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Adherence of *Actinomyces viscosus* T14V and T14AV to Hydroxyapatite Surfaces In Vitro and Human Teeth In Vivo

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Adsorption of *Actinomyces viscosus* strains T14V and T14AV to hydroxyapatite (HA) surfaces was studied, using an adsorption model based on the Langmuir adsorption isotherm. Data generally followed the adsorption model as judged by high correlation coefficients obtained for both strains to most of the treated surfaces studied. The number of binding sites for strains T14V and T14AV cells to human saliva-treated HA was similar to that for untreated HA. The affinity of strain T14V for saliva-treated HA was tenfold greater than the affinity of strain T14AV for that surface. To approximate the pellicle of the gingival crevice and margin and to determine whether adherence by strain T14V was to specific saliva or serum receptors, experimental pellicles were formed on HA by saliva/serum mixtures. The number of binding sites on the saliva/serum-treated HA remained the same as for the saliva-treated surface. Although the affinity of strain T14V cells for the saliva/serum HA surface remained generally the same as the affinity for the HA treated with saliva alone, the affinity of strain T14AV cells decreased further as the serum content increased. Strain T14V cell numbers adsorbed to serum-treated HA, and albumin-treated HA were less than those adsorbed to saliva-treated HA, indicating that the adherence by strain T14V was to specific saliva receptors. In vivo results from streptomycin-resistant mutants of both strains T14V and T14AV confirmed in vitro results using saliva-serum pellicles. Pretreatment of strain T14V with proteolytic enzymes and heat inhibited adherence to saliva-treated HA, suggesting that the adherence receptor(s) on the cell surface of strain T14V is protein in nature.

Actinomyces viscosus, a gram-positive rod, has been implicated in the etiology of gingivitis (18, 20, 27, 40) and periodontitis (19, 20). Colonization by this organism in germfree animals results in plaque formation, root surface caries, and bone loss characteristic of periodontal disease (17, 19, 20). Differences in virulence between *A. viscosus* strain T14V and its laboratory-derived avirulent variant, strain T14AV, have been reported in monoinfected germfree rats (1, 14) and conventional rats (1). It has been suggested that the prerequisite for virulence of strain T14V is its ability to attach and colonize the teeth and gingival crevice of rodents (1). The avirulent strain T14AV colonizes pits and fissures, but lacks the ability to colonize the gingival crevice region of teeth (1). The use of defective mutants to study bacterial surface components has proved a powerful tool for determining the structure or function of the surface components (43). The failure of variant strain T14AV to colonize the gingival crevice region of the tooth in germfree rats (1) and its lower order of adherence to hydroxyapatite (HA) treated with rat saliva (1) suggests that the T14V-T14AV system may be useful in understanding the mechanisms of adherence of bacteria to human teeth.

The adsorption of bacteria is influenced by the composition of the solid surface (10, 28, 6). In vivo, the teeth are covered by an acquired pellicle formed of selectively adsorbed salivary components (8, 15, 29-31, 39). The presence of adsorbed salivary components on enamel or HA surfaces alters the composition and surface characteristics of those surfaces (38) and clearly influences the selectivity of bacterial adsorption (4, 5, 11, 16, 26, 34, 35). Recent reports have suggested that the presence of these adsorbed salivary components influenced the adsorption of oral bacteria by altering the strength of adsorption bonds formed between the organism and the surface, by changing the number of receptor or adsorption sites that the surface provides for the organisms, or by a combination of the two (4, 11). Comparative estimates of these two parameters have been made by determining the adsorption of oral bacteria to HA

over a range of cell concentrations and then applying an adsorption model based on the Langmuir isotherms (4, 11, 21, 23). The model was previously found to adequately describe the adsorption of several streptococcal strains (4, 11) and two actinomyces strains (4).

The present investigation defines and compares the adsorption of *A. viscosus* strains T14V and T14AV with saliva and saliva/serum-treated HA surfaces. Adsorption isotherms for the two strains were obtained to determine whether differences reported in the adsorptive behavior and colonization were due to changes in the affinity of the organisms for treated HA surfaces or to differences in the number of binding sites available for each strain. The adsorption affinities as well as the estimated number of binding sites for both strains to saliva/serum pellicles were also examined. These in vitro data were compared with in vivo data obtained from an experiment in humans. In addition, the nature of the bacterial surface receptor(s) was studied to obtain information on the mechanism of adherence for these strains.

MATERIALS AND METHODS

Cultures and culture conditions. *A. viscosus* strains T14V and T14AV were kindly provided by B. F. Hammond, University of Pennsylvania, Philadelphia. *A. viscosus* strains T14VJ1 and T14AVT1 are laboratory-derived, streptomycin-resistant variants (200 μg/ml) of T14V and T14AV, respectively, and were isolated as previously described (41).

All cultures were stored as lyophilized stocks or as mutiple frozen stocks at −30°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol. Tritium-labeled cells of *A. viscosus* strains T14V and T14AV were prepared fresh from these frozen stocks for each experiment by growing the organisms in tryptic soy broth with dextrose (TSB; Difco) containing 10 μCi of [^{3}H]thymidine per ml (Schwarz/Mann, Orangeburg, N.Y.). Organisms from 16-h cultures were harvested by centrifugation at 1,300 × *g* for 10 min, washed twice, and suspended in 0.05 M KCl containing 1 mM potassium phosphate (pH 7.3), 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (buffered KCl) (4). Clumped organisms were dispersed with medium power 10-s pulses from a Micro-ultrasonic cell disrupter (Kontes, Vineland, N.Y.) between centrifugations and for three 10-s pulses before dilution to final concentrations. Final concentrations were determined from a plot of optical density versus concentration made in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). The plot was made from direct microscopic counts of organisms at various concentrations, using a hemacytometer (American Optical Corp., Buffalo, N.Y.).

Saliva preparation. Whole paraffin-stimulated saliva (100 ml per collection) from one donor, age 24, was collected in a container chilled in ice and heated at 56°C for 30 min to inactivate degradative enzymes (1, 4). Heat-treated saliva was clarified by centrifugation at 12,000 × *g* for 10 min (4). Sodium azide was added at a final concentration of 0.04%, and this preparation was stored at −30°C.

Serum preparation. Since the pellicle in and around the gingival crevice may contain serum components which might serve as binding sites in addition to salivary constituents (13), saliva/serum mixtures or serum alone were also used to form experimental pellicles. Blood was collected (100 ml per collection) from the same saliva donor. The blood was allowed to clot overnight at 4°C. Serum was then obtained after centrifugation of the clotted blood at 250 × *g* for 10 min. Sodium azide was added to the serum at a final concentration of 0.04%, and this preparation was stored at −30°C.

Bacterial adsorption to HA. Preparation and treatment of HA beads (BDH Biochemicals Ltd., Poole, England) with saliva, serum, saliva/serum mixtures, albumin, and buffered KCl were performed by the methods previously described by Clark et al. (4). After 90 min of incubation of HA beads with bacterial suspensions, the HA beads were allowed to settle from the mixture for 60 s. Portions, 100 μl each, were removed from the supernatants, which contained unadsorbed organisms, and placed in vials containing 10 ml of Aqueous Counting Scintillant (Amersham/Searle, Arlington Heights, Ill.). Microscopic examination of bacterial suspensions revealed that cells were evenly dispersed during all phases of the experiment. The samples were allowed to equilibrate for 2 h in the dark at 4°C and were counted on a Searle Isocap 300 scintillation counter. Portions of known numbers of ^{3}H-labeled cells were counted in a similar manner so that counts per minute could be related to bacterial cell number. Control bacterial suspensions were incubated without HA beads and counted similarly to correct for cell loss due to adsorption to the tubes. Direct counts of bacterial adsorption to HA surfaces by scanning electron microscopy (SEM) confirmed that this was a reasonably sensitive and reliable method for studying bacterial adherence to HA (unpublished data).

Calculations of parameters for bacterial adsorption to HA surfaces. Adsorption isotherms obtained by direct measurements were used to calculate the strength of the adsorption bond (i.e., affinity) and the number of binding sites, using the bacterial adsorption model described by Gibbons and co-workers (4, 11). The adsorption model is described by the equation $C/Q = 1/KN + C/N$, where C is the concentration of free cells at equilibrium, N is the maximum number of binding or "receptor" sites, and Q is the total number of cells adsorbed per unit of adsorbent. At equilibrium, the strength of the adsorption bond between the bacterial cell and adsorbent surface is described by parameter K (milliliters per cell). A plot of C/Q versus C yields a straight line if the experimental data are adequately described by the mathematical model.

Electron microscopy. HA beads with adsorbed bacterial cells were washed with three volumes of buffered KCl and placed in a vial and dehydrated through a graded acetone series. Beads were critical-point dried (Sorval, Newton, Conn.) and mounted on studs by sprinkling the beads onto double-coated tape

(Scotch, no. 666). Studs were coated with gold palladium for 3 min at 10 mA on a Hummer II (Technics, Alexandria, Va.) plater and examined in a Novascan 30 (Zeiss, New York, N.Y.) scanning electron microscope.

In vivo experiments. To determine whether in vitro bacterial adsorption mimicked adsorption to human teeth in vivo, adsorption by *A. viscosus* streptomycin-resistant strains T14VJ1 and T14AVT1 were compared for four human subjects. Both organisms were cultured overnight in TSB. Organisms were harvested by centrifugation, washed twice, and suspended in saline at a concentration of 4.0×10^9 cells per ml. Equal volumes of each cell suspension were mixed together, and dilutions of the mixture were plated in duplicate on tryptic soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing 200 μg of streptomycin per ml. These were incubated at 37°C. Proportions of colony-forming units of each strain in the mixture were determined by colony morphology and growth characteristics in TSB inoculated with one colony, and further confirmed by antigen extraction by the method of Lancefield and Perlmann (22). Antigens present in the extracts were detected by Laurell Rocket Osserman immunoelectrophoresis (24, 36) as modified by Powell et al. (37).

The six maxillary anterior teeth of four adult volunteers were cleaned by careful tooth brushing, and 1 ml of the actinomyces mixture was placed into the mouth of each subject. After 5 min, the mixture was expectorated, and the subjects thoroughly rinsed their mouths with water. The buccal surfaces of three individual teeth on the right side were sampled by forceful rubbing with calgiswabs (Inolex Corp., Glenwood, Ill.) 15 min later (42). The three anterior teeth on the left side were sampled with calgiswabs after 4 h.

All swabs were immediately placed in 2 ml of saline containing 1% Trypticase (Difco), and the actinomyces were dispersed for 1 min by a Vortex mixer. Dilutions of the resulting suspensions were plated in duplicate on tryptic soy agar plates containing 200 μg of streptomycin per ml as described above. The relative proportions of colony-forming units of strain T14VJ1 to T14AVT1 recovered from teeth were multiplied by the reciprocal of their proportions in the mixture introduced into the mouth to reflect equal opportunity of attachment (26, 41).

Enzyme treatment of *A. viscosus* cells. The influence of pretreating *A. viscosus* T14V or T14AV cells with various enzymes on their subsequent binding to saliva-treated HA was studied. Portions of cells (4×10^7 cells per ml) were treated with the following enzymes: 0.2% trypsin (type III, twice crystallized; Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) adjusted to pH 8; 0.2 to 1.0% chymotrypsin (type II, Sigma) in PBS, pH 7.8; 0.2% papain (type II, twice crystallized, Sigma) in PBS, pH 6, containing 50 mM $CaCl_2$; 0.2% protease (Type VIII, Sigma) in PBS adjusted to pH 7.8. The mixtures were incubated for 1 h at 37°C, except for the chymotrypsin which was at 25°C. After treatment, cells were collected by centrifugation and washed twice with 0.5 M NaCl and twice with buffered KCl. Washed cells were suspended in buffered KCl, and inhibition of adherence was examined. Control cells were treated similarly with each buffer but without the enzyme.

Periodate treatment and lactose competition of *A. viscosus* cells. Washed cells were suspended in 10 mM periodate in PBS at pH 6.5 and incubated for 12 h at 4°C. Cells were collected by centrifugation and washed twice with buffered KCl before the adherence assay to saliva-treated HA. Control cells were incubated in PBS without periodate. To determine whether lactose could inhibit or reverse the adherence of strain T14V to saliva-treated HA, 0.1 M lactose (Sigma) was included in reaction mixtures.

RESULTS

Bacterial adsorption to treated and untreated HA. The adsorption of strain T14V cells to saliva-treated HA was greater than to untreated HA (Table 1). This difference was not evident at low concentrations, but at initial concentrations of 4.0×10^7 cells per ml substantially more cells of strain T14V adsorbed to saliva-treated HA. Because the maximum difference in adherence between strains T14V and T14AV occurred at 4.0×10^7 cells per ml and a saturated

TABLE 1. *Adsorption of A. viscosus to HA*

HA pretreatment	Cells adsorbed[a]					
	Concn of *A. viscosus* T14V added (cells/ml)[b]			Concn of *A. viscosus* T14AV added (cells/ml)[b]		
	4×10^5	4×10^6	4×10^7	2×10^6	4×10^6	4×10^7
Untreated	4.4 ± 0.24	47.6 ± 1.2	200.0 ± 8.0	20.0 ± 0.8	35.2 ± 0.8	236.0 ± 12.0
Saliva	4.0 ± 0.12	43.2 ± 0.8	375.0 ± 12.0	8.0 ± 1.2	8.4 ± 2.0	86.0 ± 10.0
80% Saliva + 20% serum	4.0 ± 0.12	55.2 ± 0.4	400.0 ± 36.0	3.2 ± 1.2	4.8 ± 0.8	68.0 ± 4.0
20% Saliva + 80% serum	4.4 ± 3.6	48.8 ± 1.2	256.0 ± 4.0	0.8 ± 0.8	1.6 ± 1.6	24.0 ± 4.0
100% Serum			94.0 ± 10.0			
20% Saliva + 80% buffer			393.0 ± 1.0			
0.5% Albumin	1.6 ± 0.16	22.0 ± 3.6	120.0 ± 4.0			
7.0% Albumin	3.2 ± 1.2	16.0 ± 2.4	124.0 ± 4.0			

[a] Values indicate cells ($\times 10^5$) ± standard error adsorbed per 40 mg of HA.
[b] Fixed concentrations indicated were added to tubes, with total volume of 1.6 ml.

monolayer of cells was observed, 4.0×10^{7} cells per ml was utilized to study the strain T14V adherence mechanism. In contrast, the adsorption of strain T14AV cells to saliva-treated HA was lower than that to untreated HA at all initial cell concentrations investigated. The number of strain T14V cells adsorbed to saliva-treated HA was 3.6- to 5.1-fold higher than the number of strain T14AV cells adsorbed to saliva-treated HA at initial concentrations of 4.0×10^{7} cells per ml. The number of cells of both strain T14V and T14AV which adsorbed to untreated HA was similar at all initial cell concentrations.

The addition of high concentrations of serum to saliva further reduced the number of strain T14AV cells which adsorbed to HA (Table 1). The number of strain T14V adsorbed to HA treated with a mixture of 80% saliva/20% serum was similar to the number of cells adsorbed to HA treated with saliva alone. Moreover, HA treated with 20% saliva in buffered KCl adsorbed a similar number of strain T14V cells to that adsorbed by HA treated with saliva alone. However, HA treated with a 20% saliva/80% serum mixture adsorbed a fewer number of cells, suggesting that serum masks or blocks the salivary binding sites. When HA was treated with 100% serum, strain T14V adsorption was substantially reduced. These experiments were repeated with saliva and serum from different collection periods with identical results. Adsorption of strain T14V cells was inhibited to a similar extent by treatment of HA with either 0.5 or 7.0% albumin (Table 1). The number of strain T14V cells adsorbed to serum or albumin treated HA was at least threefold less than the number of strain T14V cells adsorbed to saliva-treated HA, suggesting that these cells interact specifically with adsorbed salivary components rather than serum components.

Influence of adsorbed salivary components to HA surfaces on adsorption isotherms. The differences in adsorption of strains T14V and T14AV to treated and untreated HA were also apparent when data were plotted as isotherms (Fig. 1). Isotherms of strain T14V to saliva-treated and 80% saliva/20% serum-treated HA appeared nearly identical (Fig. 1A and B) and followed Langmuir kinetics. Adsorption of strain T14V to untreated or to 20% saliva/80% serum-treated HA and of strain T14AV to untreated or saliva-treated HA also follows Langmuir kinetics HA (Fig. 1A and B). Adsorption of strain T14V to albumin-treated HA (Fig. 1B) was dramatically less than its adsorption to any other treated or untreated HA surface. It was confirmed by scanning electron microscopy that adsorption of strain T14V to saliva-treated HA was limited to a monolayer over the range used to generate the isotherms. At initial concentrations equal to or less than 4.0×10^{7} cells per ml, scanning electron microscopy observation demonstrated that monolayer adsorption had occurred (Fig. 2A and B). At concentrations of 4.0×10^{8} or greater, cell-to-cell interactions and the formation of multiple layers of cells were observed (Fig. 2C and 2D).

Graphic plots of C/Q versus C, derived from the adsorption isotherms, generally resulted in straight lines for the various HA treatments (data not shown). The generally high correlation coefficients of experimentally derived data indicate that the previously described mathematical model delineates the adsorptive behavior of the organisms studied to a satisfactory degree (Ta-

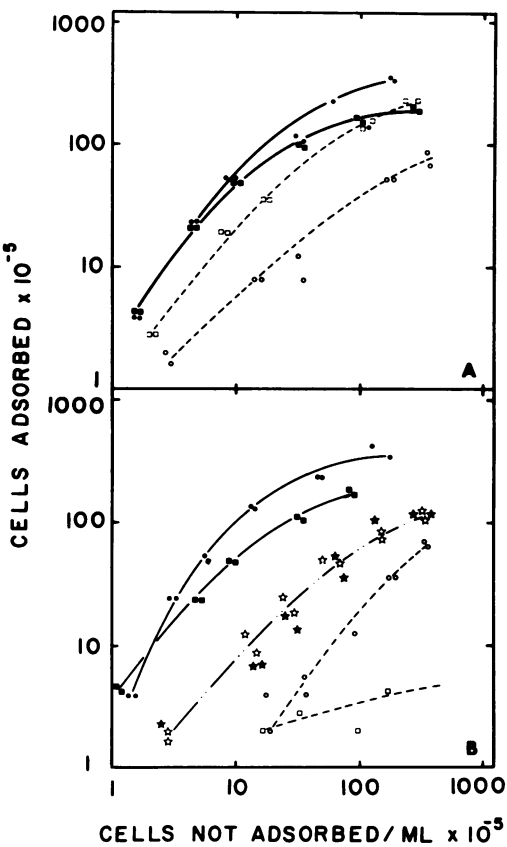

FIG. 1. *Adsorption isotherms of A. viscosus T14V and T14AV to saliva-treated, untreated, and saliva/serum-treated HA. (A) Strain T14V adsorption to saliva-treated* (●) *and untreated* (■) *HA. Strain T14AV adsorption to saliva-treated* (○) *and untreated* (□) *HA. (B) Strain T14V adsorption to 80% saliva/20% serum* (●), *20% saliva/80% serum* (■), *and to 0.5%* (☆) *and 7.0%* (★) *albumin-treated HA. Strain T14AV adsorption to 80% saliva/20% serum* (○) *and 20% saliva/80% serum* (□)*-treated HA.*

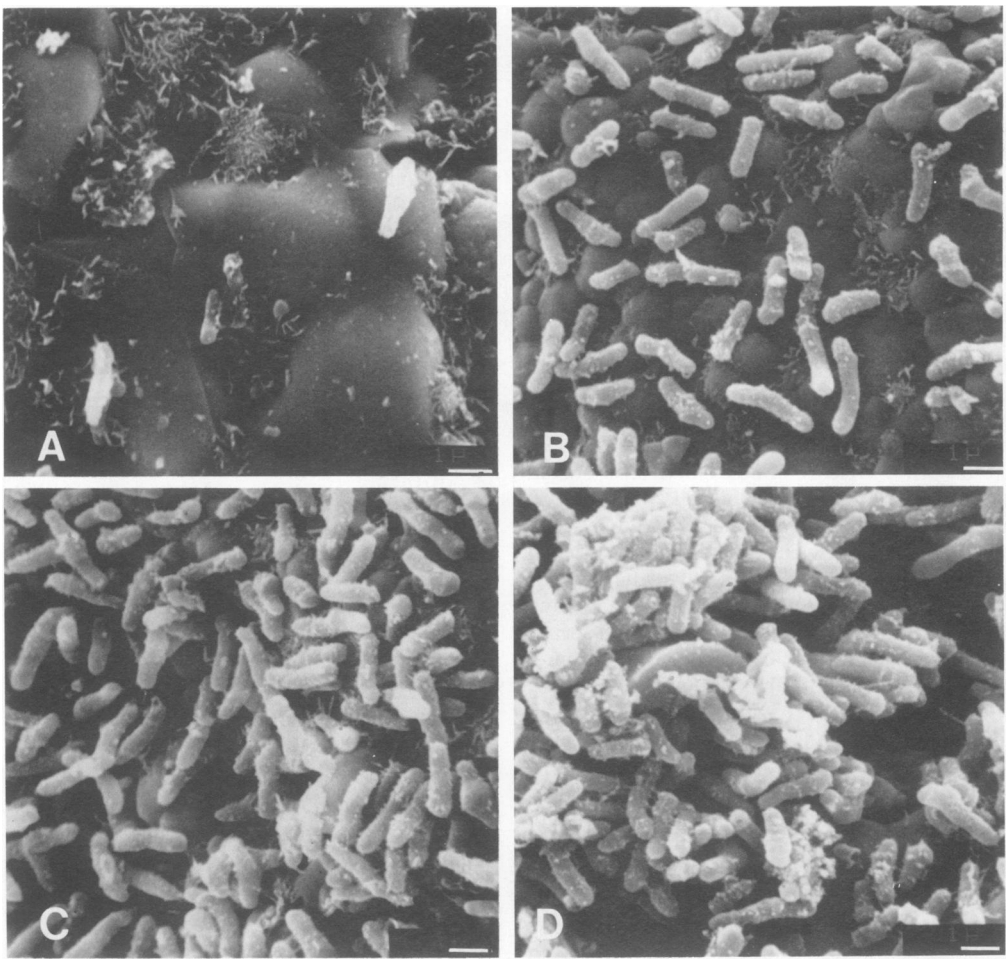

FIG. 2. *Scanning electron microscopy of various concentrations of A. viscosus T14V adsorbed to saliva-treated HA. (A) Initial concentration, 4.0 × 10^6 cells per ml; (B) initial concentration, 4.0 × 10^7 cells per ml; (C) initial concentration, 4.0 × 10^8 cells per ml; (D) initial concentration, 4.0 × 10^9 cells per ml. The bar represents 1 μm on all micrographs.*

ble 2). This did not hold true when a high percentage of cells did not adsorb to the HA, as was observed for adsorption of strain T14AV to saliva/serum-treated HA (Table 2).

The calculated number of binding sites (parameter *N*) for both strains T14V and T14AV cells to saliva-treated HA were similar to those for untreated HA (Table 2). The strength of the bacterial adsorption bonds (parameter *K*) to untreated HA was similar for both strains T14V and T14AV. The affinity of strain T14V for saliva-treated HA was tenfold greater than the affinity of strain T14AV for the same surface. The affinity of strain T14V for HA treated with saliva/serum mixtures was more than 100-fold greater than that calculated for strain T14AV cells to those surfaces.

Adsorption of *A. viscosus* to human teeth in vivo. Higher cell numbers of strain T14VJ1 than strain T14AVT1 adsorbed to human teeth in vivo (Table 3). There was an average of 6.3-fold more cells of strain T14VJ1 recovered from all teeth as compared with strain T14AVT1 15 min after introduction into the mouth of a bacterial mixture containing equal amounts of organisms. An average of tenfold more strain T14VJ1 cells than strain T14AVT1 cells were isolated from all tooth samples taken at 4 h. Therefore, during the 4-h time period, the numbers of avirulent strain T14AVT1 cells, which were adsorbed to the teeth, decreased to a greater extent than did the numbers of adsorbed virulent strain T14VJ1 cells.

Effect of enzymes, heat, and lactose on

TABLE 2. *Estimates of affinities and adsorption sites of A. viscosus T14V and T14AV on HA*

HA pretreatment	N^a		K^b		Correlation coefficient	
	T14V	T14AV	T14V	T14AV	T14V	T14AV
Untreated	2.4	3.4	2.0	1.0	0.97	0.97
Saliva	3.3	3.3	1.0	0.11	0.88	0.93
80% Saliva + 20% serum	5.0	12.0	2.0	0.016	0.96	0.67
20% Saliva + 80% serum	3.3	ND[c]	2.0	ND	0.96	ND
0.5% Albumin	1.0		1.0		0.85	

[a] Each value ($\times 10^7$) indicates number of adsorption sites per 40 mg of HA.
[b] Each value ($\times 10^{-7}$) indicates affinity constant in milliliters per cell.
[c] ND, Not determinable due to low percentage of adsorption.

adherence of *A. viscosus* cells to saliva-treated HA. The adherence of *A. viscosus* to saliva-treated HA was studied further. Heating the cells at 100°C for 15 min reduced their ability to adhere to saliva-treated HA (Table 4). Pretreatment of strain T14V or T14AV cells with protease, trypsin, and chymotrypsin inhibited their subsequent adherence (Table 4). Pretreatment of strain T14V or T14AV cells with papain increased their adherence to saliva-treated HA, indicating that enzyme may remain bound or alter the cell surface and therefore promote adherence. Pretreatment of strain T14V with higher concentrations of chymotrypsin inhibited its adherence by as much as 82%, indicating that it also was sensitive to this enzyme, as was strain T14AV. Pretreatment of both bacterial strains with periodate inhibited adherence (Table 4). This suggests that carbohydrate moieties present on the bacterial cell surface could be associated with adherence. However, periodate could also cause protein alterations which could influence adherence. To determine whether the *A. viscosus* T14V adherence mechanism mimicked the coaggregation mechanism between actinomycetes and streptococci, treatment of cells and saliva with ethylenediaminetetraacetic acid as well as strain T14V competition experiments with lactose were performed under conditions used by Cisar and co-workers (2). Pretreatment of strain T14V or T14AV or saliva with 0.1 M ethylenediaminetetraacetic acid at pH 8 had no effect on adherence in reaction mixtures containing PBS without Ca^{2+} or Mg^{2+}. Therefore, divalent cations do not appear to be essential for adherence. Incorporation of lactose into the reaction mixture did not effect adherence of strain T14V to either saliva-treated HA or to saliva-treated HA that was heated at 80°C for 10 min (Table 5).

DISCUSSION

Adsorption properties of the virulent *A. viscosus* strain T14V were observed to be very

TABLE 3. *Adsorption of A. viscosus T14VJ1 and T14AVT1 to human tooth surfaces*

Subject	Adsorption[a]			
	0.25 h		4.0 h	
	Strain T14VJ1	Strain T14AVT1	Strain T14VJ1	Strain T14AVT1
1	2.18 ± 0.17	0.47 ± 0.20	0.46 ± 0.24	0.098 ± 0.074
2	1.80 ± 0.20	0.50 ± 0.01	0.66 ± 0.24	0.050 ± 0.010
3	4.6 ± 2.0	0.66 ± 0.24	3.2 ± 1.8	0.24 ± 0.19
4	6.0 ± 3.0	0.60 ± 0.10	3.3 ± 1.7	0.43 ± 0.37

[a] Each value indicates number of cells ($\times 10^3$) ± standard error and is the mean of three teeth sampled in each subject, at the indicated times after organisms were introduced.

TABLE 4. *Effect of various pretreatments of A. viscosus cells on adherence to saliva-treated HA*

Pretreatment	% Adherence of control[a]	
	T14V	T14AV
None	100	100
60°C, 15 min	100	100
80°C, 15 min	97	100
100°C, 15 min	29	56
0.2% Papain	177	136
0.2% Protease	8	0
0.2% Trypsin	29	NT
0.2% Chymotrypsin	68	12
0.4% Chymotrypsin	47	NT
0.6% Chymotrypsin	29	NT
1.0% Chymotrypsin	18	NT
10 mM Periodate	53	13

[a] Control cells were treated separately for each enzyme. NT, Not tested.

TABLE 5. *Effect of lactose on A. viscosus T14V adherence to saliva-treated HA*

Pretreatment of saliva-treated HA	% Adherence of control	
	No lactose in suspension	0.1 M lactose in suspension
None	100	112
80°C, 10 min	102	107

different from those of its avirulent variant strain T14AV. The adsorption of strain T14V to all surfaces studied appeared to follow Langmuir kinetics, as has been previously reported for other oral bacteria (4, 11). The observation that adsorption isotherms of strain T14V to saliva-treated, untreated, and 20% saliva/80% serum-treated HA were generally similar indicated that adsorption to these three different surfaces followed similar kinetics. In addition, strain T14AV adsorption to untreated and saliva-treated HA also followed the Langmuir isotherm, although the adsorption kinetics to saliva-treated HA was of a lower order. Strain T14AV exhibited poor adsorption to HA treated with mixtures of saliva/serum, and the data exhibited a poor fit to the adsorption model, as judged by the low correlation coefficient. Application of the adsorption model requires that a significant percentage of the cells initially available adsorb to the surface (11). Since only 6% or less of strain T14AV cells initially available adsorbed to the saliva/serum HA surface, it is not likely that these data adequately described its adsorption. It is important to note that Langmuir kinetics are limited to monolayer adsorption to a surface (23). We have confirmed by scanning electron microscopy that a monolayer of strain T14V cells adsorbed to these HA surfaces from initial cell concentrations up to and including 4×10^7 cells per ml. However, at concentrations of 4×10^8 cells per ml and greater, cell-to-cell interactions were observed which lead to formation of multiple cell layers on the HA surface. Dispersed cells of *A. viscosus*, *A. naeslundii* strains, and other oral strains are known to reaggregate rapidly (7, 13, 33, 37). Aggregation between homologous or heterologous bacterial cells has been implicated in the accumulation phases of developing plaque (13). However, the mechanisms by which cells interact with adsorbed salivary components are not necessarily the same as those involved in cell-to-cell aggregation (10). The series of photomicrographs (Fig. 2) illustrate that at saturating cell densities of strain T14V cell-to-cell aggregation can occur. These aggregates may not be distinguishable in the in vitro adherence assay from initial attachment of the cell to the HA surface. Although the degree of cell-to-cell aggregation may vary from strain to strain, this possibility should not be overlooked when studying the kinetics of the initial attachment of cells to HA or other surfaces.

Calculations made from adsorption isotherms showed that HA treated with saliva or saliva-serum mixtures provided a slightly greater number of binding sites for strain T14V than did untreated HA. Although the strength of the adsorption bond between cells of strain T14V and the saliva or saliva/serum-treated surfaces was equal to or half that calculated for the untreated HA surface, similar numbers of strain T14V cells adsorbed to untreated and saliva/serum-treated HA from fixed cell concentrations of 10^4 (data not shown) and 10^5 cells per ml, whereas substantially more cells adsorbed to saliva or saliva/serum-treated HA surfaces from concentrations of 10^7 cells per ml. This is consistent with a previous observation that cell adsorption at higher initial cell concentrations was more closely related to the maximum number of binding sites, whereas at low concentration the cell adsorption was influenced by the cellular affinity of the cell for the surface (4). Although the number of binding sites for strains T14V and T14AV are similar, the affinity of strain T14AV for the saliva-treated surface was tenfold less than that calculated for strain T14V. This suggests that the impaired adsorption of strain T14AV is due to its relatively poor affinity for the saliva-treated HA surface. Assuming that both the virulent parent strain and the avirulent variant strain compete for similar binding sites on the saliva-treated HA surface, the adsorption model predicted that a decrease in adsorption would result from a reduced affinity between strain T14AV and the saliva surface rather than an alteration in the number of adsorption sites on the HA surface. The data support this prediction. Strain T14V also adsorbed as well to HA treated with a mixture of 80% saliva and 20% serum as to HA treated with 20% saliva in buffer, but did not adsorb well to serum or albumin-treated HA, suggesting that strain T14V cells interact specifically with adsorbed salivary components and not serum components.

It is significant that relative in vitro adsorption specificities exhibited by *A. viscosus* strains T14V and T14AV to saliva and saliva/serum HA surfaces were confirmed in vivo in humans by determining recoverable cell numbers from teeth after introduction of streptomycin-resistant organisms of both strains, indicating that this in vitro model mimics the adsorption specificities of human teeth in vivo. In vivo results showed that cell numbers of strain T14V averaged tenfold higher than those for strain T14AV. In addition, the recovery of strain T14AV was less than that observed for strain T14V, indicating that strain T14AV exhibited a weaker association with the tooth surface. Thus, observations based on both the in vitro adsorption model and in vivo studies in humans indicate that strain T14AV exhibits a feeble affinity for adsorbed salivary components relative to strain T14V.

Low affinity of strain T14AV for human saliva-treated HA appears to adequately explain

the differences in the adsorption observed for that strain and strain T14V in the in vitro adsorption model and on human teeth in vivo and is consistent with data obtained by Brecher and co-workers with rat saliva-treated HA and with rat teeth in vivo (1). Therefore, efforts were directed toward further understanding the mechanism of adherence and differential affinity between strains T14V and T14AV. It has been suggested that specific bacterial surface components such as the fibrils of *A. viscosus* may interact in a specific "lectin-like" manner with components comprising the acquired pellicle, thereby accounting for the specificity of bacterial attachment to teeth (9, 11, 12, 25). The present observation that heat or proteolytic enzymes inhibit the adherence of both strains to saliva-treated HA suggests that the bacterial receptor molecules are associated with proteins or glycoproteins on the cell surface. Preliminary examination by transmission electron microscopy and negative staining of heat-treated cells which did not adsorb well to HA revealed that fibrils had been removed by the treatment (not shown). Recent reports have also shown that TSB cultures of strain T14AV possess fewer surface fibrils than those of strain T14V (3, 37). The number of cell surface receptor molecules which interact with salivary binding sites could be expected to influence the affinity the cell has for the surface. This could explain the low affinity which was observed with strain T14AV for saliva-treated HA and human teeth. It was of interest that 0.2% chymotrypsin had little effect on the adherence of strain T14V cells to saliva-treated HA but abolished the adherence of strain T14AV. However, an increase in the concentration to 1.0% chymotrypsin did dramatically inhibit the adherence of strain T14V cells. This observation suggests that strain T14V may possess quantitatively more enzyme-sensitive binding material (i.e., fibrils) than did strain T14AV. Alternatively, its binding material might be less available for enzymatic digestion. McIntire and co-workers (32) and Cisar and co-workers (2) have suggested that the receptor site for cell-to-cell aggregation between *A. viscosus* strain T14V and certain *Streptococcus sanguis* strains may be associated with the surface fibrils. Cisar et al. reported that coaggregation of actinomycetes and streptococci was Ca^{2+} dependent and could be blocked by heat treatment of the streptococci; or, if coaggregation still occurred after heat treatment, it could be reversed with lactose (2). In the present study, neither lactose nor ethylenediaminetetraacetic acid influenced the adherence of strain T14V to saliva-treated HA under the conditions tested, suggesting that the adherence mechanism to saliva-treated HA is different than that involved in the aggregation phenomenon.

Brecher and co-workers have suggested that the presence of large amounts of cell-surface-associated heteropolysaccharide may contribute to the weak affinity of strain T14AV (1). Similarly, we have observed adherence inhibition by polysaccharide isolated from strain T14AV cultures (J. T. Powell, unpublished data). Moreover, when cultured in TSB, a quantitative difference in the number of surface fibrils and cell-associated extracellular material has been observed (1, 3, 37). Our laboratory has also demonstrated that strain T14AV cells grown in Socransky chemically defined medium (CDM) appear similar in cell serology, morphology, quantity of surface fibrils, and cell-associated extracellular components to cells of strain T14V grown in either TSB or CDM (37). It was further shown that cell numbers of strain T14AV cultured in CDM that adhered to saliva-treated HA were similar to those of strain T14V cultured in either TSB or CDM (J. T. Powell, T. T. Wheeler, and D. C. Birdsell, Abstr. Int. Assoc. Dent. Res. Annu. Meet. 1979, no. 997, p. 341). However, these data do not indicate whether the decrease in affinity of strain T14AV from strain T14V for saliva-treated HA is due to strain T14AV possessing more cell-associated polymer that could inhibit adherence, fewer fibrils that could act as receptors, or both. Collectively, these changes do correlate with reduced adherence.

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