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Characterization of the Adherence Properties of *Streptococcus salivarius*

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The adherence and aggregation properties of 46 human oral *Streptococcus salivarius* isolates were examined. A total of 41% of the isolates aggregated with whole human saliva, 50% aggregated with human erythrocytes, and 85% adhered to human buccal epithelial cells. Strains that aggregated with saliva and erythrocytes usually reacted with *Streptococcus* group K typing serum whereas the non-hemagglutinating strains did not. K^+ strains also adhered more strongly to human buccal epithelial cells than K^- strains. All isolates coaggregated with *Fusobacterium nucleatum* LF and *Bacteroides asaccharolyticus* 2D, 91% coaggregated with *Veillonella alcalescens* V1, and 50% coaggregated with *Veillonella parvula* V4. *S. salivarius* HB aggregated with saliva from 15 different human donors and aggregated with human erythrocytes irrespective of the blood group. This strain only weakly aggregated with rat saliva or rat erythrocytes. We isolated mutants which concomitantly lost the ability to agglutinate erythrocytes, aggregate with saliva, and bind to buccal epithelial cells, but retained their interbacterial aggregation properties. A second class of mutants lost the ability to coaggregate with *Veillonella*, but these mutants retained all of the other aggregation properties. Treatment of *S. salivarius* HB cells with pronase or subtilisin destroyed their ability to aggregate with saliva and erythrocytes and to bind to buccal epithelial cells. The unique characteristics of the aggregation and adherence reactions were suggested by differences in the rate of loss of activity during protease treatment and in the response to chemical modification. The presence of saliva did not affect hemagglutination and adherence to buccal epithelial cells. Binding of the salivary aggregating factor to the bacteria could be distinguished from aggregation on the basis that the latter required divalent cations. The factor involved in coaggregation with *F. nucleatum* LF was physicochemically different from the other factors, since it was resistant to heat and to extraction with trichloroacetic acid, aqueous phenol, sodium dodecyl sulfate, and formamide, but was sensitive to proteases and was present in both classes of mutants. Coaggregation with *V. alcalescens* was not sensitive to proteases. A variety of mono- and disaccharides had no influence on any of the reactions tested.

Attachment of bacteria to teeth and oral mucosal surfaces is thought to be an essential step in colonization of the oral cavities (16). The process is highly selective, and the in vitro adherence of bacteria to various surfaces correlates well with their natural distribution in oral cavities (14, 16). In addition to direct adherence to host surfaces, specific interbacterial aggregation among many oral bacteria has been demonstrated (10), and it has been suggested that this aggregation is a mechanism by which certain gram-negative bacteria initially colonize oral cavities (35).

Insight into the character of the interactions between the reacting surfaces would be obtained if the nature of the responsible components were known. However, information about the receptors on bacterial and host surfaces is very limited. A controversy exists about whether adherence of bacteria to host surfaces involves specific complementary chemical structures on the interacting surfaces, such as in lectin-carbohydrate interactions (9, 14, 20, 22), or is the result of electrostatic interactions (3, 31, 32), in which specificity could result from subtle differences in overall surface charges or in the density and distribution of charges on the bacterial surfaces (28, 29). Gibbons and co-workers showed that various oral bacteria bound specific blood group-reactive components from saliva and proposed this as a model to explain the specificity of adherence reactions (11, 12). They associated the receptors on epithelial cells to which some

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oral streptococci attach with blood group-reactive components (39) and also showed that the in vitro adsorption of some streptococci to buccal epithelial cells was inhibited by blood group-reactive salivary mucins (39). Blood group-reactive mucins are present in the acquired pellicle and therefore influence binding to teeth (6, 30). Lipoteichoic acid has been suggested as a cell wall component which mediates the attachment of *Streptococcus mutans* and other oral streptococci to tooth surfaces (3, 25) and acts in the binding of group A streptococci to buccal epithelial cells (1). Adherence of *Streptococcus salivarius* (17) and some strains of group A streptococci (1, 5) to buccal epithelial cells appears to be sensitive to trypsin. However, trypsin was shown to completely remove the surface appendages found on these cells and thus in addition may remove other components, such as lipoteichoic acid (1) and peptidoglycan (37). Surface proteins also seem to be involved in the binding of various oral bacteria to erythrocytes (RBC) (hemagglutination) (7, 20). A carbohydrate on *Streptococcus sanguis* 34 and a (glyco)protein on *Actinomyces viscosus* supposedly mediate coaggregation between these strains (24).

S. salivarius is the predominant *Streptococcus* species found on the dorsum of the tongue (16) and one of the earliest colonizers of infant oral cavities after birth (2). The binding of *S. salivarius* to buccal epithelial cells has been studied previously (15, 17, 39). Saliva-induced aggregation (39) and hemagglutination (23) have been reported previously, but have not been studied in detail. The aim of the present study was to survey and characterize the aggregation and adherence properties of *S. salivarius*. Mutants impaired in aggregation and adherence properties were isolated, and the reactions of cells with chemically and enzymatically modified surface structures were studied.

MATERIALS AND METHODS

Bacteria and culture conditions. *S. salivarius* strains were isolated from human oral cavities on mitis salivarius agar (Difco Laboratories, Detroit, Mich.). Preliminary identification was made on the basis of colonial morphology and was confirmed by the scheme which was outlined by Hardie and Bowden (18), supplemented with assays for acetoin and levan production (38) and urease activity (18).

To establish the presence of the streptococcal group K antigen, acid extracts were prepared by the Lancefield method, and antigen-antibody reactions were detected by counter-immunoelectrophoresis as recommended by Wellcome Reagents Ltd.

S. salivarius HB was isolated from the dorsum of a tongue. This organism was nonhemolytic, possessed K antigen, produced levan from sucrose, formed acid from glucose, trehalose, lactose, raffinose, and inulin, and hydrolyzed esculin, but did not ferment mannitol and sorbitol, did not hydrolyze arginine, was urease negative, and produced little or no acetoin from glucose. Bacteria were grown for 18 h in the medium described by Germaine et al. (8) and were harvested by centrifugation. The cells were washed twice with 0.05 M tris(hydroxymethyl)aminomethane hydrochloride–0.005 M $CaCl_2$ buffer (pH 7.0) (TC buffer) and were resuspended in the same buffer for the aggregation assays. In studies on the effect of the culture medium, the cells were grown in the chemically defined medium described by Terleckyj et al. (36), with the modifications of Wittenberger et al. (40). The cells were transferred at least six times in the defined medium before they were harvested, to insure that there was no carry-over of components from the complex medium.

Radioactively labeled *S. salivarius* HB 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 (26 mCi/mmol; The Radiochemical Centre, Amersham, England). Cells were washed twice and finally suspended in TC buffer. They 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 counts, using a Petroff-Hausser counting chamber.

Streptomycin- and rifampicin-resistant variants were obtained by subculturing strains into increasing concentrations of these antibiotics. The other bacterial strains used were human oral isolates maintained in laboratory stock cultures. Inocula were kept at −70°C in growth medium supplemented with 7% (vol/vol) dimethyl sulfoxide. *Veillonella* strains were grown in a medium containing 0.5% Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.3% yeast extract, 1.5% sodium lactate, and 0.1% (vol/vol) Tween 80. *Fusobacterium nucleatum* LF was grown in a medium containing 1.7% Trypticase, 0.3% yeast extract, 0.5% NaCl, and 0.25% K_2HPO_4; this medium was supplemented with 5 μg of hemin per ml for growth of *Bacteroides asaccharolyticus* 2D.

Aggregation and adherence assays. Aggregation was measured as described previously (22). Reaction mixtures contained 50 μl of TC buffer, 50 μl of an *S. salivarius* cell suspension (2×10^9 cells per ml), and 50 μl of aggregating substrate. Bacterial suspensions used as aggregating substrates were standardized to contain 5×10^8 cells per ml by microscopic counts. Reaction mixtures were shaken for 30 min at room temperature and observed periodically. Aggregation was scored visually on a 0 to 4 basis. A score of 4 represented rapid aggregation (less than 1 min) with complete clearing of the cell suspension, whereas a score of 1 to 3 indicated that aggregation had occurred but that aggregates were smaller and there was not complete clearing of the suspending fluid. Although this type of assay was only semiquantitative, it could distinguish among strongly aggregating cells, cells which were moderately or weakly aggregating, and cells which did not aggregate. Control experiments indicated that it was possible to accurately reproduce

aggregation scores within an experiment and from one experiment to another. To establish aggregation titers, the *S. salivarius* suspensions were serially diluted, and the last tube containing visually detectable aggregation was taken as the endpoint of the reaction. In the case of saliva-induced aggregation, the saliva was serially diluted and mixed with a constant number of cells.

Adherence to buccal epithelial cells was determined in a similar reaction mixture by using a suspension containing 10^6 epithelial cells per ml. After 30 min of incubation, the mixtures were centrifuged and washed four times to separate unattached bacteria. The washed cells were examined by dark-field and phase-contrast microscopy. Because of the large number of adhering bacteria, which often completely covered the epithelial cells, it was usually not possible to count the number of adherent organisms accurately. Therefore, the adherence was scored on a 0 to 4 basis; 0 represented less than 5 adherent bacteria per epithelial cell, 1, 2, and 3 represented up to 20, 50, and 200 bacteria per cell, respectively, and 4 represented more than 200 bacteria per cell. At least 25 epithelial cells were observed from each reaction mixture. Approximately 5 to 10% of the epithelial cells observed under the microscope did not support binding even when other cells in the field were covered with bacteria.

Binding of bacteria to hydroxyapatite. Adherence of radioactively labeled bacteria to hydroxyapatite beads (BDH Biochemicals Ltd., Poole, England) or saliva-coated hydroxyapatite beads was measured essentially as described by Clark et al. (4). Hydroxyapatite beads were washed three times to remove fines. Washed beads (40 mg) were placed in plastic vials (Provial; Cooke Engineering Co., Alexandria, Va.) and mixed with either 1 ml of buffer or 1 ml of saliva for 3 h at room temperature. The beads were washed three times with TC buffer, and 1 ml of a radioactively labeled cell suspension containing 10^7 cells per ml was added. The vials were incubated horizontally on a reciprocal shaker (120 strokes per min) at ambient temperature. After 90 min the vials were placed in a rack, and the beads were allowed to settle for 20 s. Then the beads were washed three times and transferred to scintillation vials, and the amount of ^3H was determined in a scintillation counter. Portions of known numbers of ^3H-labeled cells were counted in a similar manner in order to relate radioactive counts and bacterial numbers.

Preparation of human buccal epithelial cells and saliva. Buccal epithelial cells scraped from the buccal mucosa with a wooden applicator stick were suspended in phosphate-buffered saline (pH 7.0) containing 10 mM sodium ethylenediaminetetraacetate (EDTA), sonicated for 10 s, and washed twice in phosphate-buffered saline. Typically, the epithelial cells contained less than five adherent bacteria per cell when observed under phase-contrast microscopy. Only freshly prepared suspensions were used in these experiments.

Paraffin-stimulated saliva was collected at 4°C and clarified by centrifugation for 15 min at 20,000 × *g*. The saliva was placed in a boiling water bath for 10 min and subsequently centrifuged for 15 min at 20,000 × *g*. The supernatant was stored at 0°C for up to 1 week or at −20°C.

A mucin-enriched fraction was prepared from unheated whole saliva by a modification of the procedure of Levine et al. (21). Clarified, unheated whole saliva was dialyzed in the presence of sodium-EDTA; this was followed by dialysis against distilled water and freeze-drying. A 10-fold-concentrated solution was chromatographed on a Sephadex G-200 column. The void volume of this column was pooled and freeze-dried. The aggregating activity eluted in the void fractions of a Sepharose 4B column. Except when otherwise specified, saliva and buccal epithelial cells from the same adult donor (blood group A) were used throughout these experiments.

Mutagenic procedures. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was carried out as previously described (38). Cell suspensions were sonicated for 30 s before the start of the mutagenic procedure in order to break the streptococcal chains. Nonadherent mutants were selected by incubating the bacteria in the appropriate aggregation-adherence reaction mixture, followed by filtering them through a membrane filter (pore size, 8 μm; Millipore Corp., Bedford, Mass.) which allowed nonaggregated or nonadherent bacteria to pass but retained aggregated organisms. The filtrate was streaked on mitis salivarius agar plates, and about 25 single colonies were subcultured and tested for their aggregation and adherence properties. A portion of the filtrate was transferred to fresh culture medium, grown to an optical density at 660 nm of 0.5, and then stored at 4°C. If no mutants were obtained in the first step, the enrichment procedure was repeated with these cultures.

Modification of cell surfaces. (i) Proteolytic enzymes. Cell suspensions (absorbance at 660 nm, 5) were prepared in TC buffer at pH 7.0 (pronase, subtilisin), TC buffer at pH 8.0 (trypsin), and 0.05 M acetate buffer at pH 5.8 (pepsin) and incubated with the enzymes at 37°C for varying times. Reaction mixtures were then chilled rapidly, and the cells were washed three times with ice-cold TC buffer, pH 7.0. Enzyme activities were determined with azoalbumin as the substrate under appropriate experimental conditions.

(ii) Sonication. A 5-ml suspension of cells (absorbance at 660 nm, 5) was sonicated for 90 s at 4°C with a microtipped Biosonic probe (Bronwill Scientific Inc., Rochester N.Y.). Microscopic observation showed that the cells were not broken by this procedure.

(iii) Phenol extraction. A 5-ml amount of a cell suspension in TC buffer (absorbance at 660 nm, 10) was mixed with an equal volume of 90% aqueous phenol and shaken at room temperature for 30 min. After chilling in ice, the mixture was centrifuged, and the pellet was extracted with 45% aqueous phenol. After centrifugation the cells were washed four times with TC buffer and finally resuspended in the same buffer.

(iv) Trichloroacetic acid extraction. Extractions were carried out in trichloroacetic acid (final concentration, 10%, wt/vol) by the method of Schleifer and Kandler (33) at either 0 or 60°C.

(v) Sodium periodate treatment. Washed cells were suspended in ice-cold 0.12 M acetate buffer (pH 4.0) containing 0.05 M sodium periodate and incubated in the dark at 0°C for 4 h. Subsequently, the cells were washed three times and suspended in TC buffer. Control suspensions were incubated similarly, but sodium

periodate was omitted.

(vi) Formamide extraction. Cells were suspended in formamide and extracted twice for 15 min at 160°C (37). The extracted cells were collected by centrifugation, washed four times, and suspended in TC buffer.

(vii) Acetylation. Washed cells were suspended in 0.05 M borate buffer, pH 8.5 (absorbance at 660 nm, 5); 3 ml of an acetic anhydride-benzene mixture (1:2) was added slowly to 10 ml of cells over a 60-min time period with constant stirring. The pH was maintained between 8 and 9 by adding 1 N NaOH. At the conclusion of the reaction, benzene was removed from the surface of the suspension with a Pasteur pipette, and the cells were collected by centrifugation, washed three times with distilled water, and suspended in TC buffer, pH 7.0. Controls contained glacial acetic acid instead of acetic anhydride.

Chemicals. Trypsin (twice crystallized; 12,000 U/mg), subtilisin BPN' type VII (10.4 U/mg), pepsin (twice crystallized; 3,200 U/mg), ganglioside type III, bovine submaxillary mucin type I, bovine serum albumin, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and sugars were obtained from Sigma Chemical Co., St. Louis, Mo. Dextrans were from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; pronase (45 U/mg) was from Calbiochem, La Jolla, Calif.; and streptococcal group K grouping serum was from Wellcome Reagents, Beckenham, England. Other biochemicals were obtained from Fisher Scientific Co., Pittsburgh, Pa.

RESULTS

Aggregation and adherence reactions. A total of 65 strains of *S. salivarius* were isolated from various oral surfaces of 65 young adults. Only one isolate was obtained per subject since a preliminary investigation had shown that the majority of the isolates from a single individual were identical with respect to their adherence and aggregation properties. A total of 19 strains (29.2%) were strongly self-aggregating under the assay conditions used, and 10 strains (15.4%) self-aggregated weakly. Self-aggregation could not be prevented by adding sodium-EDTA or by varying the pH of the assay buffer. The non-self-aggregating and weakly self-aggregating strains were characterized with regard to a number of aggregation and adherence properties (Table 1). A total of 40% of these organisms aggregated with clarified whole saliva. The sensitivity of aggregating organisms to saliva-induced aggregation ranged from strains which formed visible aggregates with saliva diluted 1:128 to strains which reacted only with undiluted saliva.

All of the isolates tested coaggregated with *F. nucleatum* and *B. asaccharolyticus*. Except for three strains, all isolates coaggregated with *Veillonella alcalescens* V1, but fewer strains coaggregated with *Veillonella parvula* V4.

S. salivarius strains can be classified into two serotypes, based on possession of the streptococcal group K antigen (27, 34). Therefore, we compared serotypes and activities in aggregation and adherence assays (Table 2). A strong correlation was observed between activity of strains in host-related aggregation and adherence assays, including saliva-induced aggregation, hemagglutination, and adherence to buccal epithelial cells, and the possession of the K antigen by the strains. K^+ strains aggregated with saliva and adhered strongly to buccal epithelial cells, and only a few did not agglutinate RBC. In contrast, all K^- strains lacked saliva-aggregating ability and, except for one strain, did not hemagglutinate. Several of the K^+ strains did not adhere to buccal epithelial cells, and the few that did adhere attached in lower numbers (Table 2).

S. salivarius HB was chosen for further study because it possessed all of the aggregating activities tested. The aggregation and adherence properties of this organism are listed in Table 3. The reactivity of strain HB to saliva from 15 different donors ranged from a salivary titer of 1:4 to a salivary titer of 1:128. The differences were not related to blood group, sex, age, or time of collection. Saliva collected on different days

TABLE 1. *Adherence and aggregation properties of S. salivarius isolated from human oral cavities*

Aggregating agent	Aggregation-adherence score of:					
	4		1 to 3		0	
	No. of strains	% Of total	No. of strains	% Of total	No. of strains	% Of total
Human saliva	5	10.9	14	30.4	27	58.7
Human RBC	5	10.9	18	39.1	23	50.0
Human buccal epithelial cells	31	67.4	8	17.4	7	15.2
F. nucleatum LF	25	54.4	21	45.6	0	0.0
V. alcalescens V1	28	60.9	14	30.4	4	8.7
V. parvula V4	11	23.9	12	26.1	23	50.0
B. asaccharolyticus 2D	44	95.6	2	3.4	0	0.0

TABLE 2. *Correlation between the aggregation and adherence activity of S. salivarius and the presence of the streptococcal group K antigen*[a]

Aggregating agent	% Of strains tested with aggregation-adherence score of:					
	4		1 to 3		0	
	K^+	K^-	K^+	K^-	K^+	K^-
Saliva	25.0	0.0	75.0	0.0	0.0	100.0
RBC	41.1	0.0	35.3	6.2	23.5	93.8
Buccal epithelial cells	88.2	18.7	11.8	50.0	0.0	31.2

[a] A total of 17 K^+ strains and 16 K^- strains were tested. Because of self-aggregation, only 12 K^+ strains could be tested in the salivary aggregation test.

TABLE 3. *Aggregation and adherence properties of S. salivarius HB and HB mutants*

Aggregating agent	Aggregation-adherence score with:		
	HB[a]	HB-7	HB-V5
Human saliva	4 (64)[b]	0	4 (64)
Human saliva (neuraminidase treated)	4 (64)	0	4 (64)
Rat saliva	2 (2)	0	2 (2)
Human RBC	4 (16)	0	4 (16)
Rat RBC	2 (2)	0	2 (2)
Human buccal epithelial cells	4	0	4
F. nucleatum LF	4 (64)	4 (64)	4 (64)
V. alcalescens V1	4 (64)	4 (64)	0

[a] No aggregation was observed with *Leptotrichia buccalis* (one strain tested), *Staphylococcus aureus* (three strains), *Staphylococcus epidermidis* (one strain), *S. mutans* (three strains), and *S. sanguis* (three strains).

[b] Values in parentheses are titers of *S. salivarius* ($0 = 2 \times 10^9$ cells per ml), except for saliva, where they are titers of saliva.

from the same donor had the same aggregating activity. The aggregating activity resided in the mucin-enriched fraction, which eluted in the void volume of a Sepharose 4B column. Heating of this fraction did not decrease the activity. Neuraminidase treatment of saliva under conditions in which more than 80% of the sialic acid residues were removed did not affect the ability to agglutinate *S. salivarius* HB. Such preparations completely lost the ability to agglutinate *S. sanguis* ATCC 10557 (22). In contrast to the activity of human saliva, pilocarpine-stimulated rat saliva reacted weakly with strain HB (Table 3). This was not due to a lower mucin content of the rat saliva since the latter contained 152.0 μg of sialic acid per ml, whereas the human saliva used in these experiments contained only 20.5 μg/ml.

RBC from blood groups A, B, AB, O, Rh(+), and Rh(−) were all equally effective in the hemagglutination assay. Rat RBC agglutinated weakly.

The aggregation and adherence properties of strain HB were independent of the growth phase of the culture. Cells subcultured six times in a chemically defined medium had similar aggregation and adherence properties compared with cells grown in the complex medium.

Properties of mutants. Adherence mutants selected from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized cultures could be grouped into two principal categories (Table 3). Mutant HB-7 lost the ability to aggregate with saliva and RBC and the ability to adhere to buccal epithelial cells. HB-7-like mutants were obtained by selection for any of these aggregation-adherence characteristics. We tested a total of 38 aggregation mutants, obtained from six mutation experiments. Not all characteristics were lost simultaneously. Mutants defective in saliva-induced aggregation were obtained most frequently, followed by mutants that had also lost the ability to hemagglutinate. Mutant HB-7 retained the K antigen. The second mutational type, represented by mutant HB-V5, was obtained by selecting for the inability to coaggregate with *V. alcalescens* V1. This organism specifically lost the ability to bind to this *Veillonella* strain, but retained all other aggregation and adherence properties (Table 3). Repeated attempts to obtain mutants that were specifically impaired in the ability to coaggregate with *F. nucleatum* or *B. asaccharolyticus* failed. Mutants were not obtained from nonmutagenized cultures of *S. salivarius* HB, indicating that this parent did not consist of a mixture of several strains.

Examination of cells by electron microscopy, using a negative stain method, showed the presence of fimbria-like surface appendages on strain HB, as well as on mutants HB-7 and HB-V5. No difference in the number or distribution of the fimbriae on parent and mutant strains was observed (data not shown).

To differentiate further among the mutants, various (glyco)proteins and polysaccharides were tested for their agglutinating properties. Bovine submaxillary mucin aggregated the parent strain, strain HB, but not mutant HB-7, suggesting a common receptor for bovine submaxillary mucin and saliva. Bovine serum albumin, fetuin, desialized fetuin, lysozyme, and hyaluronic acid (all tested at a concentration of 1 mg/ml), as well as high- and low-molecular-weight soluble dextrans (T250 and T20; 2.5 mg/ml), did not aggregate *S. salivarius* HB, nor did

they inhibit any of the aggregation reactions tested.

In an experiment designed to study the effect of mutations on binding to hydroxyapatite, cell suspensions containing equal numbers of strain HB and mutant HB-7 cells were mixed with hydroxyapatite beads; 90% of both strains bound to the beads. A marked difference was noted when cells were mixed with hydroxyapatite which had been incubated previously with human saliva and then washed. In this situation 12.5% of the strain HB cells but less than 0.5% of the strain HB-7 cells bound to the beads. Phase-contrast microscopic observation of saliva-coated beads incubated with either one of the strains showed that many more strain HB cells than strain HB-7 cells were attached to the beads. Thus, both the nature and the number of receptor sites on the hydroxyapatite beads were changed by saliva.

Characterization of the receptors. *S. salivarius* HB was subjected to a number of enzymatic, chemical, and physical modification reactions in order to determine whether the receptor activities resided in different components of the cell wall and possibly to gain insight into the character of the cell wall receptors involved in the adherence and aggregation reactions. As Table 4 shows, it is possible to group the adherence and aggregation properties of strain HB into three distinct classes, which correlate with the classes based on the properties of mutants HB-7 and HB-V5. The host-related activities, including saliva-induced aggregation, hemagglutination, and adherence to buccal epithelial cells, form a class which can be clearly distinguished from coaggregation with veillonellae and *Fusobacterium*. The interbacterial aggregation systems themselves form two distinct classes which involve different receptors. The inhibition and modification reactions (Tables 4 and 5) provide tentative clues to the nature of the receptors, but more importantly, they indicate that the receptor activities reside in separate components of the cell surfaces of the organisms.

(i) Salivary aggregation, hemagglutination, and buccal epithelial cell adherence. These reactions share a number of properties and differ in others (Table 4). All three reactions are sensitive to pronase and subtilisin, acetylation, hot trichloroacetic acid, extraction with formamide and sodium dodecyl sulfate, and are insensitive to trypsin. Sensitivity to hydrolysis by proteases indicates that the receptors contain protein. The evidence presented above suggests the common nature of the three receptor activities. However, there are a number of properties of these systems which suggest that each has unique characteristics. The salivary aggregation system was the most sensitive to modification, followed by hemagglutination and then adherence to buccal epithelial cells. Salivary-induced aggregation was more rapidly destroyed by subtilisin and pronase (Fig. 1); it was also sensitive to sonication, extraction with aqueous phenol, and treatment with periodate (Table 4). Hemagglutination was resistant to periodate oxidation. Adherence to buccal epithelial cells was the only activity not affected by heating at 100°C for 15 min. Sodium chloride (200 mM) and ganglioside inhibited salivary aggregation and hemagglutination, but not buccal cell adherence (Table 5). EDTA inhibited salivary aggregation but had no effect on the other two activities.

Binding of the salivary aggregation component and agglutination are distinct events, which

TABLE 4. *Aggregation and adherence properties of modified S. salivarius HB cells*

Treatment	Aggregation-adherence score with:					
	Saliva	RBC	Buccal epithelial cells	*V. alcalescens* V1	*F. nucleatum* LF	*B. asaccharolyticus* 2D
Controls	4	4	4	4	4	4
Subtilisin (1.0 mg/ml; 5 h)	0	0	2	4	0	NT[a]
Trypsin (1.0 mg/ml; 5 h)	4	4	4	4	1	1
Pronase (1.0 mg/ml; 5 h)	0	0	0	4	0	NT
Sonication (90 s)	0	2	4	4	4	4
Aqueous phenol (45%)	1	4	4	4	4	3
Trichloroacetic acid (10%; 4 h; 60°C)	0	0	0	3	4	3
Acetic anhydride (10%)	0	0	0	4	3	4
Buffer (15 min; 100°C)	0	0	4	4	4	2
Sodium dodecyl sulfate (2%; 5 min; 100°C)	0	0	0	4	4	4
$NaIO_4$ (10 mM; 4 h; pH 4)	1	4	1	4	4	4
Formamide (15 min; 160°C)	0	0	0	1	4	NT

[a] NT, Not tested.

TABLE 5. *Effect of various compounds on the aggregation and adherence activity of S. salivarius HB*

Compound tested	Concn[a]	Aggregation-adherence score with:				
		Saliva	RBC	Buccal epithelial cells	*V. alcalescens* V1	*F. nucleatum* LF
Buffer control		4	4	4	4	4
NaCl	100	0	4	4	4	4
NaCl	200	0	0	4	4	4
EDTA (sodium salt)	20	0	4	4	4	0
Ganglioside type III	0.1	0	0	4	4	4
Carbohydrates[b]	100	4	4	4	4	4

[a] All concentrations are millimolar, except for ganglioside type III, which is given in milligrams per milliliter.
[b] Glucose, galactose, rhamnose, fucose, mannose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, glucosamine, galactosamine, and lactose were tested.

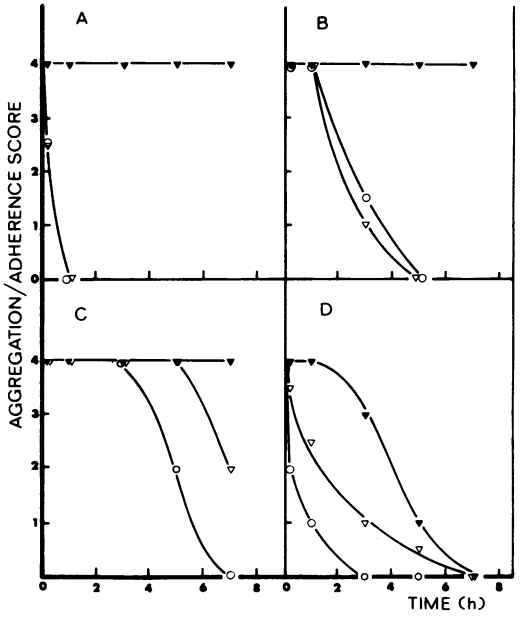

FIG. 1. *Effect of protease treatment of S. salivarius HB on aggregation and adherence properties. Cell suspensions (absorbance at 660 nm, 5) were incubated as described in the text. Samples were withdrawn at varying times, washed three times with TC buffer, and resuspended to the original density, after which aggregation and adherence properties were scored. Enzyme concentrations were adjusted to standard activity by using azoalbumin as the substrate. (A) Saliva-induced aggregation. (B) Hemagglutination. (C) Adherence to buccal epithelial cells. (D) F. nucleatum coaggregation. Symbols:* ○, *pronase;* ▽, *subtilisin;* ▼, *trypsin.*

can be distinguished from one another under the appropriate experimental conditions (Table 6). When cells were incubated with saliva in the presence of sodium-EDTA, they did not form aggregates. After centrifugation and dialysis of the supernatant to remove the inhibitor, no aggregating activity remained in these preparations. However, washing the cell pellet with buffer in the presence of the inhibitor to remove unbound salivary components, followed by suspension in buffer without the inhibitor, resulted in the immediate formation of aggregates. Thus, the active salivary component was bound to the cell surface but was not able to interact with other cells. Cells which had been incubated with subtilisin or had been boiled did not bind the aggregating factor. These data are consistent with the hypothesis that binding requires a cell wall protein. The nonaggregating mutant HB-7 did not bind the aggregating factor.

To determine whether binding of the salivary aggregation factor would interfere with the ability of cells to hemagglutinate or to bind to buccal epithelial cells, strain HB was incubated with excess saliva in the presence of EDTA before it was mixed with RBC or buccal epithelial cells. In neither case was there evidence that binding of the salivary factor interfered with attachment of the bacteria to human cells.

(ii) *Veillonella* coaggregation. The ability of *S. salivarius* HB to coaggregate with *V. alcalescens* V1 was specifically lost in mutant HB-V5. Physicochemical characterization of the cellular component(s) functioning in this reaction distinguished it from other aggregation systems. Coaggregation was resistant to protease and to all other modification reactions, with the exception that the activity decreased upon hot trichloroacetic acid treatment. Acetylation appeared to reduce the strength of binding between the aggregated cells, since they slowly disaggregated upon standing. The methods employed to extract (lipo)teichoic acid from streptococci (cold trichloroacetic acid and aqueous phenol) did not remove the activity, nor did they yield an extract that interfered with the reaction, suggesting that these substances are not involved. Culture supernatants of strain HB but not of strain HB-V5 contained a nondialyzable, soluble factor that induced aggregation of *V. alcalescens* V1.

TABLE 6. *Binding of the salivary aggregation factor to S. salivarius cells*

Treatment	Aggregation score	
	Unadsorbed aggregation activity[a]	Aggregation of washed cells
Salivary mucin fraction incubated with:		
Buffer	4 (16)	
S. salivarius HB	0	4
S. salivarius HB-7	4 (8)	0
S. salivarius HB pretreated by sonication for 90 s	2 (2)	1
S. salivarius HB pretreated with subtilisin (0.2 mg/ml; 30 min)	4 (8)	0
S. salivarius HB pretreated by boiling for 5 min	4 (8)	0
S. salivarius HB in the presence of:		
EDTA (sodium salt; 20 mM)	0[b]	4
NaCl (100 mM)	4 (8)[b]	0

[a] Aggregating activity remaining in the salivary supernatant after incubation with *S. salivarius*. Values in parentheses are titers.

[b] Measured after dialysis of the reaction supernatant.

(iii) *F. nucleatum* coaggregation. Although sensitive to proteases, including trypsin (Table 4 and Fig. 1), coaggregation with *F. nucleatum* LF was markedly resistant to all extractions and other treatments, with the exception of a slight reduction in aggregating activity upon acetylation (Table 4). This, coupled with the observation that *F. nucleatum* coaggregation was unaffected in both mutant types, clearly distinguished this reaction from the other interactions studied.

Aggregation was completely inhibited when EDTA was incorporated into the reaction mixture (Table 5) and could be restored by the addition of Ca^{2+} ions. None of the other substances tested had any effect on the coaggregation reaction.

No specific physicochemical characteristic could be attributed to the coaggregation with *B. asaccharolyticus*. Some evidence indicated that proteins on the *S. salivarius* surface were involved, since treatment with proteases and boiling partly destroyed the reactivity of the cells.

DISCUSSION

We demonstrated in this study that cell walls of *S. salivarius* possess at least three distinct components which are involved in a number of aggregation and adherence reactions. 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 *V. alcalescens* V1, was absent in mutant HB-V5 and was not sensitive to proteases. Coaggregation with *F. nucleatum* LF was mediated by a third category of receptors, which were present in both mutants and were sensitive to trypsin.

It is important to note that the activities of *S. salivarius* strains in host-related adherence and aggregation assays correlated strongly with the presence of the streptococcal group K antigen in Lancefield extracts of the strains. All K^+ strains tested aggregated in the presence of human saliva and adhered in high numbers to buccal epithelial cells, and most agglutinated human RBC. However, none of the K^- strains aggregated with saliva, and only 1 of 16 strains gave a weak hemagglutination reaction. Several K^- strains did not adhere to buccal epithelial cells or adhered only in low numbers. Since the K^+ strains cross-reacted with an antiserum prepared against *S. salivarius* HB and K^- strains did not (data not shown), the two groups probably represent serotype I and II strains, respectively, according to Sherman et al. (34) and Montague and Knox (27). Therefore, serotype should be considered in any ecological study of *S. salivarius*. As a consequence of their greater ability to adhere to human tissues, serotype I strains could be expected to have ecological advantages over serotype II strains in human oral cavities. However, the proportion of serotype I strains among our isolates was only 51%, a value which is similar to that reported by others for the oral cavity (27) and for clinical isolates from various parts of the human body (6). It is interesting that a significantly higher proportion of serotype I strains were isolated from throats (34), which may be related to greater ability to adhere there. It may be of interest to study the distribution of serotypes within human oral cavities.

Some of the discrepancies between our findings with strain HB and those of other authors who have reported on the adherence properties of *S. salivarius* may result from the use of different serotypes. It should also be noted that the strength of the various aggregation and adherence reactions varied within the same serotype.

Although the host-related aggregation and adherence reactions were clearly recognized as a single category, it is not yet clear whether only one receptor or different but closely related receptors on strain HB are involved; each receptor or each combination of receptors may mediate one of the host-related properties. If the same receptor mediates all of the reactions, some of the differences observed between the reactions could be quantitative rather than qualitative, assuming that an increasing number of receptor sites would be required in the following order: adherence to buccal epithelial cells < hemagglutination < saliva-induced aggregation. A certain degree of specificity between these reactions could also result if more than one factor were involved in each reaction. This was clearly demonstrated for saliva-induced aggregation. Calcium was not required for binding of the salivary aggregation factor to the bacteria, but was required to induce aggregation. Its role might be to form additional bonding forces between negatively charged cells or between the cells and the saliva factor. Calcium could play a similar role in other saliva-induced aggregation systems. Kashket and Donaldson (19) noted that the requirement for Ca^{2+} ions in saliva-induced aggregation of *S. sanguis* was lost upon formalinization of the cells, which suggests that the role of these ions was not to bind the salivary factor to the cells. Preliminary experiments showed the complete inhibition of saliva-induced aggregation of *S. salivarius* HB by 25 mM *N*-acetyl-D-glucosamine (T. Weerkamp and B. C. McBride, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, J4, p. 89). However, these observations could not be confirmed with other batches of *N*-acetyl-D-glucosamine and appeared to be the result of a non-dialyzable, boiling-resistant contaminant present in the original batch of carbohydrate. As little as 5 μg of the partially purified factor per ml completely inhibited saliva-induced aggregation of strain HB, but it did not affect the binding of the active salivary component to the cells or any of the other aggregation properties. Thus, the specific inhibition by this factor supports the hypothesis that more than one factor is involved in saliva-induced aggregation. In the present study it was shown that saliva did not interfere with hemagglutination or adherence to buccal epithelial cells. This supports the concept that different receptors are involved in the host-related reactions. This observation is in contrast to results reported by Gibbons and co-workers (17). These authors also reported that trypsin destroyed the ability of *S. salivarius* to adhere to buccal epithelial cells, presumably by removing the fimbriae or "fuzzy coat" (17). The discrepancies are probably due to differences in the surface properties of the isolates from the two laboratories.

The receptor in *S. salivarius* HB responsible for saliva-induced aggregation is different from that in *S. sanguis*. The latter is sensitive to trypsin (unpublished data) and presumably binds sialic acid residues (21, 22). *S. salivarius* HB cells and a variety of other K^+ strains remove the *S. salivarius* HB aggregation factor from saliva but do not remove the activity for *S. sanguis* cells (unpublished data). This confirms the observation of Gibbons and Qureshi (12) that different oral streptococci bind different mucins.

The results in this study show that a significant proportion of *S. salivarius* isolates have the potential to adhere to a variety of host surfaces in oral cavities, including soft epithelial surfaces and the pellicle on the teeth. It is obvious from studies on the growth habitat of this organism that some adherence capabilities are used either infrequently or never. What controls the expression of these properties is an interesting question and is currently under study in our laboratory. In addition to these host-related activities, *S. salivarius* HB binds strongly to a number of anaerobic bacteria. The role of these interbacterial associations in oral cavities is not known, but it can be speculated that *S. salivarius* acts as a primary colonizer and subsequently as a primary site of attachment for more fastidious, less O_2-tolerant organisms. Possibly there are other physiological consequences, such as providing lactic acid for *Veillonella* (26). Presumably this physiological interaction would be facilitated by close physical contact.

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