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Effect of Saliva on Coaggregation of Oral *Actinomyces* and *Streptococcus* Species

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Human oral actinomyces and streptococci that exhibit specific coaggregation patterns when the cells are suspended in buffer were tested for their ability to coaggregate in saliva. Of 53 paired combinations of actinomyces (*Actinomyces viscosus*, *A. naeslundii*, or *Actinomyces* sp. WVa 963) and streptococci (*Streptococcus sanguis* or *S. morbillorum*) that exhibited coaggregation in buffer, all but 4 pairs also coaggregated when suspended in saliva. Twenty-four pairs exhibited lactose-inhibited coaggregation in buffer: 19 of these were identical in saliva. The other five pairs either did not coaggregate or formed coaggregates that were not inhibited by lactose. Highly specific coaggregations known to occur with buffer-suspended cells (e.g., a streptococcal strain that coaggregates with a single strain of actinomyces) were unchanged when cells were suspended in saliva. These results indicate that the coaggregation properties of both oral actinomyces and streptococci are very similar with cells suspended in either saliva or coaggregation buffer. Thus, the potential for coaggregation among bacteria in the oral cavity is evident. The possible mechanisms which mediate coaggregation in saliva are discussed.

Saliva is a complex mixture of a wide variety of components which include proteins such as enzymes, immunoglobulins, and mucins. Molecules that represent each of these three general classes of components, lysozyme (21), secretory immunoglobulin A (1, 15), and sialoglycoproteins (14, 18), respectively, have been implicated as salivary aggregating factors causing aggregation of oral bacteria.

Saliva-mediated bacterial aggregation has been reported by numerous laboratories, and there appears to be selectivity in this phenomenon. For example, some strains of oral bacteria are agglutinated by saliva and some are not (8, 15, 23). Some that are agglutinated by unheated saliva are not agglutinated by heated saliva (18). In fact, Rosan et al. (23) reported that heating saliva above 50°C significantly reduced its ability to aggregate *Streptococcus sanguis* strains G9B and M5. Certain *S. sanguis* strains (e.g., M5 and ATCC 10556) were agglutinated strongly in experiments conducted in one laboratory (16) but weakly or not at all in studies done by others (15, 18). Furthermore, a survey of saliva samples from 150 adult humans revealed no aggregating activity for *S. sanguis* M5 with some samples, whereas others varied from weak to very strong (16). Some saliva samples that aggregated *S. sangius* M5 failed to aggregate *S. mutans* LM7. Generally, saliva that exhibited high activity for *S. sanguis* M5 also possessed high activity for other strains of *S. sanguis*, *S. mitis*, and *S. salivarius*. Thus, it appears that the mechanisms involved in saliva-mediated bacterial aggregation are multiple and ill defined.

During the course of our studies of coaggregation (intergeneric aggregation) between oral streptococci and actinomyces, we became interested in the possible effect that saliva might have on these cell-to-cell recognition reactions. Large numbers of coaggregating pairs of stock cultures (5) and fresh isolates (11–13) have been previously examined, and specific coaggregation patterns were described. Based on these patterns, six coaggregation groups of actinomyces (groups A to F) and six streptococcal coaggregation groups (groups 1 to 6) were delineated. An actinomyces (or streptococcus) coaggregation group is characterized by (i) the presence or absence of coaggregation between its members and representatives of the six streptococcal groups (or six actinomyces groups), (ii) the effect of heating cells on the ability of the heated cell type to coaggregate, and (iii) the effect of adding lactose to inhibit coaggregation. In all of these studies the coaggregation reactions were conducted with cells suspended in Tris buffer. All of the strains were of human oral origin and were tested here for their ability to coaggregate when suspended in saliva. The coaggregation properties of cells suspended in saliva were compared with those characterized previously by using cells suspended in buffer.

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MATERIALS AND METHODS

Bacterial strains. All strains were of human origin. Each of the 12 strains served as a reagent strain representing one of the six actinomyces and six streptococcal coaggregation groups. Their identity is given in footnotes *a* and *d* to Table 1. The growth medium contained tryptone, yeast extract, Tween 80, and glucose (0.2%) and was buffered to pH 7.5 with K_2HPO_4 (17). Cultures in screw-capped bottles were incubated at 37°C without shaking, and the cells were harvested and prepared for coaggregation as described previously (5).

Saliva collection and clarification. Saliva was collected in a beaker set in ice and was clarified by centrifugation at 12,000 × *g* for 30 min at 4°C. The clarified supernatant fluid was either used immediately or stored at −20°C until needed. If frozen, the clarified saliva was centrifuged as above before use.

Coaggregation assay. Reagent strains were suspended to a density of about 5×10^9 cells per ml in 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). Equal volumes of cell suspensions of a pair of the two cell types were mixed by blending on a Vortex mixer, and coaggregation was monitored by a previously described visual assay (5). The degree of result-

TABLE 1. Coaggregation patterns of buffer-suspended and saliva-suspended cells of strains representing actinomyces and streptococcal coaggregation groups

Actinomyces coaggregation group[a]	Heated cell suspension[b]		Coaggregation score[c] after reaction with streptococcal group[d]:											
			In buffer						In saliva					
	Streptococcus	Actinomyces	1	2	3	4	5	6	1	2	3	4	5	6
A	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	3[e]	4[e]	0	0	0	0	3[e]	3[e]	0	0
	No	Yes	3	4	0	3	0	0	3	1	0	0	0	0
	No	No	3	4	3[e]	4	0	0	3	1	3[e]	4	0	0
B	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	4[e]	4[e]	3[e]	0	0	0	3[e]	4[e]	3[e]	0
	No	Yes	0	2	0	0	0	0	1	1	0	0	0	0
	No	No	0	2	4[e]	4[e]	3[e]	0	1	1	3[e]	4[e]	3[e]	0
C	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	4[e]	3[e]	2[e]	0	0	0	4[e]	3[e]	1[e]	0
	No	Yes	3[e]	3	1	0	0	0	3	3	1	0	0	0
	No	No	3[e]	3	4[e]	3[e]	2[e]	0	3	3	4[e]	3[e]	2[e]	0
D	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	2[e]	2[e]	3[e]	0	0	0	0	0	2[e]	0
	No	Yes	4	4	4	4	3	3	4	2	4	4	4	4
	No	No	4	4	4	4	3	3	4	2	4	4	4	4
E	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	0	0	0	0	0	0	0	0	0	0
	No	Yes	4	4	0	4	0	0	4	4	0	3	0	0
	No	No	4	4	0	4	0	0	4	4	0	3	0	0
F	Yes	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	Yes	No	0	0	2[e]	3[e]	0	0	0	0	0	1[e]	0	0
	No	Yes	0	0	0	0	0	0	0	0	0	0	0	0
	No	No	0	0	2[e]	3[e]	0	0	0	0	2[e]	1[e]	0	0

[a] The representatives of actinomyces groups A through F used were *A. viscosus* T14V, *A. naeslundii* 1, *A. naeslundii* PK602, *A. naeslundii* PK 606, *A. viscosus* T14AV, and *Actinomyces* sp. WVa 963 (VPI D33C-25), respectively.

[b] Cell suspension was heated at 85°C for 30 min.

[c] 0 for no coaggregation to 4 for maximum coaggregation.

[d] The representatives of streptococcal groups 1 through 6 used were *S. sanguis* DL1 (NCTC 7868), H1, 34, and J22, *S. morbillorum* PK 509, and *S. sanguis* (VPI E1A-1A), respectively.

[e] Coaggregation was reversed by 0.06 M (final concentration) lactose.

ant coaggregation was assigned a score from 0 to 4. Absence of visible coaggregation, viewed as an evenly turbid suspension, was scored as 0, and the maximum coaggregation consisting of a clear supernatant and large settled coaggregates was scored as 4. All coaggregation complexes were reversed by 0.6 mM (final concentration) EDTA. Reversal of coaggregation was defined as the complete disappearance of bacterial coaggregates. The ability of lactose to reverse some coaggregations was determined after the addition of 1 M lactose to give a final concentration of 0.06 M.

For studies involving aggregation of cells suspended in saliva, a volume of buffer-suspended cells was subjected to centrifugation (12,000 × *g* for 15 min), and the pelleted cells were resuspended to the original volume with clarified saliva. Saliva-suspended cells were tested for coaggregation as described above for buffer-suspended cells. Saliva samples from three individuals were tested independently by suspending separately each partner of a coaggregation pair (*S. sanguis* 34 and *Actinomyces viscosus* T14V) and then mixing the pair together. This kind of coaggregation reaction is reversed by adding lactose, and no difference in the three saliva samples was detected. After a coaggregation reaction that is not lactose reversible was also tested and no difference in the three saliva samples was noted, the remainder of the coaggregation pairs was tested with clarified saliva from a single donor. Considering that cells suspended in saliva may aggregate and thus interfere with observations of coaggregation, we examined each suspension of cells in saliva for visible aggregation. Using whole, unstimulated, and unheated saliva, we observed a weak aggregating activity with a few of the strains but none with the others. In those few cases where weak aggregation occurred, it was easily distinguished from the much stronger coaggregation reaction.

RESULTS

Coaggregation of cells suspended in buffer and in saliva. Extensive studies aimed at defining the coaggregation properties of oral actinomyces and streptococci revealed that all *A. viscosus* strains and about 90% of *A. naeslundii* strains coaggregated with *S. sanguis* when cells were suspended in coaggregation buffer (5, 11–13). Each strain exhibited characteristic coaggregation properties, and it was of interest to determine the effect of saliva on the expression of these properties. The first coaggregating pair chosen for this comparison was *A. viscosus* T14V and *S. sanguis* 34, a coaggregation reported by McIntire et al. (19) that is inhibited by lactose (Fig. 1). Moderate coaggregation with some settled coaggregates was observed with buffer-suspended

cells, and saliva-suspended cells exhibited an equal or greater degree of coaggregation. Addition of lactose reversed coaggregation in both cases. Heating the saliva at 60°C for 30 min reduced slightly the degree of coaggregation but had no effect on the ability of lactose to reverse coaggregation. Fivefold dilution of cell suspensions in either coaggregation buffer or saliva did not change the coaggregation score or lactose reversibility. Coaggregation was detectable even at 10-fold dilutions, although the cell suspensions were low in turbidity and approached the limits of the visual assay used in this study.

Saliva-mediated aggregation versus coaggregation in saliva. To examine the possibility that saliva-mediated aggregation of oral bacteria may interfere with observations of coaggregation, each of the 12 reagent strains was suspended in saliva and examined for aggregation. A weak aggregation was observed with a few strains but most showed none. An example of weak aggregation is seen in Fig. 2. A slightly granular apearance in the saliva-suspended *S. sanguis* 34 (Fig. 2A, tube a) and *A. viscosus* T14V (Fig. 2A, tube b) was detectable but was clearly different from the obvious coaggregation that occurred when the two cell types were mixed together (Fig. 2A, tube c). Coaggregation was visible immediately after mixing (Fig. 2A, tube c) and was complete after 30 min (Fig. 2B, tube c), when nearly all of the coaggregates had settled to the bottom of the tube. On the other hand, aggregation of either the streptococcus or the actinomyces was not detectably changed after 30 min (Fig. 2B, tubes a and b) and remained weak even after standing overnight at room temperature. When viewed microscopically, cell suspensions of the streptococci and actinomyces in saliva appeared as single cells, pairs, or small clumps (Fig. 3A and B), but when mixed together, large coaggregates were formed (Fig. 3C). Addition of lactose reversed coaggregate formation and individual cells were readily observed (Fig. 3D).

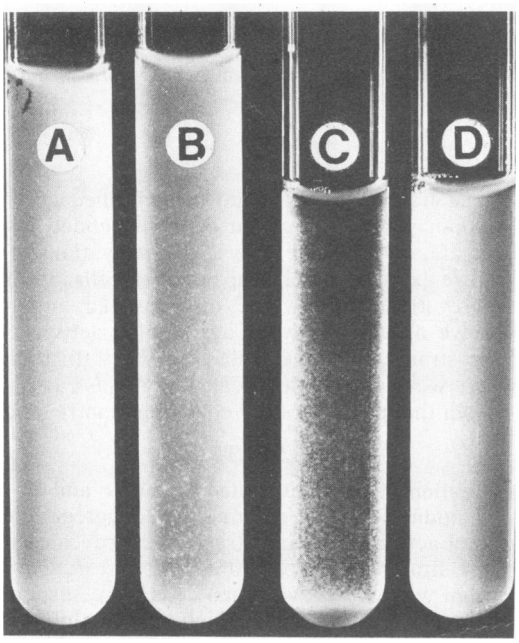

FIG. 1. Coaggregation of *A. viscosus* T14V and *S. sanguis* 34 in coaggregation buffer and in saliva. Equal volumes of cell suspensions (about 5×10^9 cells per ml; 260 Klett units, red filter) in buffer (tubes A and B) or saliva (tubes C and D) were mixed and allowed to stand for 5 min. Lactose was added to a final concentration of 100 mM to tubes A and D. All tubes were vortexed, allowed to stand for 1 to 2 min, and then photographed.

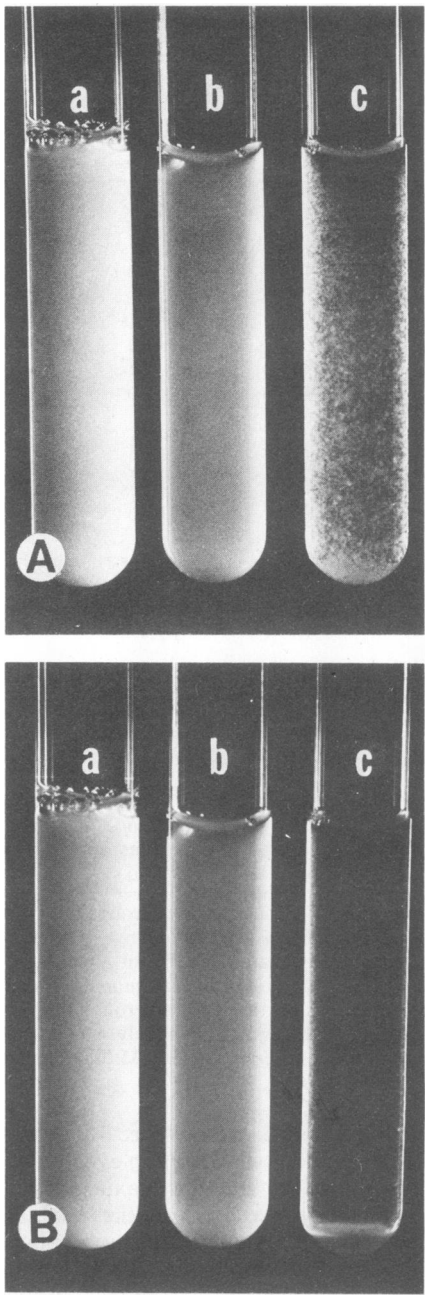

FIG. 2. Saliva-mediated aggregation versus coaggregation in saliva. *S. sanguis* 34 (tube a) and *A. viscosus* T14V (tube b) were adjusted to a cell density of about 5×10^9 cells per ml in saliva. Equal volumes of the two cell types were mixed in tube c. Each tube was mixed vigorously, and a photograph was taken immediately after mixing (A) and after standing for 30 min at room temperature (B). Identical exposure conditions for photography were used for (A) and (B). The test tube size was 13 by 100 mm.

Analysis of reagent strain coaggregations in buffer versus saliva. To investigate more thoroughly the potential effect of saliva on coaggregations between oral actinomyces and streptococci, all of the known coaggregation patterns observed in buffer were also tested in saliva (Table 1). This

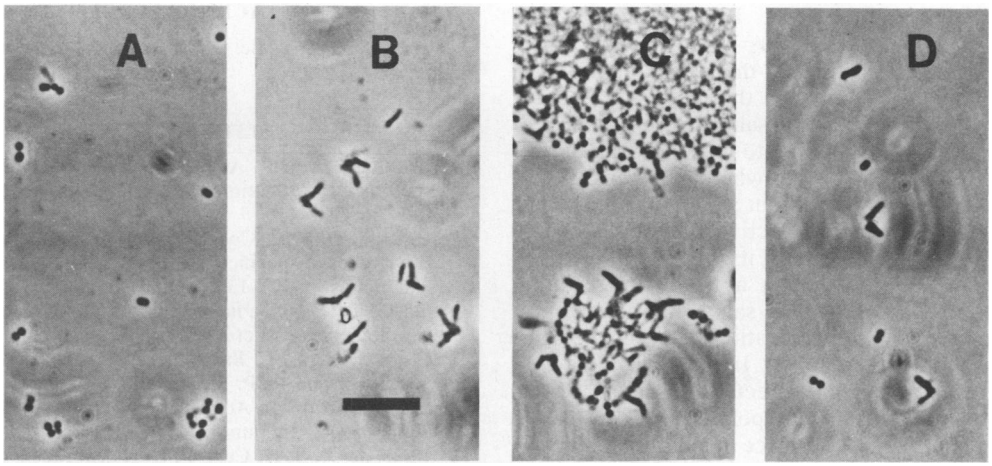

FIG. 3. Phase-contrast microscopy of saliva-suspended cells of *S. sanguis* 34 (A) and *A. viscosus* T14V (B) and a mixture of the two cell types before (C) and after (D) the addition of lactose (final concentration, 100 mM). Bar, 10 mm.

included unimodal coaggregations (heating of one cell type at 85°C for 30 min before mixing prevents coaggregation but the same treatment of the other cell type has no effect) and bimodal coaggregations (heat treatment of both cell types is required to block coaggregation). In buffer there were 144 pairings of which 53 were known to exhibit visible coaggregation. Of these, 49 occurred in saliva. Identical coaggregations were found in most pairings. It is significant that, with one exception (a very weak coaggregation between actinomyces group B and streptococcal group 1), no new coaggregations occurred in saliva that did not occur in buffer, which indicates that saliva does not mediate coaggregations with these bacteria. With two exceptions, pairing between unheated cells in both buffer and saliva exhibited the same coaggregation properties, which are: (i) coaggregations that occurred in buffer also occurred in saliva; (ii) pairs that did not coaggregate in buffer also did not coaggregate in saliva (exception noted above); and (iii) coaggregations that were lactose reversible in buffer were similarly reversible in saliva (one difference here is absence of lactose reversibility between actinomyces group C and streptococcal group 1 in saliva). In addition, in unimodal coaggregations where streptococci were heat inactivated in buffer, they were also inactivated in saliva (e.g., group 1, 2, and 6 streptococci), and where actinomyces were heat inactivated in buffer, they were also inactivated in saliva (e.g., group B actinomyces with group 3, 4, and 5 streptococci). In fact, unimodal coaggregations as a group were only slightly different with respect to coaggregation properties in buffer or saliva. The two differences were in actinomyces group F with streptococcal group 3 when the streptococcus was heated and, as noted above, the coaggregation between actinomyces group C and streptococcal group 1.

Among the five bimodal coaggregations observed in buffer, two were unchanged in saliva (actinomyces group C with streptoccal group 3 and actinomyces group D with streptococcal group 4). In the coaggregation between actinomyces group A and streptococcal group 4, the coaggregation with heated actinomyces cells did not occur in saliva. The other two cases where differences were observed involved actinomyces group D and heated streptococcal groups 3 and 4. These coaggregations were lactose reversible in buffer but absent in saliva.

Lactose-reversible coaggregations are widespread among these oral actinomyces and streptococci. In 24 of 53 coaggregating pairs in buffer, lactose-reversible coaggregation was observed, and in 19 of these, it was found in saliva. Only one of the five coaggregations altered in the presence of saliva involved unheated cells (actinomyces group C with streptococcal group 1). The others involved either heated streptococcus or heated actinomyces. Thus, saliva does not inhibit most lactose-reversible coaggregations, and of those that are inhibited most require heating the cells to see an effect.

DISCUSSION

The results of this study indicate clearly that the coaggregation reactions between actinomyces and streptococci that were described previously to occur in buffer (5, 11–13) are, with minor exceptions, unaffected by saliva. Moreover, no consistent change in coaggregation properties of coaggregating pairs could be attributed to suspending cells in saliva. Only a few unimodal and bimodal coaggregations were altered with saliva suspensions of cells. Some lactose-reversible coaggregations that occurred in buffer did not occur in saliva. Others still coaggregated but were not lactose reversible. Two coaggregations were notably reduced in saliva and may be due to the presence of sialoglycoproteins which are known to be constituents of whole saliva (6, 14). Both of these coaggregations involved streptococcal group 2 with either actinomyces group A or group D. Recent studies have indicated sialic acid-inhibited coaggregations between (1) group 2 streptococci and a bacteriophage-resistant mutant of a member of actinomyces group A (C. A. Tylenda, P. E. Kolenbrander, and A. L. Delisle, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, p. 68, D56) and (ii) a coaggregation-defective mutant (10) of group 2 and group D actinomyces (P. E. Kolenbrander, Abstr. Annu. Meet. Am. Soc. Microbiol., 1982, p. 34). These coaggregations may be mediated by a sialic acid-sensitive lectin on the streptococcus similar to the lectin recently reported on *S. sanguis* (20).

It is significant that most lactose-reversible coaggregations (19 of 24) with buffer-suspended cells were also observed with cells suspended in saliva. Some were reduced in coaggregation score but others were unchanged. Glycoproteins with galactose termini are common in whole saliva (14). Soluble molecules like these could interfere with lactose-reversible coaggregations. Inhibition by saliva of these lactose-reversible coaggregations has been reported (K. Ko-

miyama and R. J. Gibbons, J. Dent. Res. [special issue], Abstr. 836, p. 271, 1982), and it was suggested that such inhibitions decreased the likelihood that these coaggregations occur to a significant extent in the mouth (7). Those experiments showing inhibition by saliva were conducted with the actinomyces cells bound to a solid support of spermine-conjugated agarose beads, whereas our investigation was with cell suspensions without a supporting matrix. In our system saliva does not selectively inhibit lactose-reversible coaggregations nor does it inhibit all lactose-reversible coaggregations. Although a few coaggregating pairs were inhibited when tested in saliva, they were not limited to lactose-reversible coaggregations (e.g., coaggregations involving group 2 streptococci). In fact, most lactose-reversible coaggregations were unaffected by saliva.

In a recent review Cisar (2) has pointed out how such coaggregations could readily take place in a natural environment that contains soluble inhibitory molecules. The mechanism is based on the existence of multiple low-affinity lectin sites on the actinomyces cell surface. Evidence in support of this view comes from experiments with purified cell surface fimbriae from *A. viscosus* T14V, the reagent strain used here to represent actinomyces coaggregation group A. These fimbriae possess lactose-sensitive lectin activity (3, 4, 22) and mediate the lactose-reversible coaggregation with *S. sanguis* 34 (coaggregation group 3) and *S. sanguis* J22 (coaggregation group 4) (10). Purified fimbriae bind with low affinity (J. Cisar, personal communication) but are unable to agglutinate streptococcal cells. However, when the fimbriae are complexed with monoclonal (or monospecific) antibody (3) or adsorbed to latex beads (9), strong agglutination of streptococci is observed. Considering the strong coaggregation observed between actinomyces coaggregation group A and streptococcal group 3 or 4, it seems probable that this occurs by the binding of many lectin-combining sites on one actinomyces cell to many receptors on a streptococcus. A multivalent low-affinity mechanism would be expected to operate efficiently in a natural environment containing soluble inhibitor molecules such as salivary asialoglycoproteins. Whereas high-affinity lectin sites would be blocked by soluble inhibitors, a multivalent low-affinity system of cell-to-cell interactions would allow many lectin sites to remain unoccupied and therefore would be available for surface-to-surface adherence. Our results favor the latter hypothesis as an explanation for the occurrence of lactose-reversible and sialic acid-sensitive coaggregations in saliva which is rich in potential inhibitors, asialoglycoproteins containing galactose termini and sialoglycoproteins, respectively.

We observed no profound effect by saliva on coaggregation between oral actinomyces and streptococci. Moreover, the data clearly show that saliva-mediated aggregation of bacteria is quite distinct from coaggregation: coaggregation is not mediated by saliva. Where aggregation was observed, it was weak by comparison to coaggregation and it was not an obstacle in this investigation. Coaggregations in saliva exhibited the same kind of specificity reported earlier in buffer systems: (i) group E actinomyces only coaggregate with streptococcal groups 1, 2, and 4; (ii) group 6 streptococci only coaggregate with group D actinomyces; (iii) group F actinomyces only coaggregate with group 3 and 4 streptococci; (iv) streptococcal groups 1 and 2 and actinomyces group E and F participate only in unimodal coaggregations; and (v) lactose-reversible coaggregations are generally preserved. Collectively, our results are fully consistent with the likelihood that coaggregation properties studied in vitro accurately reflect those that occur in vivo.

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