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Adherence of *Streptococcus salivarius* HB and HB-7 to Oral Surfaces and Saliva-Coated Hydroxyapatite

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We compared the binding of *Streptococcus salivarius* HB and the mutant HB-7 to oral surfaces in vivo. Mutant HB-7 does not aggregate with saliva nor does it bind to buccal epithelium, but it does retain its ability to coaggregate with *Veillonella* and *Fusobacterium*. At 1 h after inoculation into the oral cavity of six volunteers, significantly more *S. salivarius* HB than HB-7 cells were found adhering to the buccal mucosa ($P < 0.05$) and to a cleaned tooth surface ($P < 0.01$); there was no significant difference in the numbers adhering to the tongue. The ratio of HB to HB-7 on the tongue increased in samples taken 1, 3, and 9 days after inoculation. The average time required to clear the mutant HB-7 from the oral cavity was 7 days, whereas that for the parent HB was greater than 20 days, and in some cases strain HB was still present 3 months after its inoculation. A representative *S. salivarius* serotype II strain, designated T3, behaved similarly to mutant HB-7 with respect to its adherence to the buccal mucosa. Strain HB adhered better to hydroxyapatite treated with human saliva than mutant HB-7; both strains adhered in similar numbers to untreated hydroxyapatite. Hydroxyapatite treated with rat saliva bound less HB than hydroxyapatite treated with human saliva, corresponding to the lower aggregating activity of rat saliva. Extraction of saliva with aggregating strains of *S. salivarius* reduced the ability of saliva to mediate attachment of strain HB to hydroxyapatite.

The microbial composition of specific ecosystems within the oral cavity is determined in part by the adherence properties of the microorganisms (12, 13, 15). Specific functional groups on bacterial surfaces are believed to interact with chemically distinct components associated with epithelial cells (14, 25, 28), acquired pellicle (1, 8, 11, 24), and other attached bacteria (9, 21). Despite the appreciation of this relationship, our understanding of the mechanisms of bacterial adherence and how they function in colonization of the oral cavity is limited. The complexity of the bacterial surface and the number of adherence properties that may be associated with a single microbial isolate (18, 26) have made it difficult to devise adequately controlled in vivo experiments. The situation is made even more confusing by the finding that supposedly similar isolates of the same species or serotype can differ markedly in their adherence properties (20, 26). Mutants defective in specific adherence properties can be useful tools to unravel such complex interactions (5, 27).

Saliva contains factors which induce aggregation of some oral organisms (11, 17, 20) and which form the pellicle modifying the bacterial binding properties of hydroxyapatite (HA) (12, 24, 27). These in vivo models reflect the processes of intrabacterial aggregation within a plaque matrix and the establishment of the initial plaque layer on the tooth pellicle. Saliva may also promote the clearance of unattached bacteria by occupying receptor sites and blocking attachment to immobilized receptors (4). The saliva-coated HA model system has been widely employed to derive kinetic parameters of the adherence process (1, 3, 8, 27), and in some instances to study more complex interactions, such as competition for binding sites (1, 3).

Streptococcus salivarius is a numerically important human oral organism which demonstrates a tropism for the tongue (7, 15, 19). The cell surface of *S. salivarius* may possess a number of different adherence and aggregation activities, including saliva-induced aggregation (28), coaggregation with *Fusobacterium* and *Veillonella* (26), binding to saliva-coated HA (1), and binding to oral epithelium (14). Both buccal and tongue epithelia support binding, but there appears to be increased binding to keratinized epithelium (25). Salivary aggregation is restricted to those organisms possessing the K antigen, i.e., serotype I (26). Recently, we demonstrated that human isolates of *S. salivarius* were heterogenous with regard to the adherence properties

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they possessed; some isolates were active in all four of the systems discussed above, whereas others were active in only one, two, or three of the systems. Based on the isolation of specific mutants and on physicochemical characterization, the receptors on the surface of *S. salivarius* HB could be classified into the following categories. The first category which mediated host-related adherence and aggregation reactions, including saliva-induced aggregation, hemagglutination, and adherence to buccal epithelial cells, was specifically absent in mutant HB-7 and involved trypsin-resistant proteins. The second category, which mediated coaggregation with *Veillonella alcalescens* VI, was absent in mutant HB-V5 and was not sensitive to proteases. Coaggregation with *Fusobacterium nucleatum* LF was mediated by a third category of receptors, which were still present in both mutants and were sensitive to trypsin.

The mutants were isolated with a view towards defining the biochemistry of the adherence activities and identifying their role in in vivo colonization. In this paper, we report on the aggregation-adherence properties of HB which are responsible for initiating binding of *S. salivarius* to oral surfaces in vivo and to HA.

MATERIALS AND METHODS

Bacteria. *S. salivarius* HB, mutants HB-7 and HB-V5, and antibiotic-resistant variants of these organisms were described previously (26). Aggregation and adherence properties of the strains are shown in Table 1. *S. salivarius* serotype I and II strains were freshly isolated from the human oral cavity as described (26). Serotype I strains possessed the streptococcal group K antigen and cross-reacted with an antiserum prepared against cell walls of strain HB. Serotype II strains did not react with either the grouping serum or the HB antiserum.

For in vivo experiments, the cells were grown overnight in the complex medium of Germaine et al. (6). Bacteria were harvested by centrifugation, washed once, and suspended in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.0) containing 5 mM calcium chloride (TC buffer), as previously described (26). The final suspensions contained single cells and short chains of streptococci. No difference in chain length was found with the various strains used in the experiments.

Radioactively labeled cells were prepared for in vitro experiments by growing the bacteria overnight in the complex medium containing 4 μCi of [^{3}H]thymidine per ml (Radiochemical Centre, Amersham, England; 26 mCi/mmol). Organisms were washed twice and finally suspended in TC buffer. The cells were dispersed by passing the suspension six times through a 21-gauge needle. Microscopic examination revealed single cells and diplococci with very few longer chains. There was no autoaggregation of cells during the experiments. Bacterial numbers were determined by direct microscopic count with a Petroff-Hausser counting chamber.

In vitro aggregation assays. Aggregation and adherence properties of the strains were assayed in vitro by observation of mixtures of cells and the aggregation substrate as described previously, and results were expressed on a scale from 0 to 4 (26). Salivary aggregation titers were determined by mixing serially diluted saliva with a fixed concentration (2×10^9 cells/ml) of cells and are represented by the highest dilution giving macroscopically visible aggregation.

In vivo experiments in humans. Adherence of rifampin-resistant strain HB^r and streptomycin-resistant strains HB-7^s, HB-V5^s, or T3^s in the human oral cavity were compared. The organisms were grown overnight, harvested, washed, and suspended to a concentration of approximately 2.5×10^9 viable cells per ml, as described above. Mixtures were prepared by mixing equal amounts of each suspension, and the proportion of each strain was determined by plating serial dilutions in duplicate on mitis salivarius agar (MS) containing either 10 μg of rifampin per ml (Sigma Chemical Co., St. Louis, Mo.) or 150 μg of streptomycin per ml (ICN Pharmaceuticals, Cleveland, Ohio). Strains HB-7^s, HB-V5^s, and T3^s were resistant to 1,000 μg of streptomycin per ml; strain HB^r was resistant to 25 μg of rifampin per ml.

Immediately before the start of the experiment, an unstimulated, whole saliva sample was taken from the subjects to determine the salivary aggregating activity, and the teeth were cleaned by careful tooth brushing. A 5-ml amount of the bacterial mixture was swirled through the oral cavity for 5 min, before being expectorated. The subject then rinsed the mouth once with water. Samples were taken at 1 h after inoculation from the buccal mucosa, the tongue dorsum, and the buccal surface of the right first mandibular molar with Calgiswabs (Inolex Co., Glenwood, Ill.); the latter was taken by forceful rubbing. A saliva sample was also taken. Swabs were immediately placed in 2 ml of

TABLE 1. *Aggregation and adherence characteristics of S. salivarius strains*

S. salivarius strain	Aggregation/adherence score with:						
	Human saliva	Rat saliva	Human buccal epithelial cells	Human red blood cells	Rat red blood cells	*V. alcalescens* VI	*F. nucleatum* LF
HB	4	1	4	4	2	4	4
HB-7	0	0	0	0	0	4	4
HB-V5	4	ND[a]	4	4	ND	0	4
T3	0	ND	2	1	ND	2	3

[a] ND, Not done.

sterile phosphate-buffered saline (PBS; pH 7.0) and sonicated for 10 s. Serial dilutions of the samples were plated in duplicate on MS agar plates containing the appropriate antibiotic, and on MS agar to estimate the total facultative streptococcal count. Plates were incubated for 24 h (antibiotic-containing plates) and 48 h (total streptococcal counts) aerobically at 37°C.

To reflect equal opportunity of adherence, the ratio of the numbers of rifampin-resistant over streptomycin-resistant bacteria was multiplied by the reciprocal of their proportions in the mixture introduced into the mouth (8).

Adherence in rats. Twelve Sprague-Dawley rats (3 to 4 weeks old) were divided into two groups. One group received a pellet diet; the other group received the sucrose diet 516S (22). At 10 days after the diet change, both groups of animals were inoculated three times with 0.1 ml of a cell suspension containing equal numbers of HB^r and $HB\text{-}7^s$. The animals were sacrificed by ether inhalation 2 h later. The tongue and the lower jaw were removed, and the molars on the left side were extracted. The molars were placed in 1 ml of sterile PBS, ground in a mortar, and sonicated for 15 s. The dorsal surface of the tongue was scraped with a dental explorer, and the scrapings were placed in 1 ml of PBS, sonicated for 15 s, serially diluted, and plated on MS agar with and without the antibiotics to determine the ratio of HB^r over $HB\text{-}7^s$ and the total streptococcal count.

Saliva was collected from Osborne Mendel rats which had been anesthetized with an intramuscular injection of Hypnorm (Philips-Duphar B.V., Amsterdam, Holland) 0.1 to 0.2 ml/100 g of body weight, and then injected subcutaneously with Carbacholum (0.01 mg/100 g of body weight). Saliva was collected on ice and centrifuged at 15,000 × *g* for 15 min, and the supernatant was stored at −70°C. Heated saliva was incubated at 60°C for 30 min before storing at −70°C.

Bacterial adsorption to HA. Adherence of radioactively labeled bacteria to HA beads (BDH Biochemicals Ltd., Poole, England) was measured essentially as described by Clark and co-workers (3, 27). HA beads were washed three times to remove fines. Washed beads (40 mg) were placed in plastic vials (Provial; Cooke Engineering Co., Alexandria, Va.), and 1.0 ml of a cell suspension containing a known number of cells (by direct microscopic count) was added. The vials were incubated horizontally on a reciprocal shaker (120 strokes/min) at ambient temperature. After 90 min the vials were placed in a rack, and the beads were allowed to settle for 20 s. The number of unadsorbed cells was determined by measuring the radioactivity present in 100-μl samples of the supernatants by liquid scintillation. Portions of known numbers of ^3H-labeled cells were counted in a similar manner to relate radioactive counts and bacterial numbers. Since there was no sign of bacterial aggregation, only the unadsorbed cells were counted, and the proportion of bound cells was calculated by subtracting the total amount of unadsorbed cells from the total input (27). Allowance was made for cells adsorbed to glass. Each value shown represents the mean of at least duplicate incubations. Similar results were obtained when the number of ^3H-labeled bacteria adhering to washed beads was determined directly, indicating that this is a valid procedure for assessing binding of this organism.

Saliva-coated HA beads were prepared by preincubating 40 mg of HA with 1 ml of freshly collected, heated, whole, paraffin-stimulated saliva (26). After 5 h the beads were washed four times with TC buffer to remove unadsorbed saliva.

Extracted saliva was prepared by suspending washed bacterial cell pellets in the saliva (80 mg [wet weight] cells/ml) and incubating the suspension with shaking at room temperature for 20 min. Subsequently, the cells were removed by centrifugation, and the supernatant was used in the assays.

Calculations of the results were carried out following the model described by Gibbons et al. (8), in which adsorption is described by the equation $C/Q = 1/KN + C/N$, where C is the concentration of free cells at equilibrium, Q is the total number of cells adsorbed per unit of adsorbent (40 mg), N is the maximal number of binding sites, and K is the "dissociation constant." The reciprocal of K is K_a, the "affinity constant," or the strength of the adsorption bond between the cell and the adsorbent. Values for N and K were obtained by regression analysis of a plot of C/Q versus C, which should give a straight line when the model adequately describes the data.

RESULTS

Adsorption to HA in vitro. The role of saliva in the binding of *S. salivarius* HB to HA was first investigated in the HA model system. Tritium-labeled *S. salivarius* HB and the nonaggregating mutant HB-7 (10^9 cells/ml) were mixed with HA beads and saliva-coated HA beads, and the number of bacteria adsorbed to the insoluble matrix was determined. The binding of HB and HB-7 to untreated HA was essentially the same (Fig. 1B); however, 34% of the HB cells and only 5% of the HB-7 cells adsorbed to the saliva-coated beads (Fig. 1A). Comparison of Fig. 1A and B shows that adsorption of saliva to HA blocks HA bacteria binding sites and actually inhibits binding of the mutant.

Adherence of strain HB to HA treated with saliva from two donors was essentially the same despite the fact that a difference in the salivary aggregation titer was 4 in one sample and 128 in the other. At a concentration of 1.4×10^9 cells per ml, 14.1% of HB cells and 8.4% of HB-7 cells adsorbed to the HA beads treated with rat saliva compared to 34.7% and 3.7%, respectively, when the HA was treated with human saliva. This correlates with the reduced aggregating activity of the rat saliva.

To determine whether the aggregating factor and the binding factor were the same, saliva was adsorbed with aggregating and nonaggregating strains of *S. salivarius*, and the adsorbed saliva was mixed with HA beads. As seen in Table 2, extraction of saliva with the aggregating organism HB reduced binding by 86% compared to

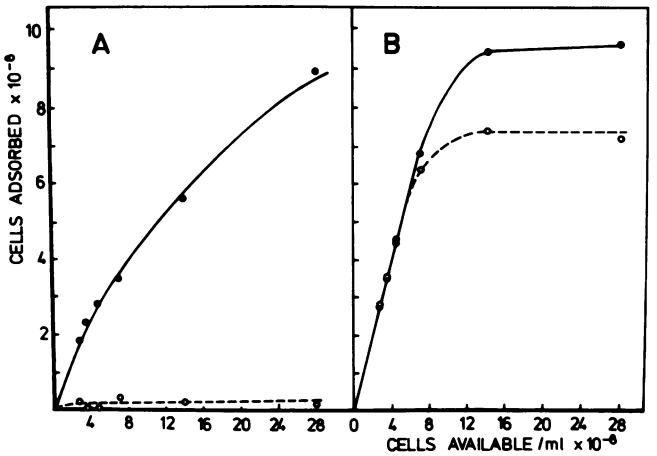

FIG. 1. *Adsorption of S. salivarius HB (●) and HB-7 (○) to HA. A, Saliva-treated HA; B, untreated HA.*

TABLE 2. *Adsorption of S. salivarius HB to HA treated with saliva adsorbed with S. salivarius*

Saliva adsorbed with:	% cells adsorbed[a] (± standard error of the mean)	Salivary aggregation titer (± standard error of the mean)
None	100	16
HB	14.5	0
HB-7	85.2	16
Serotype I ($n = 5$)	33.7 ± 11.3	2.4 ± 1.5
Serotype II ($n = 5$)	76.3 ± 7.2	12.0 ± 2.5

[a] The number of bacteria adsorbed to HA treated with unadsorbed saliva (35% of the available bacteria) was put at 100%, and the number of bacteria adsorbed to HA treated with adsorbed saliva was compared to this value.

TABLE 3. *Estimates of the affinity constants and numbers of adsorption sites for S. salivarius HB and HB-7 on HA and saliva-coated HA*

S. salivarius strain	K_a	N	K_aN	Correlation coefficient
Untreated HA				
HB	3.3×10^{-9}	7.60×10^{8}	2.51	0.99
HB-7	3.8×10^{-9}	8.53×10^{8}	2.48	1.00
Saliva-treated HA				
HB (titer 1: 2)	4.2×10^{-9}	5.42×10^{8}	2.27	1.00
HB (titer 1: 32)	1.8×10^{-9}	1.18×10^{9}	2.12	0.98

a reduction of 15% when saliva was extracted with the nonaggregating mutant HB-7. Similar experiments were performed with a number of *S. salivarius* serotype I and II strains. Serotype I organisms removed significantly more of the aggregating activity from the saliva (Student t test, $P < 0.01$), and the extracted salivas were significantly less effective ($P < 0.01$) in mediating binding to HA beads. The nonaggregating strains (serotype II) were similar to HB-7 in their ability to remove the binding factor.

The affinity constant (K_a) and the number of binding sites (N) can be calculated if the bacterial adsorption characteristics are expressed appropriately by the Langmuir adsorption isotherm. Binding of HB and HB-7 to untreated HA and binding of HB to saliva-coated HA fit the model. In the case of binding to HA, the affinity constants and number of binding sites were basically the same for both organisms (Table 3). Binding of HB to saliva-coated HA is characterized by a reduced affinity constant and a similar number of binding sites. The combined binding measurement K_aN was slightly higher for HB bound directly to HA. Binding of HB-7 to saliva-coated HA did not conform to the Langmuir adsorption isotherm, possibly because binding of a significant proportion of the available cells to the HA is a prerequisite for the validity of the model (8).

Adherence to human oral surfaces. To investigate the in vivo role of in vitro defined aggregation and adherence properties, mixtures of parent (HB^r) and mutant ($HB\text{-}7^s$) strains were introduced into the oral cavity of volunteers, and the ratio of the strains adhering to various oral surfaces was determined. Significantly higher numbers (paired observation analysis, Student t test) of *S. salivarius* HB^r were found adherent to tooth surfaces and to the buccal mucosa at 1 h after their introduction into the oral cavity of six volunteers (Table 4). The increase in the ratio of HB^r over $HB\text{-}7^s$ in individual subjects varied from 6- to 90-fold on the tooth surface, and from 6- to 270-fold on the

TABLE 4. *Adherence of S. salivarius HB and HB-7 to surfaces in the human oral cavity*

Site	Subject[a]	CFU recovered from[b]:		Ratio HB/HB-7[c]	Mean ± standard error of the mean
		HB	HB-7		
Tooth surface	1	0.16	0.026	5.78	
	2	0.42	0.06	6.54	
	3	0.41	0.022	17.10	24.51 ± 13.3
	4	2.50	0.15	15.33	($P < 0.01$)
	5	0.27	0.022	11.59	
	6	0.65	0.0067	90.70	
Buccal mucosa	1	0.11	0.0029	36.73	
	2	0.50	0.053	8.83	
	3	1.20	0.017	67.15	66.50 ± 42.3
	4	1.50	0.22	6.29	($P < 0.05$)
	5	0.80	0.10	7.40	
	6	2.80	0.0096	272.60	
Tongue dorsum	1	41.7	22.1	1.77	
	2	63.0	25.9	2.27	
	3	12.6	6.7	1.76	2.70 ± 0.63
	4	9.7	4.1	2.21	
	5	11.4	4.4	2.42	
	6	4.1	0.66	5.80	
Saliva	2	110.0	83.0	1.23	
	3	18.0	3.5	4.80	3.77 ± 0.69
	4	29.0	8.0	3.14	($P < 0.05$)
	5	48.0	8.0	5.61	
	6	3.3	0.75	4.06	

[a] Subjects are arranged in order of increasing salivary aggregating activity. The salivary aggregating activities for each subject were: 1-16, 2-32, 3-32, 4-64, 5-128, 6-256.

[b] Each value ($\times 10^6$) indicates the number of colony-forming units (CFU) per sample or milliliter of saliva at 1 h after introduction into the oral cavity.

[c] Corrected for the relative proportion of strains HB and HB-7 in the original mixture (ratio 1.07).

buccal mucosa. The ratio of organisms in saliva was much lower, ranging from a one- to a six-fold difference. The mutant did not differ significantly (paired observation analysis) from the parent in its ability to bind to the dorsal surface of the tongue. The ratios of HB^r over HB-7^s observed on the tooth surface and the buccal mucosa were significantly higher than the ratio in saliva, indicating that these values reflect adherence to these surfaces and not contamination from saliva.

At the time of inoculation, the saliva aggregating titers of the volunteers ranged from 2 to 32 for strain HB^r and were 0 for HB-7^s. There was no correlation between the difference in cell ratio on the buccal mucosa and the salivary aggregating activity of the subjects. However, some correlation may be observed between salivary aggregating activity and the ratios of cells found on the tooth surface (Table 4), in that lower salivary aggregating activity generally resulted in a lower ratio of HB^r over HB-7^s.

To test whether the difference in binding activity might have been the result of a greater susceptibility of strain HB-7^s to human salivary defense factors, the mixture of strains HB^r and HB-7^s was incubated at 37°C for 2 h in freshly collected human whole saliva. The total number of viable cells increased during the incubation, but the ratio of HB^r to HB-7^s did not change. Thus, saliva did not have a preferential effect on the viability of either HB^r or HB-7^s.

Since the dorsum of the tongue is quantitatively the most important adherence site for *S. salivarius*, adherence to this surface was followed over a prolonged time period. The ratio of HB^r over HB-7^s progressively increased over the observation period (Fig. 2), and strain HB^r colonized in all volunteers. Strain HB-7^s was cleared from the oral cavity more rapidly (average clearing time, t_c, = 7 days, range 3 to 9 days) than strain HB^r (t_c = 20 days, range 9 to 90 days). Ten rifampin-resistant *S. salivarius* isolates obtained from volunteer 6 3 months after the introduction of the organism into the oral cavity were assayed for their aggregation and adherence properties and were found to be identical to those of the original *S. salivarius* HB^r which had been stored at −70°C during that period.

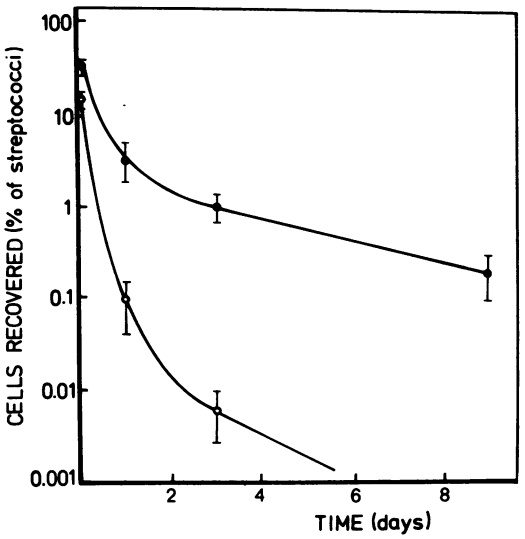

FIG. 2. *Colonization of the dorsum of the human tongue by S. salivarius HB^r and HB-7^s. A 5-ml amount of a mixture containing 2.5 × 10^9 bacteria per ml of each strain was swirled through the oral cavity of six volunteers for 5 min, before being expectorated. Samples of the bacterial flora adhering to the dorsum of the tongue were taken at intervals and analyzed for the content of viable S. salivarius HB^r and HB-7^s and total aerobic streptococci. Each value shown is the mean of six volunteers. Vertical lines represent two times the standard error of the mean.* ●, *S. salivarius HB^r*; ○, *S. salivarius HB-7^s.*

The adherence properties of a number of mutants, modified cells, and serotype II organisms were examined in volunteer number 5 (Table 5). This individual had saliva with an *S. salivarius* HB aggregating titer of 128. New experiments were initiated only after the oral cavity was shown to be free of rifampin- and streptomycin-resistant *S. salivarius*. Incubation of HB^r with subtilisin destroys its salivary aggregating activity but does not interfere with binding to buccal epithelium, thereby producing an organism which should be insensitive to the specific effects of the salivary aggregating factor. Analysis of the in vivo experiment shows that the subtilisin-treated cells (-sub) adhere more effectively to buccal mucosa than the untreated cells when they are compared to HB-7^s. The ratio of HBsub^r over HB-7^s on the dorsum of the tongue after 30 min was not significantly higher compared to untreated cells. To investigate competition between a foreign strain and the resident *S. salivarius* flora, a mixture of strain HB^r and an *S. salivarius* strain isolated from the oral cavity of volunteer 5 was introduced. This strain, designated T3 (Table 1), was considered representative of the majority of *S. salivarius* strains resident in the oral cavity at the time of the experiment, since 18 isolates from various oral surfaces in this individual were essentially similar to T3 with respect to their aggregation and adherence properties. Strain T3 is a serotype II strain and is characterized by the inability to aggregate with saliva and weak adherence to buccal epithelial cells and thus resembles mutant HB-7. Table 5 shows that the ratio of strain HB^r over T3^s was higher on the dorsum of the tongue when compared to experiments in which HB^r was introduced together with HB-7^s. Strain T3^s behaved similarly to HB-7^s with respect to adherence to the buccal mucosa.

S. salivarius HB-V5, a mutant which has specifically lost the ability to aggregate with *Veillonella*, behaved like the parent with regard to binding to both buccal and tongue surfaces. The different binding characteristics of HB, HB-7, and HB-V5 emphasized the unique nature of the genetic lesions.

Adherence studies analogous to the human experiments were performed in conventional Sprague-Dawley rats fed either a pellet or a sucrose diet. Rats were chosen for this experiment because the binding capacity of rat tongue epithelial cells and the salivary aggregating activity of rat saliva are considerably lower than in the equivalent human systems. Despite the lower salivary aggregating activity, more HB than HB-7 bound to the tooth surface (Table 6), but the ratio of 3.8 ($P < 0.01$) was considerably lower than the ratio of 24.5 observed in comparable human experiments. Analysis of the numbers adherent to the tongue showed an HB/HB-7 ratio of 6.5. Unlike the human experiment, the differences observed in binding to the rat tongue are statistically significant ($P < 0.05$).

Comparison of the values obtained within the two diet groups showed higher ratios in animals receiving the low sucrose pellet diet, but these differences were not statistically significant at a 95% confidence level (Student *t* test). Control incubations of cell mixtures in rat saliva for 2 h did not affect the ratio of HB^r to HB-7^s or decrease the viability of the cells.

DISCUSSION

Determining how specific microbial adherence or aggregation mechanisms function in the colonization of the oral cavity is a difficult task, not only because of the complexity of the oral environment but also because of the number of adherence properties associated with a single bacterial isolate. In the study reported here, it has been demonstrated that mutants lacking defined adherence properties are useful in delineating how these properties function in the ini-

TABLE 5. *Adherence of S. salivarius to the dorsum of the tongue and buccal mucosa*

Strains inoculated	Sampling time (h)	Tongue dorsum			Buccal mucosa		
		Adsorption[a]		Ratio[b] r/s	Adsorption[a]		Ratio[b] r/s
		r	s		r	s	
A HB[r]/HB-7[s]	0.5	26.2	7.3	3.43	40.1	3.3	11.6
	5.0	0.58	0.092	6.00	0.032	0.009	3.38
B HBsub[r]/HB-7[s]	0.5	33.6	5.9	5.17	51.1	0.42	110.6
	5.0	0.35	0.013	24.5	0.106	0.009	10.7
C HB[r]/T3[s]	0.5	17.2	0.5	10.1	15.3	0.20	22.5
	5.0	0.16	0.003	157.0	0.005	0.00075	2.0
D HB[r]/HB-V5[s]	0.5	5.3	5.8	1.05	5.5	3.0	2.10
	5.0	0.103	0.168	0.70	0.02	0.009	2.55

[a] Numbers represent the percentage of facultative streptococci. r, Rifampin-resistant; s, streptomycin-resistant.

[b] Ratio corrected for relative proportions of the strains in the inoculum.

TABLE 6. *Adherence of S. salivarius HB and HB-7 in the rat oral cavity*

Group	Adsorption[a]					
	Teeth			Tongue dorsum		
	HB	HB-7	Ratio[b]	HB	HB-7	Ratio[b]
I ($n = 6$)	34.6 ± 8.6	17.6 ± 5.2	4.37	3.68 ± 1.51	0.86 ± 0.31	9.51
II (sucrose) $n = 6$	43.7 ± 10.8	28.7 ± 8.5	3.38	1.80 ± 0.96	1.20 ± 0.53	3.33
Total	39.1 ± 6.4	23.1 ± 4.0	3.76	2.74 ± 0.85	1.03 ± 0.26	6.52

[a] Numbers represent the mean of the cells adsorbed ($\times 10^3$) ± the standard error of the mean at 2 h after introduction of the cells into the oral cavity.

[b] Corrected for the relative proportion of the strains in the original mixture.

tial binding of a cell to an immobilized surface in vivo. More specifically, mutant HB-7 has proven to be a good tool to study adherence, provided that the adherence system under examination accounts for a significant proportion of the adherence events taking place during the experimental period.

After their introduction into the human oral cavity, *S. salivarius* HB and HB-7 were found adhering to the buccal mucosa, the dorsal surface of the tongue, and the teeth. The greater proportion of the parent HB cells adhering to the buccal surfaces and teeth correlates with the observation that the mutant HB-7 is unable to bind to buccal cells or saliva-coated HA in vitro.

The results obtained for adherence to the human tongue dorsum were somewhat surprising. Although strain HB adheres very well to tongue epithelial cells and HB-7 does not, adhesion of HB to the human tongue dorsum measured in vivo was not significantly better than that of HB-7. A possible explanation for this observation is that a large proportion of the initial adherence events represent adhesion to resident bacteria rather than adhesion to the epithelial cells. This idea is supported by the finding that significantly more HB bound to the comparatively sparsely populated rat tongue (18), possibly because more epithelial surfaces were available in the rat, thus conferring an advantage on strain HB. The ratio in rats might have been greater if rat epithelium had a greater affinity for HB (Table 1). Further support in favor of this idea comes from the observation that the normal *S. salivarius* flora consists of approximately equal numbers of both binding and nonbinding strains. On the other hand, the slower rate of clearance of HB and the increase in the ratio of HB to HB-7 over time suggest that possession of the additional adherence properties confers a colonization advantage on the parent, although other factors, such as growth rate, could be important (2). Both HB and HB-7 had similar growth rates in vitro. The possibility that *Veillonella* was providing the majority of the binding sites was discounted when it was found that strain HB-V5, a mutant unable to aggregate *Veillonella*, adhered as well as HB. Work is in progress to identify the organisms on the tongue which might be responsible for binding *S. salivarius*.

The large number of antibiotic-resistant streptococci found in saliva at 1 h after inoculation and rinsing suggests that many of the initial binding reactions are reversible and supports the reversible binding theory postulated from in vi-

tro experiments (13). The cells in the saliva would be derived from teeth, soft tissue, and from epithelial cells sloughed into the oral cavity. The fate of the organisms which are dissociated from the immobile surfaces would be dependent on their binding properties, their location in the oral cavity, and the flow of saliva. Cells with a greater affinity for the immobilized receptors would have a better opportunity to reattach, and this selective process would serve to magnify differences in the distribution of the two strains (13).

Bacteria in the oral cavity are continually subjected to the influences of saliva; in some instances, specific salivary agglutinins may become immobilized on host tissue or attached bacteria where they can mediate attachment. The agglutinins can also have the opposite effect, blocking receptors on bacterial surfaces and promoting clearance of organisms. Studying the influence of saliva in vitro is complicated by the fact that both binding and clearing activities occur at the same time, but the relative importance of the two will vary, depending on the amount of pellicle exposed, the microbial nature of the dental plaque, and the degree to which salivary constituents have been modified by host and bacterial enzymes. Studying attachment to saliva-coated HA beads circumvents these problems and makes it possible to identify the components involved in binding (1).

A number of observations strongly suggest that the binding of *S. salivarius* to saliva-coated HA is mediated by the salivary agglutinin. The mutant HB-7 does not aggregate with saliva and does not bind to saliva-coated HA. Unlike the parent, the mutant will not adsorb either salivary agglutinin or the binding activity and, in this respect, resembles the nonaggregating serotype II isolates. The weak aggregating activity of rat saliva correlates with the relatively small number of binding sites on rat saliva-treated HA.

The *S. salivarius* salivary aggregating titer varied considerably from one individual to another, but this difference was not reflected in adherence to saliva-coated HA. This can be explained by the finding that even the lower activity human saliva retained most of its aggregating activity after reaction with HA beads, indicating that there is an excess of the aggregating factor.

The validity of the in vitro assay as a reflection of the binding activity of natural pellicle was substantiated by the finding that HB bound significantly better than HB-7 to the tooth surface in vivo; the assumption was that the same salivary agglutinin is involved in both in vitro and in vivo situations.

The affinity and number of binding sites determined by Langmuir adsorption isotherms have been combined by Appelbaum and coworkers (1) into an expression (K_aN) which characterizes the binding capabilities of an organism in a particular adherence system and makes it possible to compare one organism with another. The K_aN calculated for *S. salivarius* HB is very similar to the value reported for *Streptococcus sanguis* by Appelbaum et al. (1), indicating that these organisms should have equivalent opportunities to bind, and raises the question of why there should be preferential colonization of the tooth by *S. sanguis* (15). Recent studies in our laboratory (unpublished observations) have shown that equal numbers of aggregating strains of *S. salivarius* and *S. sanguis* can be found on the tooth at 1 h after inoculation. Presumably, other factors influencing colonization come into effect after the initial adherence events.

An important observation was made by Clark and Gibbons (4), who reported that the presence of saliva in the incubation medium strongly reduced the attachment of *Streptococcus mutans* to saliva-coated HA. Because HB is aggregated by saliva, we could not do this experiment but if, as was pointed out, similar components are involved in aggregation and adherence, an inhibitory effect of saliva is conceivable and would be operating in vivo. A saliva effect antagonistic to adherence was observed when we tested the adherence of subtilisin-treated HB cells which had lost the saliva-aggregating ability but retained the ability to adhere to buccal epithelial cells. Higher numbers of the subtilisin-treated cells relative to HB-7 attached to the buccal mucosa than in experiments where untreated HB cells were used. Since no inhibitory effect of saliva on the attachment to buccal epithelial cells was observed in vitro (26), the increased ability of the nonaggregating cells to adhere is presumably a reflection of a lower rate of clearance from the oral cavity (13).

In the experimental situation in vivo, it is obvious that a balance exists between binding and clearing and that this balance will be affected by conditions, such as inoculum size, volume, and length of time the inoculum is in the oral cavity. Although our experiments demonstrate that under the conditions used here, strong aggregation and adherence properties of *S. salivarius* are factors contributing to its ability to bind to oral surface, it should be noted that, in the natural situation, this balance may be different. The existence of such a balance may be one of the reasons that *S. salivarius* serotype I strains, which adhere relatively

strongly to human oral tissues but are aggregated by saliva, are found among human oral isolates in similar proportions to the less well-adhering but nonaggregating serotype II strains (23). Another compensating factor was suggested recently by Howell and co-workers (16), who reported an immunological pressure on an *S. salivarius* strain implanted in gnotobiotic rats, which led to the selection of less well-adhering variants.

Previous studies on the adherence of streptococci to oral tissues in vivo have shown that tissue tropisms exist which mirror observations of isolation frequency from these sites (13). In this study we have compared the adherence of mutants lacking defined attachment characteristics with wild-type strains and have shown that the loss of adherence capabilities results in a reduction of the numbers of the organisms found on the buccal epithelium and the teeth. These results emphasize the role of specific adherence mechanisms and indicate the importance of vigorous definition of all the adherence characteristics of bacterial isolates used to study adherence reactions in vivo where the complexity of the environment makes interpretation of results difficult.

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