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Cell-to-Cell Interaction of *Streptococcus sanguis* and *Propionibacterium acnes* on Saliva-Coated Hydroxyapatite

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Cell-to-cell interaction (coaggregation) between *Propionibacterium acnes* PK93 and *Streptococcus sanguis* DL1 was measured on saliva-coated hydroxyapatite beads (SHA) at bacterial concentrations between 1.3×10^6 and 6.7×10^8 cells per ml. Four hundredfold more DL1 than PK93 cells adhered to the saliva-coated beads, and the adherence of *S. sanguis* was proportional to cell input. SHA precoated with 3×10^8 DL1 cells bound 75 to 80% of available PK93 cells at all input amounts tested, up to an input of 8×10^7 cells. Adherence of PK93 to DL1-coated SHA approached saturation at an input of approximately 10^9 PK93 cells, when 1.5×10^8 bound. The coaggregation on SHA occurred either in buffer or saliva and was inhibited by *N*-acetylgalactosamine and by lactose; the attachment of DL1 to SHA was not inhibited by these sugars. *S. sanguis* 34 and heat-treated DL1 cells, neither of which form coaggregates with PK93, attached to SHA, but such cells did not bind PK93 cells. The findings of this study indicate that bacteria unable to attach to saliva-coated hydroxyapatite can indeed adhere to such a surface by strong lectin-mediated cell-to-cell interactions with bacteria already attached to the surface.

The initial stage in the bacterial colonization of the tooth is the adherence of certain species to a coat of salivary molecules (the acquired pellicle) adsorbed to the enamel surface (for a review, see references 7 and 9). After the establishment of this initial dental plaque, the colonization of other bacteria is determined by their ability to adhere to the developing plaque. The concept of surface-supported interbacterial adherence was demonstrated by Bladen et al. (2), who reported that veillonellae which did not attach to a wire surface did attach to an *Actinomyces viscosus* coating on this surface. The adherence of one bacterial species to another to form highly specific coaggregations was first observed by Gibbons and Nygaard (8); these interactions were suggested to be important in the sequential colonization of the tooth by different bacteria. Cell-to-cell interactions between distinct bacteria have also been reported to occur in vivo (7, 10, 23). Since the early study by Gibbons and Nygaard (8), numerous coaggregations between different genera of bacteria have been identified which show a high degree of specificity (3, 4, 6, 11–16), involve lectinlike proteins (3, 4, 12–17), and occur in the presence of human saliva (11, 14, 18). McIntire et al. (21) were first to report that coaggregations between oral actinomyces and streptococci involved a lectin-carbohydrate interaction which was inhibited by lactose. To date, lactose-sensitive coaggregations have been the most common type observed between the different species and strains of actinomyces and streptococci studied (4, 13, 15). In a recent study (15) which investigated intergeneric coaggregations of oral isolates of *Actinomyces* and *Actinomyces*-like microorganisms with streptococci, a lactose-sensitive coaggregation between *Propionibacterium acnes* and coaggregation group 1 streptococci (of six groups identified) was reported. The purpose of the present report is to describe a method to measure a galactoside-sensitive, intergeneric coaggregation between *P. acnes* PK93 and *Streptococcus sanguis* DL1 on saliva-coated hydroxyapatite beads (SHA). In addition, the influence of human saliva on this surface-supported coaggregation was studied.

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MATERIALS AND METHODS

Bacterial strains. *P. acnes* PK93 and *S. sanguis* DL1 were harvested from complex media at early stationary phase as described previously (12, 15). To obtain ^{3}H-labeled cells, the media also contained [*methyl*-^{3}H]thymidine (10 μCi/ml; New England Nuclear Corp., Boston, Mass.). The bacteria were washed three times and suspended in coaggregation buffer (4) (CAB; 1 mM Tris hydrochloride [pH 8.0]–0.1 mM $CaCl_2$–0.1 mM $MgCl_2$–0.15 M NaCl–0.02% NaN_3). The suspensions were passed through a 27-gauge needle several times to disrupt bacterial aggregates and chains. The resulting preparations primarily contained units of less than three cells. To substantially reduce soluble bacterial components, the bacteria were centrifuged at 5,000 × *g* for 10 min and resuspended in fresh buffer to the proper concentrations just before use. By direct microscopic counting, bacterial suspensions with an A_{660} of 1.2 were estimated to contain approximately 3×10^9 to 5×10^9 cells per ml by using a Stasar II spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Saliva collection. Paraffin-stimulated, human whole saliva was collected in beakers placed in ice and clarified by centrifugation at 12,000 × *g* for 15 min. Saliva was pooled from two or three persons and either used within 2 h of collection or rapidly frozen in dry ice, stored at −20°C, and thawed before use.

Aggregation assay. Saliva-induced bacterial aggregation was measured as the rate of sedimentation of aggregates in 40% saliva (vol/vol in CAB) by recording changes in turbidity at 660 nm by using a Stasar II spectrophotometer. Bacteria (approximately 2×10^9 cells) and saliva were mixed in a final volume of 0.4 ml in a semimicrocuvette. Controls contained CAB in place of saliva.

Coaggregation assay. Equal amounts (approximately 10^9 cells) of the two bacterial species in a final volume of 0.4 ml were mixed vigorously for 10 s (4) in semimicrocuvettes. In some experiments, 40% saliva replaced CAB. Coaggregation was determined by measuring the change in turbidity at 660 nm caused by sedimentation of coaggregates.

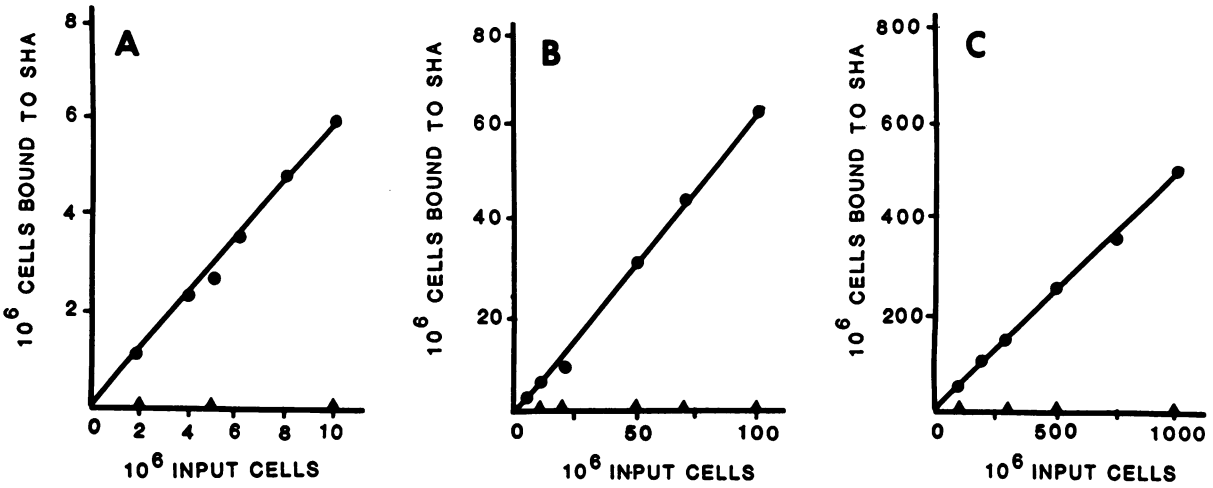

FIG. 1. Adherence of ^{3}H-labeled *S. sanguis* DL1 (●) and ^{3}H-labeled *P. acnes* PK93 (▲) cells to SHA at cell inputs between 2×10^6 and 1×10^9 cells per 1.5 ml. HA (50 mg) were precoated with whole human saliva before treatment with bacteria as described in the text.

SHA adherence assays. Various amounts of ^{3}H-labeled bacteria suspended in CAB were mixed with SHA, and the number of radioactive bacteria adhering to the SHA was determined by a modification of a previously described method (5). Briefly, 50 mg of hydroxyapatite beads (HA) (BDH Biochemicals, Poole, England) was placed in 1.5-ml plastic microcentrifuge tubes (Sarstedt Inc., Princeton, N.J.) and washed three times with CAB to remove fines. Clarified saliva (1.5 ml) was mixed at room temperature with the washed beads on a rotator for 60 min (19 rpm; Drummond Scientific Co., Boomall, Pa.). The SHA were then washed three times with CAB to remove nonadsorbed or loosely adsorbed salivary material. The washed SHA were rotated at room temperature with 1.5 ml of ^{3}H-labeled bacteria for 30 min. The bacteria-coated SHA were allowed to settle (approximately 5 s), the supernatant fluid was removed within 30 s, and the SHA were washed three times with CAB to remove nonadherent and loosely adherent bacteria. The SHA were then transferred with distilled water into a screw-cap vial. All but 0.5 ml of the water was removed, 10 ml of Hydrofluor (National Diagnostics, Somerville, N.J.) was added, and the ^{3}H-labeled bacteria adhering to the SHA were counted in a liquid scintillation counter. To measure surface-supported coaggregation, nonlabeled bacteria were allowed to bind to SHA. After three washes with CAB, the SHA with attached bacteria were then mixed for 30 min on the rotator with various amounts of ^{3}H-labeled coaggregation partner. The number of disintegrations per minute of ^{3}H-labeled bacteria adhering to the bacteria-coated SHA was measured after transferring the SHA to vials as described above. In the present experiments, for 10^9 ^{3}H-labeled DL1 and ^{3}H-labeled PK93, the range was estimated by liquid scintillation counting to be from 2.7×10^5 to 3.2×10^5 and from 3.9×10^5 to 4.5×10^5 dpm, respectively. The number of ^{3}H-labeled bacteria bound to the SHA was determined from the total disintegrations per minute of mixtures of known amounts of ^{3}H-labeled cells and SHA and the disintegrations per minute associated with the SHA (5). By this method, the numbers of cells bound are considered to be approximations. Experiments to measure the adherence of several different concentrations of ^{3}H-labeled *P. acnes* PK93 directly to SHA and to *S. sanguis* DL1-coated SHA and experiments to measure the adherence of ^{3}H-labeled DL1 to SHA were performed at least three times. All other types of experiments were carried out at least two times. Many of the assays were done with duplicate or triplicate samples. In almost all instances, standard deviations of less than 9.5% were determined for replicate samples or repeat experiments. Results of representative experiments are shown. Saliva collected from three individuals tested separately or as a pool produced similar results in SHA adherence and coaggregation experiments.

RESULTS

Adherence of *S. sanguis* DL1 and *P. acnes* PK93 to SHA. The adherence of *S. sanguis* DL1 and *P. acnes* PK93 to 50 mg of SHA was tested over a 500-fold range of bacterial concentrations at cell inputs between 2×10^6 and 1×10^9 cells per 1.5-ml reaction volume (Fig. 1). The lower concentrations shown in Fig. 1A are those reported to be present in human saliva for some types of oral bacteria. Within each cell concentration range (Fig. 1), the attachment of *S. sanguis* appeared proportional to cell input, in that 50 to 60% of the cells bound. In contrast, ^{3}H-labeled *P. acnes* cells adhered poorly (Fig. 1). At an input of 10^9 PK93 cells, only 1.1×10^6 bound, approximately 400 times less than the number of DL1 cells bound at the same cell input.

Adherence of PK93 to DL1-coated SHA. Taking advantage of the 400-fold difference in the adherence of the two coaggregation partner strains to SHA, we tested the ability of PK93 to adhere to SHA which were precoated with 3×10^8 DL1 cells (Fig. 2). The number of *P. acnes* cells bound to DL1-coated SHA was proportional up to an input of 8×10^7 PK93 cells, when approximately 6×10^7 (75%) of the bacteria bound. Above this cell concentration, the relative proportion of PK93 attached to DL1-coated SHA decreased until saturation was approached at an input of 10^9 PK93, when only 1.5×10^8 cells (15%) adhered (Fig. 2C). However, when the same amount of SHA (50 mg of HA) was precoated with 3×10^9 DL1 cells, the number of PK93 cells attached was again proportional to an input of at least 10^9 PK93 (Fig. 2C). At this input, approximately 8.5×10^8 PK93 cells (85% of input number) bound to the beads.

Effect of lactose and GalNAc on the adherence of PK93 to DL1-coated SHA. The coaggregation of *P. acnes* PK93 and *S. sanguis* DL1 is inhibited by galactosides and is prevented by heating (85°C for 30 min) DL1 but not PK93 cells (J. E.

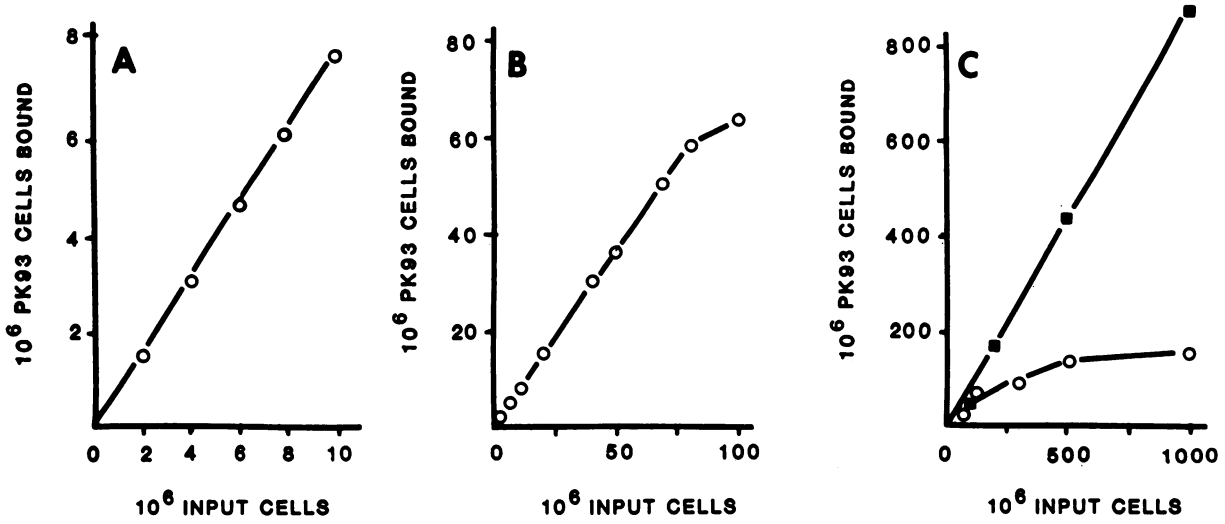

FIG. 2. Adherence of ^{3}H-labeled *P. acnes* PK93 to *S. sanguis* DL1-coated SHA at PK93 cell inputs between 2×10^6 and 1×10^9 cells per 1.5 ml. SHA were coated with 3×10^8 (○) or 3×10^9 (■) unlabeled DL1 cells before treatment with ^{3}H-labeled PK93. Coating with 3×10^8 DL1 and with 3×10^9 DL1 cells was done by treating SHA with 5×10^8 and 4×10^{10} cells, respectively.

Ciardi, G. McCray, P. E. Kolenbrander, R. N. Andersen, and A. Lau, J. Dent. Res. **64**:332, abstr. no. 1414, 1985). After the attachment of unlabeled DL1 cells to SHA, the adherence of ^{3}H-labeled PK93 cells to the surface-associated DL1 in the presence of lactose or *N*-acetylgalactosamine (GalNAc) was compared with that observed in the presence of mannose, one of many sugars found not to inhibit coaggregation. Mannose at a concentration of 150 mM did not change the level of adherence of PK93 cells to DL1-coated SHA observed for buffer controls (Fig. 3A). At a PK93 input of 10^7 cells, 50 mM GalNAc caused a 96% inhibition of adherence, whereas 150 and 100 mM lactose caused 67 and 43% inhibition, respectively. At the higher cell input of 10^8 PK93 (Fig. 3B), inhibition by 100 mM lactose and 50 mM GalNAc was somewhat less, 27 and 92%, respectively. In contrast, adherence of *S. sanguis* DL1 to SHA was not affected by either 200 mM lactose or 50 mM GalNAc (data not shown).

Adherence of ^{3}H-labeled PK93 cells to SHA coated with *S. sanguis* 34 and heat-treated DL1 cells. To test the unlikely possibility that the precoating of SHA with bacteria modified the surface environment to allow or promote the binding of PK93 cells by a mechanism other than interbacterial adherence, we examined the effect of precoating the SHA with *S. sanguis* 34 or with heat-treated DL1, neither of which forms coaggregates with *P. acnes* PK93. PK93 cells did not attach to SHA that were coated with 4×10^7 cells of the noncoaggregating strain *S. sanguis* 34 (Fig. 4). At higher PK93 cell inputs of between 2×10^7 and 1×10^8 cells, PK93 still did not adhere to the *S. sanguis* 34-coated SHA (results not shown). For comparison, the number of PK93 cells bound to SHA coated with 3×10^7 DL1 cells was proportional up to a PK93 input of 10^7 cells, when 3×10^6 PK93 bound. In other experiments (Table 1), heat-treated DL1 cells adhered to SHA (10 mg of HA) in lower numbers than did untreated cells. However, the heat-treated cells attached to the SHA

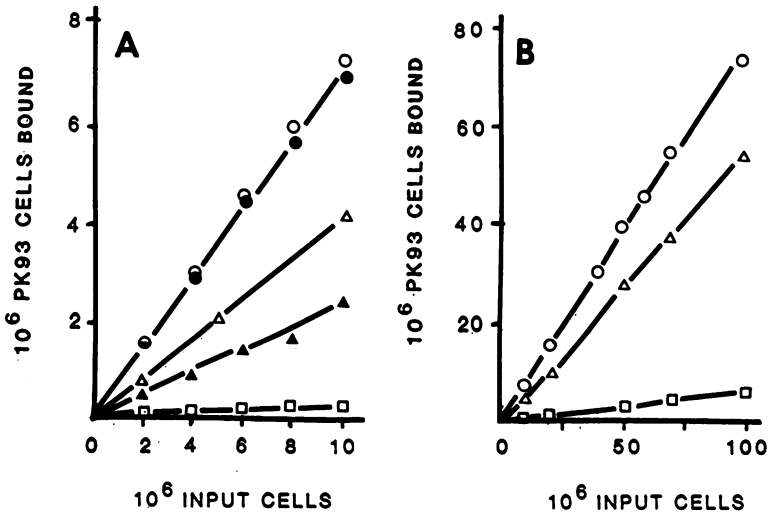

FIG. 3. Effect of mannose, lactose, and GalNAc on adherence of ^{3}H-labeled *P. acnes* PK93 cells to *S. sanguis* DL1-coated SHA. SHA were coated with 3×10^8 unlabeled DL1 cells before treatment with ^{3}H-labeled PK93 cells in the presence of CAB (○, control), 150 mM mannose (●), 100 mM lactose (△), 150 mM lactose (▲), and 50 mM GalNAc (□).

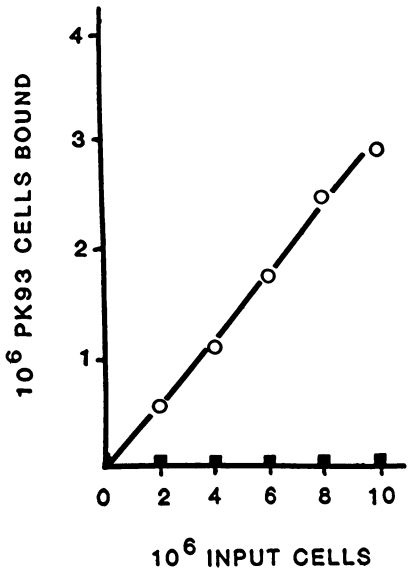

FIG. 4. Adherence of ^{3}H-labeled *P. acnes* PK93 to SHA coated with 4×10^7 *S. sanguis* 34 cells (■) or 3×10^7 *S. sanguis* DL1 cells (○).

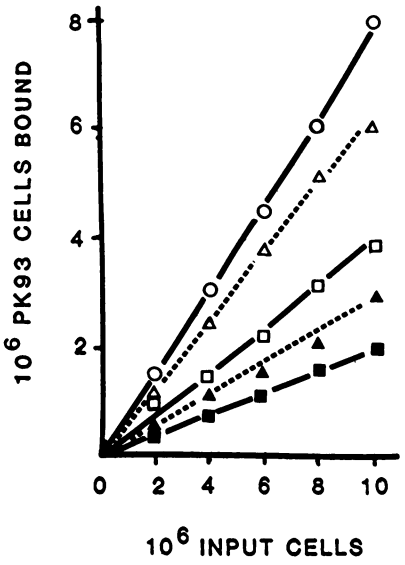

FIG. 5. Effect of saliva and GalNAc plus saliva on adherence of ^{3}H-labeled *P. acnes* PK93 to *S. sanguis* DL1-coated SHA. SHA were coated with 3×10^8 DL1 cells before treatment with ^{3}H-labeled PK93 cells in the presence of CAB (○, control), 40% saliva (△), 100% saliva (□), 50 mM GalNAc in 40% saliva (▲), and 50 mM GalNAc in 100% saliva (■).

bound few, if any, *P. acnes* cells. Two sequential incubations of DL-1-coated SHA with PK93 showed that there was a small additional binding of PK93 in the second incubation. Under the present assay conditions, the coaggregation of PK93 and untreated DL1 on SHA was stable, in that a second incubation with ^{3}H-labeled PK93 showed little, if any, exchange of bound unlabeled bacteria with free radioactive cells.

Effect of saliva and saliva plus GalNAc on adherence of PK93 to DL1-coated SHA. After the attachment of unlabeled DL1 cells to SHA, the adherence of ^{3}H-labeled PK93 cells to the surface-associated DL1 in the presence of 40% saliva or whole saliva was compared to that observed with buffer (Fig. 5). At an input of 10^7 PK93 cells, the presence of 40% saliva and whole saliva in the assay still supported the attachment of 75 and 50%, respectively, as much PK93 to DL1-coated SHA as that observed with buffer. At the same PK93 input, the addition of 50 mM GalNAc to samples containing either concentration of saliva caused approximately a 50% inhibition of PK93 binding. In other experiments with an input of 5×10^7 PK93, 40% saliva and whole saliva supported the attachment of 80 and 64% of the *P. acnes* cells, respectively, when compared with buffer controls (results not shown). In these experiments, 50 mM GalNAc inhibited PK93 adherence in 40% saliva by 30% and in whole saliva by 40%.

TABLE 1. Adherence of PK93 cells to SHA coated with heat-treated DL1 cells[a]

First bacterium	Second bacterium	Third bacterium	^{3}H-labeled cells (10^6) bound to SHA (mean ± SD)
^{3}H-labeled DL1			205 ± 10.6
Δ^{3}H-labeled DL1[b]			104 ± 1.3
^{3}H-labeled PK93			2.6 ± 0.4
DL1	^{3}H-labeled PK93		201 ± 2.5
ΔDL1	^{3}H-labeled PK93		8.8 ± 9.4
DL1	PK93	^{3}H-labeled PK93	5.9 ± 0.3
ΔDL1	PK93	^{3}H-labeled PK93	1.4 ± 0.5

[a] SHA assays with 10 mg of HA in 0.4-ml plastic Microfuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.) were carried out as described for 50 mg of HA except that 0.4-ml volumes were used. SHA were treated with 5×10^8 bacterial cells per 0.4 ml. Each result is the mean ± standard deviation of two to four independent determinations.

[b] Δ, Bacteria heated at 85°C for 30 min.

Effect of aggregates and unbound coaggregates on SHA adherence. Saliva-induced aggregation of the bacteria was investigated to rule out possible influences of aggregation on coaggregation or adherence to SHA. *P. acnes* PK93 did not aggregate in human saliva, and aggregates of *S. sanguis* DL1 cells took 2 h to completely sediment (Fig. 6B). In contrast, coaggregates of PK93 and DL1 sedimented in 5 to 10 min (Fig. 6), and SHA coated with bacteria sedimented in 5 s (Fig. 7). Also, experiments in which preformed coaggregates made with 5×10^8 cells of each partner strain were mixed with SHA showed that less than 4% of ^{3}H-labeled DL1 and 0.5% of ^{3}H-labeled PK93 coaggregates were associated with the SHA after three washes with CAB. Thus, results obtained with the adherence assay represent bacterial attachment to the bacterial coating on SHA without a significant contribution from either free aggregates or unbound coaggregates.

DISCUSSION

Although strains of *P. acnes* are isolated from both supragingival and subgingival human plaques (22), these bacteria are considered poor colonizers of plaque when compared with *S. sanguis* strains. This difference might, in part, be explained by our findings that a *P. acnes* strain, in contrast to an *S. sanguis* strain, does not bind significantly to saliva-coated surfaces (Fig. 1, Table 1).

The adherence of bacteria to SHA is widely used as a model to study the initial adherence to the tooth surface (1, 5, 9). Reports of studies using this method indicate that *S.*

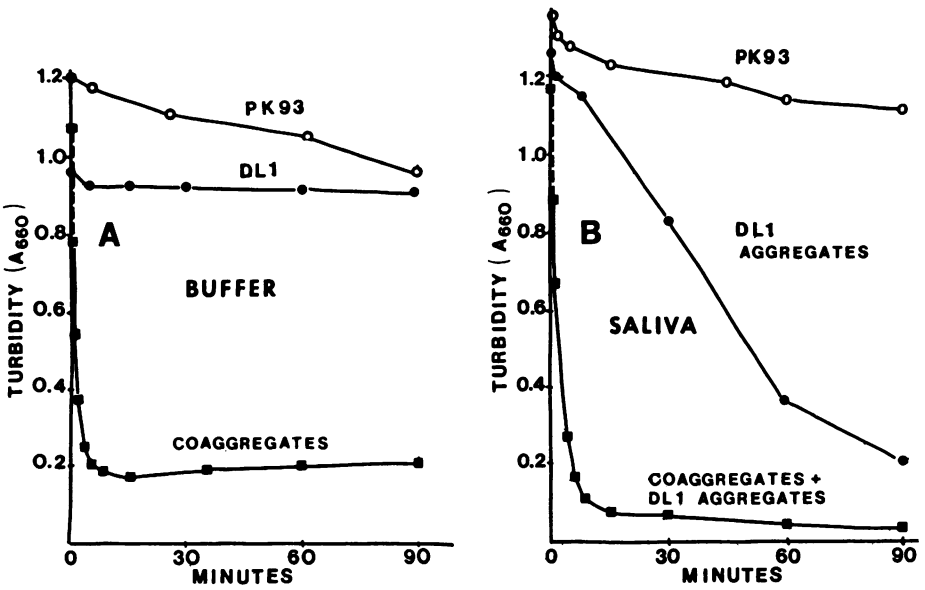

FIG. 6. Time-dependent formation of coaggregates of *S. sanguis* DL1 and *P. acnes* PK93 in CAB (A) or saliva (B) and formation of saliva-induced aggregates of *S. sanguis* DL1. Each bacterium or a mixture of the coaggregation partners was mixed in a 1.0-ml cuvette as described in the text. Sedimentation of cells was measured as a decrease in turbidity measured at 660 nm. Cells were suspended in CAB (BUFFER) or 40% saliva (SALIVA).

sanguis, a species considered an initial colonizer of tooth plaque, binds well to SHA (1, 5). The SHA method employed in our study also showed a substantial binding of *S. sanguis* DL1 but little, if any, attachment of *P. acnes* PK93 cells. Whereas the adherence of PK93 to DL1-coated SHA was inhibited by lactose and GalNAc, the binding of DL1 to SHA was not inhibited by these sugars. Therefore, this method was ideal for an investigation of the surface-associated, lactose-sensitive coaggregation between strains PK93 and DL1. Indeed, cell-to-cell interactions of PK93 with cells attached to SHA occurred both in buffer and in saliva. The ability of *P. acnes* PK93 to attach to DL1-coated SHA in the presence of saliva we consider far more significant than the reduced adherence when compared with buffer controls (Fig. 5) because it supports the contention that such an interaction is possible in vivo. The reduced adherence was expected, in that salivary glycoproteins which contain galactosides are potential inhibitors of galactoside-sensitive interactions between bacterial species (17). However, the exact mechanism of this reduced adherence is unknown at this time. The addition of 50 mM GalNAc in the assay was a less effective inhibitor of *P. acnes* binding to DL1-coated SHA in the presence of saliva (Fig. 5) than in the presence of the buffer (Fig. 3). Either enzymatic modification of GalNAc or its interaction with salivary components could, in part, account for its reduced effectiveness. Saliva-induced aggregate formation did not influence these results because strain PK93 did not form aggregates (Fig. 6).

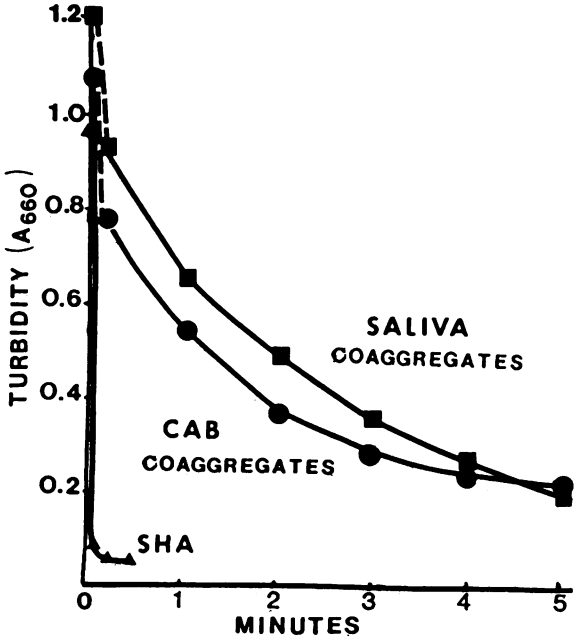

FIG. 7. Relative sedimentation rates of bacterium-coated SHA (▲), DL1-PK93 coaggregates formed in CAB (●), and coaggregates formed in saliva (■). See the legend to Fig. 6.

Two studies (19, 20) measured the binding of bacteria to SHA when the two coaggregation partners were suspended in buffer and added simultaneously to SHA. In one of these studies (19), the investigators also precoated HA simultaneously with a bacterial strain and saliva followed by exposure of the bacterium-and-saliva-coated HA to the coaggregation partner. All of the strains of *A. viscosus*, *S. sanguis*, and *S. mitis* employed in the two studies bound effectively to SHA and aggregated in human saliva. In contrast, our study involved a coaggregation partner strain that neither aggregated in saliva nor bound significantly to SHA. Thus, neither of these potentially interfering properties complicated the interpretation of the results of the SHA adherence system described here.

The results of the present study support a unique mechanism for in vivo adherence of *P. acnes* which involves a galactoside-sensitive coaggregation of this species with *S. sanguis* cells attached to the pellicle-coated tooth. Further support for this concept is that *P. acnes* PK93 cells do not

form coaggregates with other species of oral bacteria considered early colonizers of tooth surface plaque (unpublished data). The in vitro method described here should prove useful in investigating the mechanisms by which a bacterium that interacts poorly with salivary molecules can adhere to a saliva-coated surface by strong cell-to-cell interactions with bacteria already attached to the surface.

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