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Surface Properties of *Streptococcus sanguis* FW213 Mutants Nonadherent to Saliva-Coated Hydroxyapatite

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Seventeen mutants of *Streptococcus sanguis* FW213 nonadherent to saliva-coated spheroidal hydroxyapatite were isolated after mutagenesis with ethyl methanesulfonate, nitrosoguanidine, nitrous acid, hydroxylamine, or 2-aminopurine. Enrichment for nonadherent mutants was accomplished by successive adsorptions of the adherent strains to saliva-coated hydroxyapatite. After enrichment, variant colonial morphology on tryptic agar was used as a screening technique for selection of nonadherent mutants, with loss of colonial opacity frequently associated with loss of adherence ability. These mutants were further characterized for additional surface properties, including twitching motility, saliva-induced aggregation, coaggregation with *Actinomyces* species, surface hydrophobicity, and presence of fimbriae. Results from these assays indicated that the nonadherent mutants fell into six phenotypic groups. A correlation between the loss of adherence ability, a decrease in cell fimbriation, and a decrease in surface hydrophobicity is apparent.

The ability of a bacterium to cause infection depends on its ability to colonize the host. The first step in the colonization process is the attachment of the organism to host tissue (8). Virulent strains of bacteria that adhere well to host tissue become avirulent when they lose their ability to adhere (1, 6). The determination of the mechanisms of bacterial adherence is important in the control of disease processes.

The mechanism(s) by which *Streptococcus sanguis* adheres to teeth and to saliva-coated hydroxyapatite beads is not fully understood. Several reports suggest that *S. sanguis* has cell surface proteins which may play a role in adherence (7, 9, 17, 27). Several investigators suggest there are at least two binding sites, a high-affinity site and a low-affinity site, and that sialic acid may play a role in the high-affinity site (10, 23). Other reports suggest that hydrophobic interactions play a role in the adherence of *S. sanguis* to smooth surfaces (25, 26). Since Scatchard plots of the adherence of *S. sanguis* suggest positive cooperativity, Nesbitt et al. (25) suggest that the protein-protein interactions of adherence may be stabilized by hydrophobic bonds.

This paper reports the isolation of nonadherent mutants (ad$^-$) of *S. sanguis* and phenotypically groups them according to surface properties. The data suggest there is a correlation among fimbriae, hydrophobicity, and the ability of *S. sanguis* to adhere to smooth surfaces.

MATERIALS AND METHODS

Strains. *S. sanguis* FW213 was used as the parent strain for mutagenesis. It was obtained from R. M. Cole at the National Institutes of Health, Bethesda, Md. It contains an unusual 10.4-kilobase plasmid that was used to monitor the isogenicity of the mutants. *Actinomyces viscosus* T14V and *Actinomyces naesulundii* strains W1544, I, and W826 were obtained from J. Cisar at the National Institutes of Health.

Media. Overnight cultures (12 to 15 h) of the parent strain and mutants were grown on plates containing tryptose blood agar base (Difco Laboratories) and 5% defibrinated sheep blood. Broth-phase cultures were grown in Todd-Hewitt broth (TH) (Difco). The colonial morphologies of the mutants were determined on agar plates containing Trypticase soy broth and dextrose (BBL Microbiology Systems) and 1.5% type IV powdered agar (Sigma Chemical Co.) (TS). Twitching motility was determined on blood agar plates having twice the volume of media as a normal blood agar plate to help conserve moisture.

Growth conditions. For each strain used, dimethyl sulfoxide (Fisher Scientific Co.; 5% final concentration) was added to 1-ml portions of broth-grown log-phase cells, and these portions were frozen immediately at −70°C in a Revco freezer. The same preservation process was used for strain FW213 when it was originally received. Frozen cells were removed, streaked onto a blood agar plate, and incubated aerobically for 12 to 15 h in 5% CO_2 at 37°C (standard conditions). This plate, stored at room temperature, became the stock plate for all experiments performed that week. New stock plates were made weekly from a frozen vial, ensuring that cells used were only one passage from the original.

Overnight confluency blood agar plates made from stock plates were incubated under standard conditions. The broth-grown log-phase cells used in some procedures were prepared as follows. Cells from an overnight confluent blood agar plate were harvested into TH broth to a concentration of ca. 1.5×10^8 cells per ml and grown aerobically at 37°C to 5.5×10^8 cells per ml as determined by turbidity.

Mutagenesis. *S. sanguis* FW213 cells were harvested from overnight confluent blood agar plates into appropriate buffers and sonicated for 15 s at 95 W in a Branson sonifier with an ultrasonic cuphorn. Phase microscopy showed that after this sonification step the chains were disrupted to greater than 90% single cells. Strain FW213 was then mutagenized with 50 μg of *N*-methyl-*N'*-nitrosoguanidine (NG) per ml for 35 min (22); 600 μg of 2-aminopurine (2AP) per ml for 12 h (22); 0.03 M ethyl methanesulfonate (EMS) for 5 min (20); 0.05 M nitrous acid (HNO_2) for 12 min (15); or 0.1 M hydroxylamine hydrochloride (NH_2OH) for 5 min (16) by published methods. All chemicals were purchased from Sigma. Survival curves were prepared, and the 1/*e* dose (37% survival) was chosen for NG, 2AP, and NH_2OH. A cell survival of 0.01%

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was chosen for HNO_2 since this has been determined to be the critical range for effective mutagenesis with this agent (22). A cell survival of 75% was chosen for EMS since increasing time resulted in a plateau followed by another exponential phase of killing. Although efforts were taken to reduce the probability of multiple mutations, the curves for NG and 2AP were complex and produced a shoulder which extrapolated to 2 and 1.5, respectively.

Adherence to saliva-coated hydroxyapatite. (i) Clarification of saliva. To minimize the batch-to-batch variability of adherence factors in saliva, large pools (ca. 3.0 liters) of paraffin-stimulated saliva from over 200 volunteers were collected on ice. The saliva was clarified by centrifuging at 17,000 $\times$ g for 10 min at 4°C (12). The supernatant was heated to 60°C for 30 min to inactivate enzymes that destroy the adherence factor (12). Sodium azide was added to a final concentration of 0.05%, and the clarified saliva was frozen in 10-ml portions at −30°C.

(ii) Hydroxyapatite. Spheroidal hydroxyapatite (SHA) beads (catalog no. 44225; BHD Chemicals, Ltd., Poole, England) were suspended in 0.067 M phosphate buffer (pH 6.0) and settled for 5 min. The supernatant containing the fines was aspirated. This washing was repeated 4 times. The remaining SHA beads were suspended in phosphate buffer and filtered through a 12-μm polycarbonate membrane filter (Nucleopore Corp., Pleasanton, Calif.). The beads were allowed to dry and were distributed in 40-mg portions by using a calibrated scoop (±1 mg).

(iii) Preparation of SC-SHA. To prepare saliva-coated SHA (SC-SHA), 1 ml of pooled clarified saliva was added to tubes containing 40 mg of SHA, and the tubes were incubated with rocking sufficient to keep the beads in suspension for 1 h at 37°C. The beads were allowed to settle, and the supernatant was removed and discarded. The beads were then washed three times in phosphate buffer.

(iv) Enrichment technique. Log-phase FW213 cells grown in TH broth were used for each enrichment procedure. Cells were washed, mutagenized, sonicated, and suspended in TH broth. The cells were incubated for 2 h at 37°C with 5% CO_2 to allow for at least two cell divisions to incorporate mutations into daughter cells. The broth culture was then centrifuged, and the cells were suspended to 10^9 cells per ml in 0.067 M phosphate buffer (pH 6.0). Samples were removed, sonicated, diluted, and plated on TS agar to determine the number of colonial morphology mutants before the enrichment technique.

Two milliliters of the mutagenized cell suspension described above were sonicated and added to each of five tubes of 80 mg of SC-SHA. These tubes were rocked gently at 4°C for at least 1 h to allow for maximum adsorption of cells to the SC-SHA. At the end of 1 h, the beads were allowed to settle, and the supernatants from each tube were removed and pooled. The beads were discarded, and the pooled supernatants were sonicated and redistributed to five new tubes of 80 mg of SC-SHA and allowed to rock again for 1 h at 4°C. These enrichment passes were continued until the cell concentration in the supernatant reached 2×10^8 cells per ml, a cell concentration at which the number of cells adhering to SC-SHA is significantly reduced (18). The pooled supernatants were centrifuged, suspended to 2 ml (10^9 cells per ml), added to one tube containing 80 mg of SC-SHA, and rocked as before. Passes were continued until the free cell concentration again fell to 2×10^8 cells per ml or lower. Samples of the cell suspension were then diluted, sonicated, and plated on TS agar.

After 48 h of incubation at 37°C in 5% CO_2, colonies were examined with a Bausch & Lomb stereo dissecting microscope (model STEREOZOOM 7). Light was indirectly reflected through the bottom of the agar plate by using a concave microscope mirror (31). Photographs of selected colonial morphologies were taken in the same light with a Zeiss stereo dissecting microscope with an attached Olympus OM-1 35-mm camera. This isolation procedure was repeated several times for each mutagen used. Mutants were frozen at −70°C soon after isolation.

(v) Strain FW213 standard curve adherence assay. The strain FW213 standard curve adherence assay was a modification of that developed by Gibbons et al. (11). Broth-grown log-phase strain FW213 cells were labeled in TH broth with 2.0 μCi of [^{3}H]thymidine per ml. Labeled cells were centrifuged and washed three times with 0.067 M phosphate buffer (pH 6.0). Cell suspensions ranging from 4×10^7 to 6×10^9 cells per ml were made in phosphate buffer and sonicated for 15 s at 95 W in a Branson sonifier with an ultrasonic cuphorn. Portions (100 μl) were removed to determine the specific activity of the cells. One milliliter of each cell concentration was added to duplicate tubes of SC-SHA and incubated for 1 h at 37°C with gentle rocking. The beads were allowed to settle for 1 min, and 200 μl of the supernatant was removed to determine the free cell concentration. The beads were then washed three times in buffer and transferred to scintillation vials to determine the number of cells bound to SC-SHA. All samples were counted in a Beckman scintillation counter (model LS 7500). The number of bound cells was plotted against the number of free cells.

(vi) One-point adherence assay. Strain FW213 and each mutant strain tested for adherence were grown and radiolabeled as described above. The labeled cells were centrifuged, washed three times with phosphate buffer, and suspended to 10^9 cells per ml (ca. 50% adherence level). Sonified cell suspensions were added to 40 mg of SC-SHA, incubated, washed, and counted as above. The number of cells bound for each mutant strain was compared to the number of cells bound to strain FW213 in that experiment and expressed as a percentage of the adherence of strain FW213.

Biochemical characterization. Ad^- strains were tested biochemically and compared with the parent strain, FW213. Carbohydrate fermentation was determined in phenol red broth base (Difco) with 1% (wt/vol) filter-sterilized inulin, lactose, manitol, raffinose, salicin, and sorbitol (all from Sigma). Acid production was examined daily for up to 7 days. Arginine hydrolysis was tested in Moeller decarboxylase broth with 1% L-arginine (BBL). Esculin hydrolysis was detected on an agar slant containing 4.0% heart infusion agar (Difco), 0.1% esculin (Sigma), and 0.05% ferric citrate (Sigma). In addition, each mutant was screened for the presence of a 10.4-kilobase plasmid associated with the parent strain.

Twitching motility. Cells from a 12 to 15 h confluency plate were diluted in saline and spread on TW blood agar, sealed with tape, and incubated aerobically for 7 days at 37°C in 5% CO_2 under high humidity. Colonies were screened for twitching motility (tw^+) by using a stereo dissecting microscope with oblique lighting. Those colonies possessing a thin, translucent, wavy spreading edge moving away from the colony center were classified as tw^+ (13), whereas those without this zone were classified as tw^-.

Saliva-induced aggregation. (i) Clarification of saliva. Saliva was clarified and stored as described above with the exception that the saliva was not heated to avoid degradation of the aggregation factor (28).

(ii) Assay. The aggregation assay was adapted from that of McBride and Gisslow (21). Overnight confluent blood agar plates (ca. 4×10^9 cells) for each strain tested were harvested into 1 ml of aggregation buffer (0.05 M Tris hydrochloride [pH 7.2] containing 0.005 M $CaCl_2$) and kept in a 37°C water bath until tested. Aggregation testing was performed in 12-well concave agglutination slides (well size, 16 mm in diameter and 1.75 mm deep). The test wells contained 30 μl each of buffer, bacterial suspension, and undiluted saliva, whereas control wells contained only buffer and bacteria. Covered slides were rotated for 5 min on a slide rotator (Thomas rotating apparatus no. 3623) at 150 rpm and examined macroscopically for bacterial aggregates by using an MIC microviewer (Rayco) with illumination from beneath. Negative strains showed no aggregation in either the control or test well. Positive strains showed aggregation in the test well but not in the control well. Those strains that showed aggregation in both the test well and control well were termed saliva-independent aggregators. Positive strains were retested in the same manner by using twofold dilutions of saliva, with the highest dilution showing visible aggregates recorded as the endpoint.

Coaggregation. The ability of selected mutants to coaggregate with strains of *A. viscosus* and *A. naeslundii* was adapted from the method of Cisar et al. (3). For each strain tested, cells from an overnight confluent blood agar plate were harvested into 5 ml of coaggregation buffer ($CaCl_2$ [10^{-4} M], $MgCl_2$ [10^{-4} M], NaN_3 [0.02%], NaCl [0.15 M] dissolved in 0.001 M Tris adjusted to pH 8). The cells were washed 3 times in 5-ml volumes of coaggregation buffer and suspended to 2×10^9 cells per ml. Portions of each cell suspension (0.15 ml) were added to 0.15 ml of each *Actinomyces* strain (2×10^9 cells per ml in buffer). Tubes were allowed to stand overnight at room temperature, vortexed as described above, and read again. After reading, 1 drop of 1 M lactose was added to each tube showing coaggregation, and the tube was vortexed for 10 s and examined for reversal of coaggregation.

Estimation of surface hydrophobicity. A modification of the method of Lindahl et al. (19) was used to estimate the relative surface hydrophobicity of the cells. Dilutions of ammonium sulfate (pH 6.8) from 4.0 to 0.2 M were made in 0.002 M sodium phosphate buffer (pH 6.8) in 0.2 M increments. Ninety-six-well V-bottomed microtiter plates (Dynatech Laboratories) were used for all tests. Each well received 0.03 ml of the appropriate salt dilution. For each strain tested, cells from a confluent blood agar plate were harvested into 1 ml of 0.002 M sodium phosphate buffer at 4°C to an approximate concentration of 4×10^9 cells per ml. Cells (0.03 ml) were placed in each dilution of salt, and the plates were rocked gently for 2 min and then read on an MIC viewer (Rayco) with illumination from beneath. Visible aggregates were scored as positive, and the lowest molarity giving this pattern was defined as the endpoint. A control well containing buffer and cells was also run. Those strains that aggregated in the control well were classified as autoaggregators and were excluded from further testing. After initial screening, each strain was tested a second time in 0.033 M increments within the critical range.

Electron microscopy. *S. sanguis* FW213 fimbriae are very hydrophobic and resist ordinary negative staining techniques. Log-phase FW213 cells were washed three times in phosphate-buffered saline (pH 7.4) and suspended to their original volume. Cells (0.8 ml) were mixed with 0.2 ml of preimmune antiserum (16 μg/ml) and incubated at 4°C for 2.5 h. These cells were then concentrated by centrifugation and suspended in 25 μl of phosphate-buffered saline. The sample was mixed with an equal volume of 3% phosphotungstic acid (pH 7.4) and allowed to settle on a carbon-coated grid for 1 min. The excess fluid was removed.

TABLE 1. Numbers of nonadhering mutants of *S. sanguis* FW213 obtained with selected mutagenic agents

Mutagen	No. of postenrichment colonies screened	No. of nonadherent mutants isolated
NG	1,505	1[a]
2AP	1,567	0[a]
EMS	1,191	12
Nitrous acid	1,145	2
Hydroxylamine	1,154	0

[a] One nonadherent mutant from NG and one from 2AP were isolated from screening an equivalent number of preenrichment colonies.

RESULTS

Isolation of nonadherent mutants. Putative ad$^-$ mutants were selected for adherence testing on the basis of variant colonial morphology on TS agar (see Table 2). The morphology of strain FW213 on TS agar after 48 h of incubation can be described as medium sized, round, smooth edged, slightly raised, and opaque (Fig. 1A). A variety of colonial morphologies were noted, but the most common type isolated was a transparent colony that was flat compared with strain FW213 (Fig. 1B). Many of the differences in colonial morphology, although subtle, were easily discernible with the stereo microscope. A screening of 6,922 postenrichment colonies resulted in the selection of 73 variant colonial morphology mutants. When tested for adherence, 12 of these mutants showed levels of adherence less than that of the control. Upon subsequent replating, three variants, VT326, VT328, and VT300, gave rise to two distinct morphologies, each of which was nonadherent. Two of these ad$^-$ mutants (VT345 and VT343) had colonial morphologies resembling that of the parent strain and would have been missed on initial isolation. Two ad$^-$ mutants were isolated from preenrichment plates. The adherence levels of all of the ad$^-$ mutants ranged from 2 to 34%, with greater than 80% of the mutants showing adherence levels less than 20% of that of the parent strain (see Table 2).

Relative effectiveness of mutagenic agents. The alkylating agent EMS was the most effective mutagen, inducing six times as many ad$^-$ clones as the other alkylating agent, NG, or the deaminating agent HNO_2, the next most effective mutagens. NH_2OH was totally ineffective in producing nonadherent strains (Table 1).

Biochemical characterization. All the mutants remained alpha hemolytic on blood agar, maintained the 10.4-kilobase plasmid of the parent, and conformed to the fermentation characteristics of the parent strain, although 12 of the mutants had delayed fermentation of inulin.

Adherence to SC-SHA. The ability of *S. sanguis* FW213 to adhere to SC-SHA was tested by the adherence method of Gibbons et al. (11). The Langmuir adsorption isotherms derived from these data show the curvilinear relationship and saturation of sites characteristic of typical specific binding in this model (Fig. 2). A plot of C (the number of free cells)/Q (the number of bound cells) versus C yielded a straight line with an r value of 0.99. The calculated number

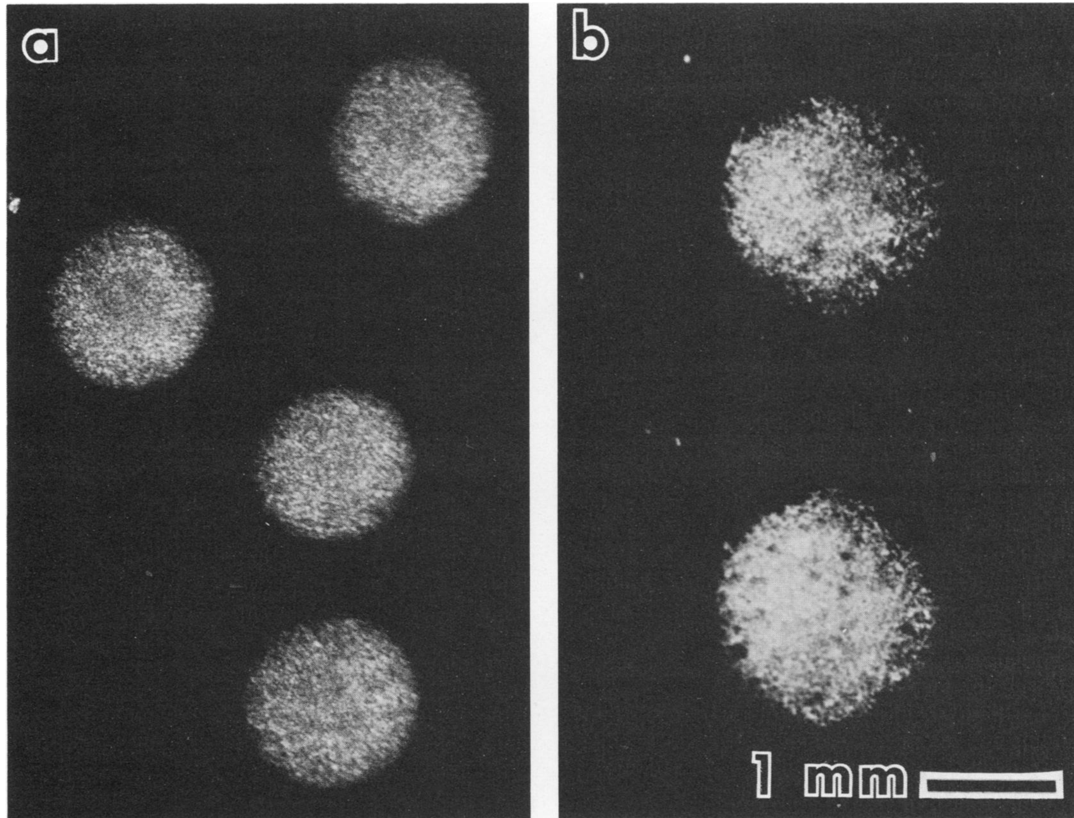

FIG. 1. Mutant colonial morphology. Strains were grown on TSA plates for 48 h at 37°C in 5% CO_2. Photographs were taken with a Zeiss stereo dissecting microscope with an attached Olympus OM-1 35 mm camera. Light was indirectly reflected up through the bottom of the agar plate by using a concave microscope mirror. All colonies were photographed at the same magnification. (a) Parent FW213; Medium sized, round, smooth edges, slightly raised, and opaque. (b) Mutant VT378; Large colony depicting the more typical transparent, flat colony.

of binding sites (N) was 2.79×10^8, whereas the affinity (K) was 1.22×10^{-9}. Saturation of the binding sites was reached when Q approached 3×10^9 cells per ml. In contrast, the ad$^-$ mutants displayed the straight line characteristic of nonspecific binding (Fig. 2).

Twitching motility. Twitching motility is a characteristic translocation of bacteria on agar surfaces that has been reported to be associated with polar fimbriae (13). Since twitching motility is a bacterial surface phenomenon, each mutant was tested for twitching motility and scored as a percentage of colonies that were tw$^+$ (Table 3). Five ad$^-$ mutants displayed a twitching phenomenon different from that of the parent strain. Three strains (VT330, VT331, and VT345) showed a thin, translucent edge, but this edge was very even compared with the normal, irregular (wavy) edge. These strains were classified as intermediate twitchers. Only two mutants, VT344 and VT342, were tw$^-$.

Estimation of surface hydrophobicity. Cell surface hydrophobicity is a characteristic repeatedly associated with bacterial adherence (25). Precipitation with $(NH_4)_2SO_4$ is one method that may be used to estimate and quantitate the relative surface hydrophobicity of bacterial cells (19). This salting out effect has been shown to be indirectly proportional to the hydrophobicity of the cell. Strain FW213 salts out at 2.8 M $(NH_4)_2SO_4$ (Table 3). Thirteen of the ad$^-$ mutants tested required greater than 4.0 M $(NH_4)_2SO_4$ to precipitate the cells. Four of the mutants (VT345, VT324, VT379, and VT380) were salted out with significantly less $(NH_4)_2SO_4$ (1.47 M, 0.90 M, 0.70 M, and 0.63 M) than was required for the parent strain, and based on this method of estimating hydrophobicity they were classified as more hydrophobic than strain FW213.

Coaggregation. A number of isolates of *S. sanguis* are capable of coaggregating by a lactose reversible interaction with selected strains of *A. viscosus* and *A. naeslundii* (3). Strain FW213 did not display this coaggregation phenomenon (Table 3). One of the nonadherent mutants (VT346) coaggregated with *Actinomyces* isolates of both group A (*A. viscosus* T14V and *A. naeslundii* W1544) and group B (*A. naeslundii* I and *A. naeslundii* W826). Since the coaggregation was stronger with group B *Actinomyces* and was lactose reversible, this mutant was placed with the Cisar group 3 coaggregators (3). All other ad$^-$ mutants were negative for coaggregation when tested against these *Actinomyces* species.

Saliva-induced aggregation. Many strains of *S. sanguis*, but not strain FW213, are capable of aggregating in the presence of heated and unheated saliva (21, 28). Three of the ad$^-$ mutants, (VT330, VT331, and VT346) displayed saliva-mediated aggregation (Table 3). The relative amount of aggregation factor present on each positive strain was indicated by the greatest dilution of saliva at which aggregates were still visible. One strain (VT346) still aggregated in saliva at a dilution of 1:128, and two strains (VT330 and VT331) aggregated only to a dilution of 1:4.

Electron microscopy. *S. sanguis* has long, delicate peritrichous fimbriae when negatively stained and viewed in an electron microscope (Fig. 3A). All but two of the ad$^-$

mutants (VT345 and VT324) lost the fimbriae (Fig. 3B) characteristic of the parent strain.

DISCUSSION

The ability of *S. sanguis* to colonize teeth depends on its ability to adhere and form plaque. Since *S. sanguis* has been shown to be the first organism to adhere to teeth (2), has one of the highest affinities for saliva-coated tooth surfaces (14), and makes up a major portion of total plaque accumulation, these experiments to isolate mutants defective in adhesive ability were undertaken to gain some insight into the mechanisms involved in adhesion of this organism.

The first problem encountered was the lack of a selection technique for nonadherent mutants. In hope of obtaining a variety of mutant types, we selected an enrichment procedure (removing ad^+ cells by binding to SC-SHA) which did not selectively bias one mutant type over another. The enrichment procedure was helpful, resulting in a 25-fold increase in ad^- cells. A screening of ca. 7,000 postenrichment colonies resulted in the selection of 73 colonial morphotypes (approximate frequency, 4×10^{-4}), of which 15 showed adherence levels less than that of the control (adherent mutant frequency, 8×10^{-5}). The screening of an equal number of preenrichment colonies resulted in only two nonadherent isolates. The enrichment technique using glass flasks and 1% sucrose to isolate nonadherent mutants of *S. mutans* (24), although more effective than the technique described above, cannot be used for *S. sanguis*. The isolation of *S. sanguis* mutants by partitioning (9) selects for mutants that are hydrophylic. Although our data indicate that 76% of the mutants had decreased hydrophobicity and support the observation that hydrophobic interactions promote adhesion of the bacterial cell to host tissue (25), four of the mutants had a significant increase in hydrophobicity and still cannot adhere.

Changes in colonial morphotype have been used as a screening technique for isolation of nonadherent mutants by many investigators (24, 29, 30). We also found it useful, and during these investigations we recognized that a loss in colonial opacity was frequently associated with nonadherence. Consequently, it introduced a bias in the screening process, since transparent colonies were preferentially selected. Since two of our mutants had wild-type morphology, it was clear that nonadherent cells do not need to be colonial morphotypes. This differs from the observations of Murchison et al. (24); they found that all tested *S. mutans* colonies with wild-type morphologies had parental adherence characteristics.

All of the mutants isolated came from either different enrichment experiments or displayed differences in colonial morphology. Thus, we believe that each mutant is of independent origin. The most common type of mutant isolated lacked fimbriae and had decreased surface hydrophobicity. The association of loss of fimbriae with decreased hydrophobicity is not surprising since it has been reported that the hydrophobicity of a bacterial cell surface is often related to the possession of fimbriae (32). The loss of fimbriae in all but

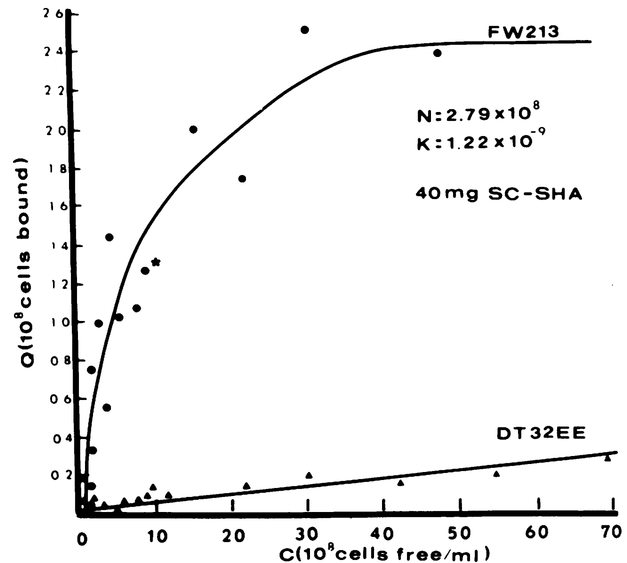

FIG. 2. Langmuir adsorption isotherms of *S. sanguis*. Broth-grown log-phase cells were labeled with [^{3}H]thymidine in TH broth. Washed, labeled cells in phosphate buffer were added to 40 mg of SC-SHA. The beads were incubated on a mixer at 37°C for 1 h. The beads and adsorbed cells were allowed to settle, and the supernatant was removed. The amounts of radioactivity associated with the beads and supernatant were determined in a Beckman LS7500 liquid scintillation counter, and the counts were converted to the number of cells bound to 40 mg of SC-SHA (Q) and number of free cells (C). *S. sanguis* FW213 (●) displayed the curvilinear relationship characteristic of specific binding. The straight line shown by VT321 (▲) suggests that these cells bind nonspecifically to SC-SHA.

TABLE 2. Colonial morphology of *S. sanguis* FW213 and its nonadherent mutants

Strain	Mutagenic agent	Morphology on TS agar
FW213 (parent strain)		Medium sized, round, smooth edge, slightly raised, opaque.
VT321	EMS	Medium, slight wavy edge, less opaque, striated.
VT324	EMS	Medium, slight wavy edge, transparent, "spokes".
VT325	EMS	Medium, less opaque center with transparent edge.
VT344[a]	EMS	Small, irregular edge, transparent.
VT345[a]	EMS	Like FW213.
VT342[b]	EMS	Small, transparent, flat.
VT343[b]	EMS	Like FW213 but larger.
VT377	EMS	Medium, irregular edge, "flower" shaped, transparent sections.
VT378	EMS	Large, feather edge (thin), less opaque.
VT329	EMS	Medium, opaque center, transparent edge.
VT379	EMS	Medium, rough colony, irregular edge, less opaque, more raised in center.
VT380	EMS	Medium, irregular transparent edge, opaque center.
VT330[c]	HNO_2	Small, more raised in center, opaque.
VT331[c]	HNO_2	Medium, transparent, round, slightly raised.
VT346	2AP	Medium, very irregular shape, less opaque, more raised in center.
VT360	NG	Medium, very transparent, raised rim.
VT361	NG	Medium, less opaque, striated.

[a,b,c] These mutants were originally isolated as a striated colony that gave rise to two colonial morphologies, both of which were nonadherent.

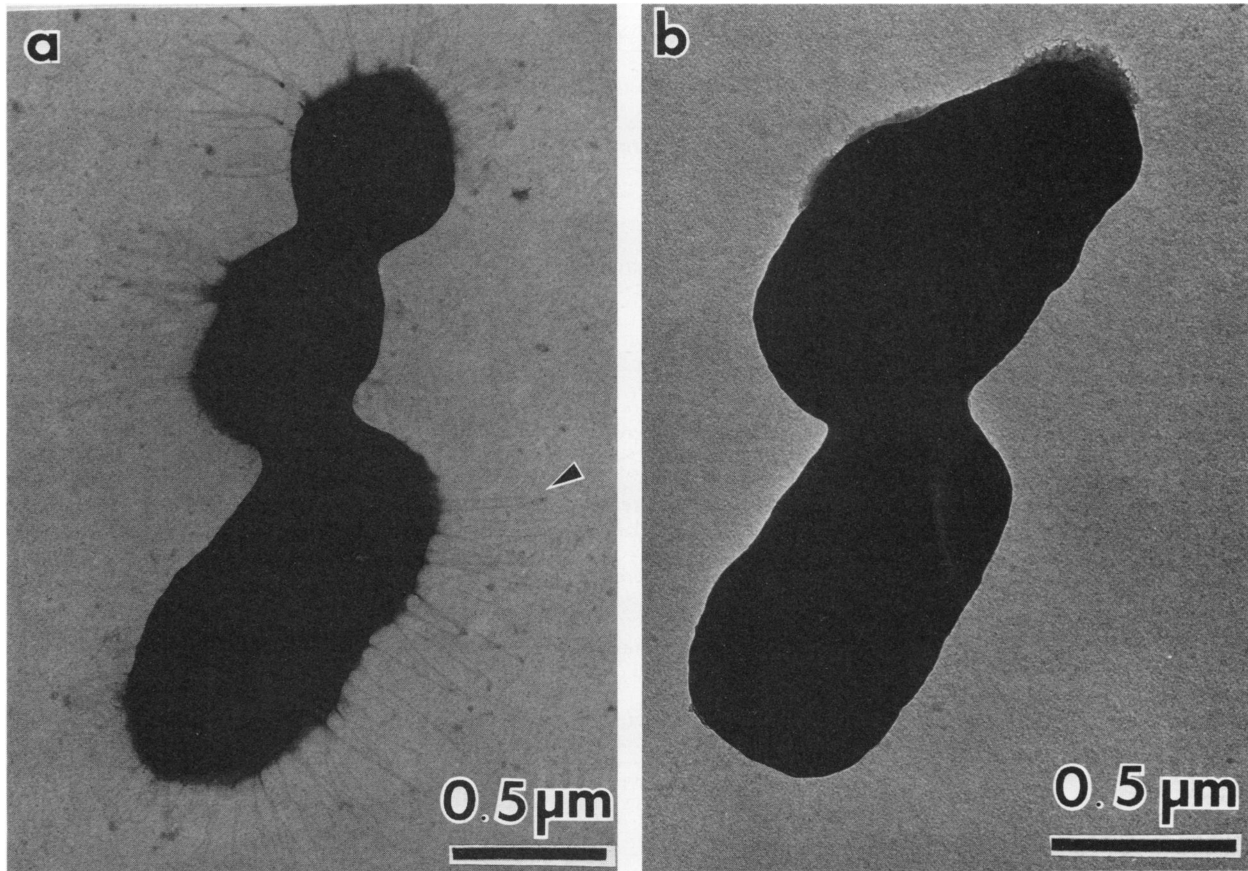

FIG. 3. Electron micrographs of *S. sanguis* FW213 and a typical mutant. Cells were grown in TH to the late log phase of growth. Grids were made, negatively stained with phosphotungstic acid, and observed in a Phillips 300 transmission electron microscope. (a) Parent strain FW213; Cells possess peritrichous fimbriae (arrow) that are as long or longer than the width of the cell (650 nm). (b) VT321; Typical mutant cells that do not have fimbriae.

two mutants implies that fimbriae play an important role in the adhesion of *S. sanguis* to SC-SHA.

The mutagenic agents were not equally effective in inducing mutations to ad$^-$. Twelve of the 17 ad$^-$ mutants were induced by the alkylating agent EMS, making it six times more effective than any of the other agents. The other alkylating agent, NG, only produced two ad$^-$ mutants. The deaminating agents HNO_2 and NH_2OH also differed in their ability to induce mutation to ad$^-$, with HNO_2 inducing two mutants and NH_2OH inducing none. The base analog 2AP was not very effective either. One mutant was selected from preenrichment colonies, and none were selected from the postenrichment colonies. EMS is widely used as a mutagenic agent because it is known to produce a high frequency of mutations at relatively high survival rates (20). This study supports these observations. Swaney et al. (30) isolated type 1 pili-negative mutants of *Escherichia coli* by using many of the same mutagenic agents. In contrast to our findings, the base analog 2AP was the most effective, with 23 mutants derived, and 15 more mutants were isolated with another base analog, 5-bromouracil. The least effective agents in their study were the alkylating agents NG (six mutants) and EMS (nine mutants). The investigators did not indicate whether care was taken to ensure that all mutants were of independent origin. Their high number of isolates by using base analogs may have been due to siblings that arise because of the long growth period necessary for incorporation of the base analogue (22). Using only EMS and HNO_2, Murchinson et al. (24) isolated 38 *S. mutans* mutants that varied from the parent strain in adherence and aggregation and were selected on the basis of variant colonial morphology. Of these, 31 were from EMS mutagenesis and 7 were from HNO_2 mutagenesis. Although each of these studies indicates differences in the effectiveness of the mutagenic agents used, it is difficult to evaluate these studies since the numbers of colonies screened and the corresponding frequencies of isolation for each agent are not indicated.

Since the survival curves for EMS indicated a single mutation, it is surprising that two isolates, VT342 and VT344, were both ad$^-$ and tw$^-$. These data imply that twitching motility and adhesive ability have a gene in common. In further support of this hypothesis, Gibbons et al. (9) isolated a spontaneous hydrophilic mutant of *S. sanguis* by using partitioning in hexadecane for selection. This mutant had lost hydrophobicity, fimbriae, twitching motility, and adhesive ability. It would have been classified in our phenotypic group II. Henriksen and Henrichsen (13) suggest that twitching motility is mediated by polar fimbriae. We were unable to confirm this observation since our ad$^-$ tw$^+$ mutants do not appear to have fimbriae. Interestingly, Cole et al. (4), Fives-Taylor (7), and Gibbons et al. (9) have all reported peritrichous fimbriae on *S. sanguis*. Micrographs by Henriksen and Henrichsen (13) show polar fimbriae but no peritrichous fimbriae. Further studies are under way to

TABLE 3. Phenotypic groups of mutants of *S. sanguis* FW213 nonadherent to SC-SHA

Group	Mutant strain	% Adherence	% Twitching motility colonies	Relative hydrophobicity[a]	Coaggregation	Saliva-inducted aggregation	Presence of fimbriae
	FW213 (parent strain)	100	100	Normal (2.8 M)[a]	−	−	+
I	VT321	11					
	VT325	8					
	VT377	2	100	Decreased (>4.0 M)	−	−	−
	VT378	2					
	VT360	11					
	VT361	4					
	VT343	7					
	VT329	15					
II	VT344	2		Decreased (>4.0 M)			
	VT342	11	0		−	−	−
III	VT345	11	70[b]	Increased (1.47 M and 0.9 M)			
	VT324	16	100		−	−	+
IV	VT330	27	96[b]	Decreased (>4.0 M)			
	VT331	24	94[b]		−	+	−
V	VT379	8		Increased (0.63 M and 0.7 M)			
	VT380	16	100		−	−	−
VI	VT346	34	100	Decreased (>4.0 M)	+	+	−

[a] The concentration of $(NH_4)_2SO_4$ required to precipitate the cells is shown in parentheses.
[b] Intermediate twitching zone, i.e., has the transparent zone associated with twitching motility but lacks the irregular, wavy margin.

determine whether this discrepancy is due to phase variation, cultural conditions, or electron microscopy techniques or a combination of these factors.

The group III mutants are particularly interesting since they produced fimbriae and yet were unable to adhere to SC-SHA. Since the relative surface hydrophobicity of the cells was significantly increased, it seems probable that these two mutants have mutant fimbriae with more nonpolar groups exposed. Our laboratory is in the process of cloning the fimbrial genes, and these mutants will prove very useful in elucidating the functional adherent regions of the fimbriae.

The group IV and group VI mutants present unique problems in that they gained the properties of coaggregation or saliva-coated aggregation or both not found in the parent strain. The only plausible explanations for gaining a property are that strain FW213 is a coaggregation-deficient, aggregation-deficient mutant and this is a back mutation, that strain FW213 has this gene or genes turned off and the new mutation reversed it, or that the site or sites in strain FW213 are masked by other structures and the new mutation exposed the site. We have no data at present to suggest which of these explanations is most likely. Since these mutants do not have fimbriae, these data suggest that saliva-induced aggregation and coaggregation of *S. sanguis* with *Actinomyces* strains are not mediated by *S. sanguis* fimbriae. This hypothesis is further supported by Cisar et al. (3), who have demonstrated that coaggregation is a lectin-like activity and that the type 2 fimbriae on *A. viscosus* is required. The group VI mutant, VT346, is both coaggregation and agglutination positive. Since it was induced by 2AP, which gave a complex survival curve, it is likely that this mutant phenotype is the result of two mutations, one involving the loss of fimbriae and the other involving loss of the aggregation-coaggregation properties. It is more difficult to explain the group IV mutants VT330 and VT331, which acquired the property of saliva-induced aggregation, since the survival curves for HNO_2 indicate single-hit kinetics. Perhaps the mutation that resulted in the loss of fimbriae exposed aggregation sites that already existed on strain FW213. These two mutants may be closely related since they were originally isolated as a single, striated colony that gave rise to two distinct morphologies when subcloned.

In summary, these data indicate that: (i) the fimbriae of *S. sanguis* play an important role in the adhesion of this organism to SC-SHA; (ii) a loss of fimbriae is associated with a loss in surface hydrophobicity; (iii) adherence activity and twitching motility, although mediated by separate genes, do have at least one gene in common; and (iv) coaggregation and saliva-induced aggregation are not mediated by fimbriae.

The grouping of these mutants by surface properties has allowed us to place them into six phenotypic groups (Table 3). Immunological (5) and genetic analyses of these mutants have begun. They will prove invaluable in understanding the molecular basis of adhesion as well as of fimbrial regulation and control.

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