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Intrageneric Coaggregation among Strains of Human Oral Bacteria: Potential Role in Primary Colonization of the Tooth Surface

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Of the 122 human oral bacterial strains tested from 11 genera, only streptococci and a few actinomyces exhibited coaggregation among the strains within their respective genera. Eight of the ten streptococci showed multiple intrageneric coaggregations, all of which were inhibited by galactosides. The widespread intrageneric coaggregation among the streptococci and the less extensive coaggregation among the actinomyces offers an explanation for their accretion on cleaned tooth surfaces and their dominance as primary colonizers.

Streptococci and actinomyces are primary colonizers of freshly cleaned tooth surfaces (27, 30, 31). The relative predominance of streptococci and actinomyces on a tooth root surface four hours after cleaning is 67 to 85% and 8 to 16%, respectively (27). Similarly, on a clean enamel surface, they constitute 47 to 82% and 4 to 32%, respectively, of the initial population. Besides growth of initially attached cells, bacterial repopulation would be promoted by intrageneric interactions among streptococci and among actinomyces as well as by intergeneric coaggregation between streptococci and actinomyces. After the primary colonizers cover the tooth surface, further accretion of dental plaque may occur by intergeneric coaggregation involving other genera and the primary colonizers.

Such intergeneric coaggregations between genetically unrelated partners have been observed with most of the more than 700 human oral bacterial strains tested so far (11, 12, 17). A simple visual assay has been used to screen strains for their ability to be coaggregation partners (3, 15). Each strain has its own specific partners, but often several isolates of one genus exhibit the same set of partners, which is a property that has been used to organize isolates into coaggregation groups. The parameters tested and used to characterize the coaggregation groups are (i) ability of members of a pair to recognize each other, (ii) inhibition of coaggregation by the addition of lactose, (iii) inhibition of coaggregation by heat or protease treatment of one or both partners, and (iv) simultaneous loss of ability by a coaggregation-defective mutant to recognize all members of a partner coaggregation group. By using these parameters, six coaggregation groups of streptococci and six coaggregation groups of actinomyces were identified (3, 19–22). More than 300 streptococcal and actinomyces strains have been surveyed; 84% coaggregate, and of these 84%, 92% exhibit lactose-inhibitable coaggregation. The remaining 400 or more strains are members of 12 additional genera, and many of their coaggregations are also inhibited by lactose and related galactosides (11, 12, 17).

From among this larger group of strains, 122 strains were chosen to test for intrageneric coaggregation. The species and some of the strain designations we previously reported are listed in Table 1. Strains of *Porphyromonas gingivalis* were grown in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.), and strains of *Selenomonas* were grown in a supplemented brain heart infusion broth (7). All other strains were grown in modified Schaedler broth (1). *Rothia* strains were grown aerobically at 37°C. All other cells were grown at 37°C under anaerobic conditions with the GasPak system (BBL Microbiology Systems). Cultures in the late exponential or early stationary phase of growth were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Cells were washed in coaggregation buffer and stored in the same buffer at 4°C until used. Coaggregation buffer 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).

A radioactivity-based assay (13, 14) was used to examine the inhibitory capabilities of sugars on coaggregation. The effects of temperature (85°C for 30 min) and protease (0.5 mg/ml of cell suspension, 50°C for 60 min) treatments were determined as previously described (17).

More than 100 strains from 10 genera besides *Streptococcus* (see below) were examined. Intrageneric coaggregation was found with only one strain of *Actinomyces naeslundii* serotype I, strain PK606, the reference strain for actinomyces coaggregation group D. It coaggregated with one other *A. naeslundii* serotype I, two strains of *Actinomyces gerencseriae* (formerly identified as *Actinomyces israelii* serotype II [9]), and one strain of *Actinomyces meyeri*, PK81 (formerly designated *A. israelii* CROB 2052 [18]), out of a total of 22 actinomyces examined. Coaggregations between the two serotype I *A. naeslundii* strains, PK606 and PK29, and between strain PK606 and *A. meyeri* PK81 were inhibited by lactose.

The strains tested from the other genera numbered 2 actinobacilli, 8 capnocytophagae, 5 eubacteria, 13 fusobacteria, 5 porphyromonads, 10 prevotellae, 2 rothias, 20 selenomonads, and 25 veillonellae. Even with the fusobacteria, no intrageneric coaggregation was observed, although intergeneric coaggregations occur between fusobacteria and members of all of the other genera (17).

In contrast, 8 of the 10 streptococci coaggregated (Table 2). All coaggregations were completely reversed by the addition of 60 mM *N*-acetyl-D-galactosamine (GalNAc), and most were also reversed by lactose. *Streptococcus* SM PK509 was a partner of all but the noncoaggregating strains H1 and J22. The two representatives of streptococcal coag-

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TABLE 1. List of species examined

Species	Strain(s) reported in earlier studies	Reference
Actinobacillus actinomycetemcomitans	Y4, N27	17
Actinomyces gerencseriae[a]	PK16, ATCC 23860	9, 16, 18
Actinomyces israelii	ATCC 12103	17, 18
Actinomyces meyeri	PK81	17, 22
Actinomyces naeslundii[a]	T14V, PK29, PK947, PK606, PK984	17
Actinomyces serotype WVA 963[a]	PK1259	17
Actinomyces odontolyticus serotype II	PK48	17
Capnocytophaga gingivalis	DR2001, ATCC 33624	17, 18
Capnocytophaga ochracea	ATCC 33596	17, 32
Capnocytophaga sputigena	ATCC 33612	17
Eubacterium nodatum		
Fusobacterium nucleatum	PK1594	14, 17
Porphyromonas gingivalis[b]	PK1924, 381	14, 17
Prevotella buccalis[b]		16
Prevotella denticola[b]	PK1277	16, 17
Prevotella intermedia[b]	PK1511, strain of 4197 group	17
Prevotella intermedia[b]	strain of 8944 group	16
Prevotella loescheii[b]	PK1295, PK1298 (VPI D1C-20)	16, 17
Prevotella oris[b]		16
Prevotella veroralis[b]		16
Rothia dentocariosa	PK44	17
Selenomonas flueggei	PK1958	17
Selenomonas infelix	PK1956	17
Selenomonas noxia	PK1967	17
Selenomonas sputigena	PK1559	17
Streptococcus gordonii[c]	DL1, PK488, ATCC 10558	2, 10, 15, 17
Streptococcus oralis[c]	H1, 34, J22, ATCC 10557	10, 13, 17
Streptococcus sanguis[c]	C104, ATCC 10556	10, 13, 17
Streptococcus SM[c]	PK509	13, 17
Veillonella atypica	PK1910	8, 17
Veillonella dispar	PK2503	17
Veillonella parvula	PK1915	8

[a] The actinomyces strains used as reference strains to represent the six actinomyces coaggregation groups A to F are, respectively, T14V, PK29, PK947, PK606, PK984, and PK1259. *A. naeslundii* T14V was previously called *A. viscosus* T14V. All *A. viscosus* serotype II strains have been reclassified as *A. naeslundii* genospecies II, and *A. israelii* serotype II strains have been reclassified as *A. gerencseriae* (9).

[b] Human oral strains previously classified as members of the genus *Bacteroides* have been reclassified as *Porphyromonas* (29) and *Prevotella* (28) strains. *Prevotella intermedia* was formerly *Bacteroides intermedius*.

[c] The streptococcal strains used as reference strains are DL1, H1, 34, C104, J22, PK509, and PK488, which represent the six streptococcal coaggregation groups 1, 2, 3, 3, 4, 5, and 6, respectively. The streptococcal strains DL1, H1, C104, 34, J22, and PK488 were originally classified as *S. sanguis*. Only strain C104 remains as *S. sanguis*. *Streptococcus* SM PK509 was formerly classified as *Gemella morbillorum* and originally as *Streptococcus morbillorum*. Strains ATCC 10556, ATCC 10557, and ATCC 10558 were obtained from the American Type Culture Collection. Strains ATCC 10556 and ATCC 10558 are the type strains for *S. sanguis* and *S. gordonii*, respectively. The human oral viridans streptococci have been recently reclassified (10), and the new identification scheme has been incorporated into the other routinely used methods of polyacrylamide gel electrophoresis of soluble proteins and analysis of cellular fatty acids (7, 24, 25).

gregation group 3, *Streptococcus oralis* 34 and *Streptococcus sanguis* C104, coaggregated with strains ATCC 10558, DL1, PK488, and ATCC 10556. In all of the coaggregations described above, strains 34 and C104 were resistant to heat and protease treatments, while their partners were inactivated by the treatments (data not shown). In the remaining coaggregations, *Streptococcus* SM PK509 was the heat- and protease-insensitive partner.

Each of the coaggregating streptococcal pairs and the actinomyces pairs was tested for its ability to coaggregate in the presence of saliva. Whole clarified saliva (20) was added to each cell suspension to give 50% saliva before the coaggregating partners were mixed. Strain ATCC 10557 agglutinated and could not be tested for coaggregation. The remaining saliva-suspended strains exhibited results nearly identical to those found with buffer-suspended cells. All coaggregations between streptococci were inhibited by 60 mM GalNAc, and the two lactose-inhibitable coaggregations between the buffer-suspended actinomyces (see above) were also lactose inhibitable with saliva-suspended actinomyces. Thus, saliva does not seem to cause any significant change in the ability of these bacteria to coaggregate. Similar results had been observed in an extensive study of intergeneric coaggregations between streptococci and actinomyces (20).

Further characterization of the GalNAc-inhibitable coaggregations was done by using a radioactivity-based assay in which *S. oralis* 34 was radioactively labeled and its partner cells were unlabeled (Table 3). The sugar was added to the assay mixture containing only one cell type. After vortex mixing, the other cell type was added, and the percentage of input *S. oralis* 34 cells detected in coaggregates was determined. Sugars were tested at several concentrations, and the inhibition with 15 mM sugar is shown in Table 3. GalNAc was the best inhibitor of all coaggregations. In some coaggregations, lactose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, galactose, and rhamnose showed 11 to 23% inhibition. Little or no inhibition was observed with D-galacturonic acid, *N*-acetyl-D-glucosamine, D-fucose, fructose, L-mannose, D-mannose, D-glucose, or sucrose even at 150 mM, the highest concentration tested.

The most unexpected result of this investigation of 122 oral bacterial strains was that intrageneric coaggregation occurred so infrequently. Such coaggregation is a property restricted to most of the streptococcal strains tested and to a

TABLE 2. Intrageneric coaggregations among reference strains of streptococcal coaggregation groups, *S. sanguis* ATCC 10556, *S. oralis* ATCC 10557, and *S. gordonii* ATCC 10558[a]

Strain	Coaggregation score								
	Streptococcus SM PK509 (5)	*S. gordonii*			*S. sanguis*		*S. oralis*		
		ATCC 10558	DL1 (1)	PK488 (6)	ATCC 10556	C104 (3)	34 (3)	ATCC 10557	H1 (2)	J22 (4)
PK509 (5)	0	2	1	2	3	4	4	3	0	0
ATCC 10558		0	0	0	0	4	4	0	0	0
DL1 (1)			0	0	0	3	3	0	0	0
PK488 (6)				0	0	4	3	0	0	0
ATCC 10556					0	3	3	0	0	0
C104 (3)						0	0	0	0	0
34 (3)							0	0	0	0
ATCC 10557								0	0	0
H1 (2)									0	0
J22 (4)										0

[a] The method for assigning coaggregation scores has been described (15). All coaggregations were completely reversed by the addition of GalNAc to a final concentration of 60 mM. The strains are identified in Table 1. Numbers in parentheses indicate the coaggregation group for which the strain is the reference strain.

few of the actinomyces. Even the fusobacteria, which coaggregate with the widest range of genera tested so far (17), did not coaggregate with other fusobacteria.

It is probably no coincidence that only the primary colonizers exhibited intrageneric coaggregations among their strains. It would be to their advantage to be capable not only of intergeneric coaggregation but also of intrageneric coaggregation. We propose that intrageneric coaggregation is an important phase in the accretion of bacteria on a cleaned tooth surface. After cleaning, the tooth surface is immediately coated with the acquired pellicle, a mixture of salivary components of host and bacterial origin. Some of the components have been identified and appear to mediate initial attachment of bacteria to the tooth surface (6). Besides these important host-derived substances, saliva also contains bacterial cell wall fragments and other cell surface components. It is possible that some initial bacterial attachment occurs by the interaction of a primary colonizing cell with cellular debris that is suspended in saliva and hence may become part of the acquired pellicle. It should be noted that the initially adherent bacteria need not be viable or metabolically active. Living cells, dead cells (cells stored in azide-containing coaggregation buffer), and cell walls mediate coaggregation (11, 12) and adhere to saliva-coated spheroidal hydroxyapatite, a model surface of the human tooth (2, 4, 5, 18, 23, 26).

TABLE 3. Inhibition of streptococcal intrageneric coaggregation by sugars[a]

Sugar tested (final concn, 15 mM)	% Inhibition of coaggregation of *S. oralis* 34 with:				
	PK488	DL1	PK509	ATCC 10556	ATCC 10558
Lactose	4	14	4	14	3
N-Acetyl-D-galactosamine	100	88	31	82	100
Methyl-α-D-galactopyranoside	0	12	0	0	0
Methyl-β-D-galactopyranoside	2	14	3	21	10
D-Galactose	1	9	23	14	0
D-Galacturonic acid	0	2	6	0	3
N-Acetyl-D-glucosamine	0	0	0	0	0
L-Rhamnose	2	1	0	11	0
D-Fucose	0	9	0	0	3

[a] *S. oralis* 34 was radioactively labeled with ^{14}C-uracil, and the various partners were unlabeled. The specific radioactivity was about 10^3 bacteria per cpm. Input radioactivity was between 10,000 and 13,000 cpm. Coaggregation in control tubes containing the partners but no sugar was normalized to 100% coaggregation. The percent inhibition by sugar was calculated relative to the control value of coaggregation in the absence of sugar. Duplicate tubes were assayed. The average value is reported, and the assay has a variability of ±5% of the average value (13, 14).

Our studies included three widely used streptococcal strains, *S. sanguis* ATCC 10556, *S. oralis* ATCC 10557, and *Streptococcus gordonii* ATCC 10558, so that comparisons could be made with our reference strains of streptococcal coaggregation groups 1 to 6. The observation that they also exhibit intrageneric coaggregation indicates that this property is a genetically stable property that is not lost by culture manipulations or by extended-storage conditions associated with stock cultures deposited in the culture collection in 1946. Besides intrageneric coaggregation, these streptococci also coaggregate with actinomyces. When tested against the reference strains of the actinomyces coaggregation groups, strains ATCC 10556 and ATCC 10558 coaggregated only with *A. naeslundii* PK606 (reference strain for actinomyces coaggregation group D), which is identical to the partner specificity exhibited by *S. gordonii* PK488, the reference strain for streptococcal coaggregation group 6 (11, 12). In contrast, strain ATCC 10557 coaggregated with all actinomyces except *A. naeslundii* PK984, the reference strain for actinomyces coaggregation group E. This coaggregation pattern is very similar to that of reference strains of streptococcal coaggregation group 3. Thus, it appears that strains from the American Type Culture Collection (Rockville, Md.) can be accommodated within our current classification scheme (11, 12).

The intrageneric coaggregations between the streptococci are very specific and are shown diagrammatically in Fig. 1. *Streptococcus* SM PK509 is a partner of all the others, but the mechanisms appear to be slightly different. While GalNAc inhibits all interactions, other sugars inhibit coaggregation with different efficacies, and tests of partner specificity and protease sensitivity indicate differences in the mechanisms of the interactions. The observation that only a few sugars inhibit most coaggregations suggests that adhesins mediating these coaggregations recognize carbohydrate receptors by functionally related mechanisms. The potential role of the adhesin-receptor interactions in surface colonization by human oral bacteria will become clearer when purified adhesins and carbohydrate receptors are available

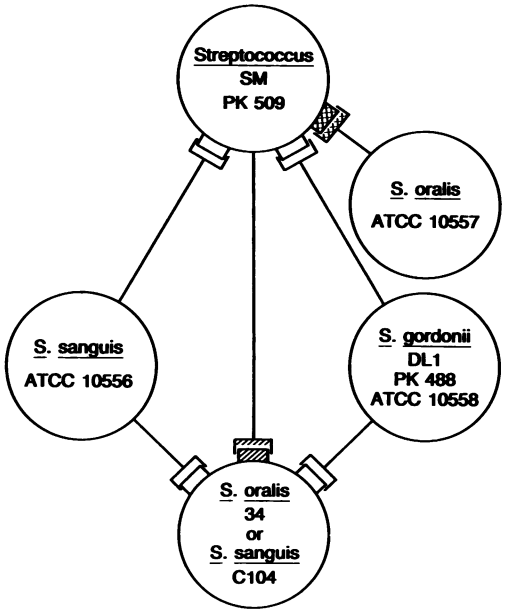

FIG. 1. Diagrammatic representation of the intrageneric coaggregations among human oral viridans streptococci. Each interaction is depicted as a complementary set of symbols. Identical symbols do not necessarily represent the same structure but are used here to show the simplest model. The symbols attached to circles with stems represent heat- and protease-inactivated components which are probably lectinlike proteins, since GalNAc inhibits all of these interactions. The complementary symbols without stems (i.e., attached directly to circles) represent components which are resistant to heat and protease and which are thought to be carbohydrate-bearing receptors. The symbols (open, hatched, and crosshatched) are drawn to be similar but not identical. See text for details.

for study and can be used either to mediate or to interdict colonization by the oral bacteria.

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