unlabeled/labeled cell ratio of 3.

N-acetyl-D-galactosamine at concentrations greater than 100 mM (37). L-Rhamnose is an ineffective inhibitor of all of the other coaggregating pairs discussed above. In accord with the sugar-inhibition profiles, purification of the *S. sanguis* H1 hexasaccharide receptor involved in this third kind of interaction revealed two rhamnose residues but no *N*-acetyl-D-galactosamine (1), whereas the repeating hexasaccharide receptor on *S. sanguis* 34, which is involved in the first two kinds of interactions, contains two *N*-acetyl-D-galactosamine residues (23, 26).

The coaggregating pair studied here represents a fourth kind of sugar-inhibition profile for lactose-inhibitable coaggregations. A comparison of the structures of the most inhibitory sugars with the least inhibitory sugars indicates that the galactose configuration of the hydroxyls (*cis* hydroxyls) on C-3 and C-4 may be important for bacterial recognition. Both L-rhamnose and *N*-acetyl-D-glucosamine, whose hydroxyls on C-3 and C-4 are in the two *trans* configurations, have no inhibitory activity. Sugars that are methylated at either C-1 or C-6 (methyl galactosides or D-fucose, respectively; Table 2 and Fig. 3B) remain inhibitory. Thus, each lactose-inhibitable pair mentioned above appears to respond to a related group of sugars slightly differently, which probably reflects structural differences in carbohydrate receptors as well as heterogeneity of the interactions between different receptors and their specific lectin adhesins on partner cell types. The general character of microbial recognition of carbohydrate sequences in animal glycolipids has been extensively studied by Karlsson (11). Many of the human large intestinal bacteria, including members of the genera *Bacteroides* and *Fusobacterium*, bind to lactosylceramide and apparently recognize lactose, whether it is terminally or internally located in the glycolipid molecule (10).

The apparent corncob morphology associated with coaggregations involving fusobacteria in the presence of an excess number of partner cells (Fig. 1) is intriguing and is reminiscent of the structures seen in the early electron microscopic observations of human dental plaque (9, 21). Corncobs are simply different morphological manifestations of coaggregations; they are formed by the same interacting cell-surface components that mediate formation of amorphous mats or clumps. The corncob morphology is a variation of the intergeneric rosettes, which are composed of a central cell(s) that is surrounded by cells belonging to a different genus (13a, 16). Depending on the proportion of the two cell types, a mixture of streptococci and fusobacteria can form rosettes with a central streptococcal cell surrounded by spindle-shaped fusobacteria (P. Kolenbrander and R. Andersen, unpublished observations) or corncobs in the presence of a 40-fold excess of streptococci (12). The information obtained from the studies of the mechanism of corncob formation between *S. sanguis* and *F. nucleatum* (12, 19, 20) will be very helpful in investigating the mediators of the interaction between *P. gingivalis* PK1924 and *F. nucleatum* PK1594.

Protease treatment of the coaggregation mediator(s) on *F. nucleatum* PK1594 prevents coaggregation with the porphyromonad partner, which suggests that the fusobacterium bears a proteinaceous adhesin. The differential effects of 37 and 50°C are probably due to different types of surface components. Although protease treatment at either temperature had no effect on the porphyromonads, heating at 85°C had a dramatic effect. Unheated porphyromonads coaggregated in a fully lactose-inhibitable interaction, but heated cells exhibited only partially inhibitable coaggregation with untreated fusobacteria. This unusual change in coaggregation after heating of the porphyromonads may be a common occurrence among fusobacteria and their coaggregating gram-negative partners. In the accompanying survey, we observed the same phenomenon with *F. nucleatum* PK1594 (and certain other fusobacteria) and *Selenomonas flueggei*, *Veillonella dispar*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga sputigena*, *Capnocytophaga gingivalis*, *Bacteroides denticola*, *Bacteroides intermedius*, and *Bacteroides loeschei* but not with various gram-positive partners (18).

In the same survey six of the nine strains of *P. gingivalis* tested with *F. nucleatum* PK1594 exhibited lactose-inhibitable coaggregation (18). None of the *P. gingivalis* strains coaggregated with any of the more than 100 potential coaggregation partner strains used in the survey and representing 13 genera. No coaggregation was seen between pairs of *P. gingivalis* strains. This absence of coaggregation with other oral bacteria except fusobacteria confirms and extends our previous results, where no partners for *P. gingivalis* strains were found among human oral streptococci and actinomyces (17). Thus, the availability of fusobacteria in the subgingival ecological niche may be a prerequisite for a successful colonization by *P. gingivalis*, whose numbers are elevated in plaque samples taken from sites exhibiting active destructive periodontal disease (5). *F. nucleatum* is the most frequently detected species in active sites (5).

Coaggregation with a wide variety of partners may play an important role in the maintenance of fusobacteria in the oral cavity, considering that fusobacteria adhere very poorly to human cheek epithelial cells (8) and perhaps to other oral tissues as well. Coaggregation permits adherence with ensuing formation of mixed cell-type networks and gratuitous colonization of oral surfaces through the adherent properties of other members of the network.

The results presented here and in the accompanying report (18) extend the list of oral bacteria participating in lactose-inhibitable coaggregations. Inclusion of the two presumed periodontal pathogens examined here on the list enhances the likelihood that this kind of coaggregation is a common form of interaction among the changing populations of bacteria from primarily gram-positive bacteria found in a state of periodontal health to gram-negative bacteria associated with progressively more severe stages of periodontal disease (5, 22, 35).

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