Phenotypic Characterization of *Streptococcus sanguis* Virulence Factors Associated with Bacterial Endocarditis

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Certain strains of *Streptococcus sanguis* adhere (Adh⁺) selectively to human platelets and, in plasma, induce them to aggregate (Agg⁺) into in vitro thrombi. In this study, we examined 18 recent endocarditis and dental plaque isolates of microorganisms that were biotyped as *S. sanguis* for coexpression of platelet interactivity phenotypes with another possible virulence factor in bacterial endocarditis, dextran synthesis. Detectable production of extracellular glucosyltransferase ranged from 0.2 to 66 mU/mg of culture fluid for 10 representative strains tested. Production of extracellular or cell-associated glucosyltransferase, fructosyltransferase, and soluble or insoluble dextrans was not necessarily coexpressed with platelet interactivity phenotypes, since the levels of production of soluble and insoluble dextrans varied among representative Adh⁺ Agg⁺ and Adh⁻ Agg⁻ strains. Analysis of a second panel of 38 fresh dental plaque isolates showed that *S. sanguis* distributes in a reproducible manner into the possible phenotype groups. Strains with different platelet interactivity phenotypes were distinguished with a panel of four murine monoclonal antibodies (MAbs) raised against Adh⁺ Agg⁺ strain 133-79 and screened to rule out artifactual reactions with antigenic components in culture media. The MAbs reacted selectively with Adh⁺ Agg⁺ strains in a direct-binding, whole-cell, enzyme-linked immunosorbent assay and also inhibited their interactions with platelets. Analysis of minimal tryptic digests of many strains, including variants that failed to bind the MAbs, suggested that some noninteractivity phenotypes possess cryptic surface determinants. Since the ability to adhere to platelets and induce them to aggregate is relatively stable, these traits may be useful in a phenotyping scheme for these Lancefield nontypeable streptococci.

The virulence of viridans streptococci in the pathogenesis of bacterial (or infective) endocarditis (BE) remains an enigma. These bacteria, typically *Streptococcus sanguis* and *S. mutans*, arise in the oral cavity and are believed to enter the bloodstream as a result of trauma as bland as the manipulations of oral hygiene (19, 22). These bacteremias may infect sites of underlying pathologic changes of heart valves (1, 4, 11, 26, 35), such as nonbacterial thrombotic vegetations (NBTV) (10, 20, 27, 28). Adhesion to NBTV may be insufficient to explain the virulence of certain viridans streptococci in BE. In the rabbit model of BE, NBTVs are formed in response to an indwelling catheter before induction of experimental bacteremia (11, 27). On the damaged heart valves, adherent bacteria soon become embedded and protected in newly formed thrombi or platelet vegetations. Consequently, streptococci capable of initial adhesion and rapid induction of thrombosis are likely to be more virulent in clinical disease. As many as half of all cases of BE have been attributed to viridans streptococci, with *S. sanguis* identified as the vector three to four times more frequently than *S. mutans* (25, 34). This association may reflect the large proportion of these microorganisms in the oral flora and the frequency of these bacteremias in comparison with those that arise from other organs and tissues. The specificity of infection may also reflect special virulence traits of these bacteria.

Synthesis of dextrans by streptococci has been suggested to be associated with virulence in BE. Dextrans may in-

crease adhesion of bacterial cells to valvular tissues (23, 24) and NBTV (10, 11, 27, 28). However, when many strains and species of BE-associated viridans streptococci were compared for the ability to adhere to platelet-fibrin clots in vitro to model NBTV, no simple relationship emerged between (i) the ability to adhere and synthesize dextrans and (ii) clinical disease (5).

Certain strains of *S. sanguis* from dental plaque and blood cultures of confirmed cases of BE have been shown in vitro to adhere to and induce thrombotic aggregation of human platelets, with stability and specificity apparently independent of the actual synthesis or binding of dextrans (14, 16). There are significant and reproducible differences between strains as agonists in induction of aggregation of normal platelets (31). These differences are stable over time. Platelet-*S. sanguis* interactions are mediated by at least two distinct antigens on the bacterial surface (8, 16). A trypsin-sensitive adhesin (class I antigen) binds the bacterial cell to the platelet. Platelets are triggered to aggregate by the platelet aggregation-associated (or class II) antigen, which is immunologically cross-reactive with platelet-interactive determinants on type I and III collagens. Since many streptococci also synthesize cell-associated and extracellular dextrans (12, 24, 25, 27, 28), coexpression with platelet-interactive antigens may have important implications for virulence in BE.

In this report, expressions of platelet interactivity phenotypes and dextran synthesis were compared. Production of extracellular glucosyltransferase (GTF), cell-associated GTF, and soluble or insoluble dextrans was not necessarily

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coexpressed with platelet interactivity phenotypes. Strains that carried surface determinants associated with these phenotypes could be distinguished with monoclonal antibodies (MAbs). Analysis of minimal tryptic digests of variant strains suggested that some noninteractivity phenotypes, however, possess cryptic surface determinants. Since the ability to adhere to platelets and induce them to aggregate is relatively stable, these traits may be useful in a phenotyping scheme for these Lancefield nontypeable streptococci.

MATERIALS AND METHODS

Bacterial strains. All strains were purified from mitis salivarius and blood agar plates maintained for up to 72 h at 37°C in 5% CO₂. Strains were stored at -70°C in skim milk and classified before study as *S. sanguis* by the biotyping scheme of Facklam (9). *S. sanguis* 133-79 and 10556 have been described previously (8, 14, 16, 31). Strain 133-79 was isolated from a blood culture in a confirmed case of BE and was obtained from R. R. Facklam, Centers for Disease Control, Atlanta, Ga. Strain 10556 was originally obtained from the American Type Culture Collection. Other reference strains and recent isolates of *S. sanguis* from human dental plaque (strains L74, L14, L22, L31, L13, L59, L52, L50, LS4123, LS4124, FW213, and 10558) were the kind gift of W. F. Liljemark, University of Minnesota, Minneapolis. Strains E1219, S1219, and NR133 were variants derived from the parental strain, *S. sanguis* 133-79.

Additional strains were isolated with sterile cotton swabs from the buccal surfaces of both mandibular first molars of 25 children who had recently completed antibiotic therapy for recurrent otitis media (Park-Nicollet Medical Center, Minneapolis, Minn.) and a healthy-child control group ($n = 24$) after informed consent was obtained from a parent or guardian. Consent procedures were approved by the Committee on the Use of Human Subjects in Research of the University of Minnesota. Therapy involved 10 days of antibiotic coverage with amoxicillin, sulfamethoxazole-trimethoprim, or erythromycin ethylsuccinate-sulfisoxazole acetyl. The children who received antibiotics ranged in age from 36 to 72 months. When patients were grouped as 3, 4, or 5 years old, the 12 males and 23 females distributed generally into groups of 4 each, males and females. The control group was selected from a pool of healthy children who were scheduled for yearly physical examinations, showed the same age distribution, and received no antibiotic within 24 months before sampling.

Selection of variants. Variants of *S. sanguis* 133-79 were selected on the basis of antibiotic resistance or spontaneous loss of platelet interactivity. Streptomycin-resistant strain S1219 was selected from a Todd-Hewitt broth (THB) agar plate supplemented with 50 µg of streptomycin per ml. Streptomycin-resistant colonies were readily obtained by this method (G. Manjula and M. C. Herzberg, Annu. Meet. Am. Soc. Microbiol. 1987, abstr. no. D-68, p. 83). Erythromycin-resistant strain E1219 was obtained from liquid culture in THB supplemented with 20 µg of erythromycin per ml and purified on THB agar plates supplemented at the same concentration. Very few colonies were obtained.

A spontaneous variant (NR133) of *S. sanguis* 133-79 which exhibited minimal adhesion to platelets was obtained by incubating freshly washed bacteria with equal concentrations of washed outdated human platelets (P. H. Krebsbach, G. D. MacFarlane, A. H. Soberay, and M. C. Herzberg, J. Dent. Res. 66:351, 1987). Platelet-bacterial agglutinates were removed by centrifugation at $55 \times g$ for 5 min at 4°C, and

nonagglutinated bacteria were reincubated with additional samples of washed platelets. This procedure was repeated four times. The bacteria remaining in the supernatant were plated on mitis salivarius agar and blood agar and purified. The phenotypic characteristics of this strain remained stable after subculturing more than 10 times and storage for 3 years at -70°C.

Strains were biotyped by the scheme of Facklam (8) and characterized by platelet-bacterial adhesion and induction of platelet aggregation (14, 15). Bacterial cells tested for platelet interactions were grown from skim milk frozen stocks in THB overnight at 37°C in an atmosphere of 5% CO₂. Cells were then harvested by centrifugation and washed three times in 0.01 M sodium phosphate buffer (pH 7.0) with 0.9% sodium chloride (PBS). Harvested, washed cells were sonicated for 8 s at 50 W (model W185; Heat Systems-Ultrasonics, Plainview, N.Y.) to disperse aggregates, increasing the number of CFU per milliliter. Bacteria were then suspended to an optical density at 620 nm (OD₆₂₀) of $1.0 (2 \times 10^9$ cells per ml). To establish colony morphology, strains were plated on (i) THB agar supplemented with either 5% sucrose or 5% glucose, (ii) mitis salivarius agar, or (iii) blood agar. In addition, cells of all strains were minimally digested for 3 min with 50 µg of L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-trypsin per ml (8, 14, 15), and the resulting surface protein digests were compared by silver-stained (21) gradient (5 to 10%) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (18).

Analysis of GTF activity. Strains were grown in tryptone-yeast medium (TYF) containing 2.5% tryptone, 1.5% yeast extract, 0.5% K₂HPO₄, 0.1% MgSO₄, and 1.0% fructose (sucrose free) which was ultrafiltered through a membrane with a molecular size cutoff of 5.0 kilodaltons (Amicon Corp., Lexington, Mass.). To concentrate secreted bacterial products, bacteria were cultured after inoculation from a starter culture in dialysis tubing (cutoff, 12.0 to 14.0 kilodaltons) containing 50 ml of TYF medium which was immersed in 500 ml of the same medium. Cultures were incubated with gentle shaking in a water bath at 37°C. Following overnight growth, bacteria were removed by centrifugation and the culture supernatants were made 0.02% in NaN₃ and 1.0 mM in phenylmethylsulfonyl fluoride to inhibit proteolysis. These culture supernatants, or culture supernatants concentrated eightfold and dialyzed against imidazole buffer (0.02 M, 0.05 M NaCl, pH 6.5) by ultrafiltration (cutoff, 10.0 kilodaltons) (Centricon 10; Amicon Corp.), were used for the GTF enzyme studies and gel electrophoresis analyses described below.

To determine the GTF levels secreted by *S. sanguis* strains, cells were inoculated into 15 ml of TYF, grown overnight at 37°C in 10% CO₂, and harvested by centrifugation. Cells were then washed twice in sterile TYF containing 40 µg of chloramphenicol per ml, 20 mM NaF, and 10 mM ε-aminocaproic acid. After sonication (4×30 s at 400 W), the cells were suspended in PBS containing chloramphenicol, NaF, and ε-aminocaproic acid to an OD₅₄₀ of 1.5 (approximately 2×10^9 cells per ml).

GTF and fructosyltransferase (FTF) assays were similar to those described by Robrish et al. (27) and Germaine et al. (12). For GTF activity, culture supernatants (300 µl) were incubated with a reaction mixture (300 µl) containing 200 mM glucosyl[U-¹⁴C]sucrose, 40.0 µM dextran (9.0 kilodaltons), and 0.02% NaN₃ in 20.0 mM imidazole hydrochloride (pH 6.5). After incubation at 37°C for 4 to 6 h, the reactions were stopped by addition of 900 µl of ice-cold methanol. To assay cell-associated GTF, 250 µl of each cell suspension

was incubated for 8 h with an equal volume of radioactive substrate. Radioactive glucans were precipitated with methanol over glass fiber filters in a vacuum manifold. Following two washes with 5.0 ml of methanol, radioactive polymers on the filters were quantitated in a scintillation counter. Activity was expressed in milliunits, a milliunit being equal to the amount of enzyme activity required to incorporate 1.0 nmol of radioactive glucose into methanol-insoluble polymer in 1.0 min at 37°C. For FTF activity, the reaction mixture contained 200 mM 1-fructosyl[³H]sucrose and 0.02% NaN₃ in the same imidazole buffer. A milliunit of FTF activity represented the amount of enzyme activity required to incorporate 1.0 nmol of radioactive fructose into methanol-insoluble polymer in 1.0 min at 37°C. For both enzymes, specific activity of culture supernatants was expressed as milliunits per milligram of culture supernatant protein as determined by the dye-binding assay of Bradford (2).

To estimate the amount of water-insoluble and water-soluble radioactive glucans made by the *S. sanguis* culture supernatants, GTF assays were conducted as described above, except that reactions were stopped by placement in an ice bath. Samples were then centrifuged at 20,000 × *g* for 30 min, and supernatants were assayed for methanol-insoluble radioactive glucans. These samples were compared with duplicate samples which were not centrifuged before addition of methanol. The percentage of water-insoluble radioactive glucans was then determined by using the formula $100 - [(cpm \text{ for centrifuged samples} \times 100)/cpm \text{ for noncentrifuged samples}] = \text{percent water-insoluble glucans}$.

For electrophoretic display of extracellular GTF and FTF, supernatants were diafiltered with a buffer containing 0.02 M imidazole and 0.05 M NaCl and concentrated eightfold with ultrafiltration spin tubes (Centricon 10; Amicon). After being mixed 1:1 with 2× SDS-PAGE treatment buffer (nonreducing), samples were incubated for 1.5 h at 37°C to allow for denaturation by SDS. Samples (75 µl) were loaded onto duplicate discontinuous SDS-PAGE gels (4% stacking, 7.5% separating) as described by Laemmli (18). Following electrophoresis, gels were either stained for protein with silver nitrate as described by Morrissey (21) or assayed for GTF and FTF activities by being washed with 50.0 mM acetate buffer (pH 5.5) and incubated with 200 mM sucrose–20 µM dextran (9.0 kDa)–1.0% Triton X-100–NaN₃ in acetate buffer for 24 h at 37°C. Gels incubated for enzyme activity were then stained for carbohydrate with the periodic acid-Schiff reagent (22). Protein standards for electrophoresis (pre-stained and normal high molecular weight) were obtained from Bio-Rad Laboratories, Richmond, Calif.

Platelets and platelet interactions. For use in the bacterial adhesion assay, human platelet ghosts were prepared as previously described (8, 14–16). Briefly, outdated platelets were washed in 0.02 M Tris hydrochloride (pH 7.25)–1% EDTA and suspended to an OD₆₂₀ of ~1.0 in PBS. For platelet aggregation experiments, fresh platelets were obtained as platelet-rich plasma anticoagulated with acid citrate glucose from healthy, medication-free volunteers by venipuncture by procedures approved by the Committee for the Use of Human Subjects in Research at the University of Minnesota.

Platelet-bacterial adhesion was determined by mixing 105 µl each of equal concentrations (~4 × 10⁹/ml; determined spectrophotometrically at a λ of 700 nm) of outdated washed platelets and washed bacteria in V-well microtiter plates and incubating them at 4°C for 30 min as described earlier (14). The plates were then centrifuged at 55 × *g* for 5 min at 4°C. Supernatant (100 µl) was diluted 1:2 into flat-well plates, and

the OD₇₀₀ was determined in an enzyme-linked immunosorbent assay (ELISA) reader. Percent adhesion is calculated as previously described, with the following equation: % adhesion = $100 \times \{1 - [OD_{\text{test}}/(OD_{\text{bacteria}} + OD_{\text{platelets}}/2)]\}$.

For platelet aggregation studies, equal numbers of washed bacteria were added to platelets in platelet-rich plasma at 37°C in a stirred cuvette in a recording aggregometer. Light transmission was recorded for 25 min or until platelet aggregation was completed. Strains were considered to be aggregation negative if aggregation did not occur within 20 to 25 min (31). The lag time to onset of aggregation was computed as the elapsed time from addition of bacteria to the upward deflection of the tracing that indicated increasing light transmission and platelet aggregation.

Tryptic digests of *S. sanguis* strains. Minimal tryptic digests of harvested and washed cells of *S. sanguis* were prepared essentially as described previously (8, 15, 16). Cells were suspended to approximately 2 × 10⁹/ml in PBS. After being warmed to 37°C for 30 min, the bacterial slurry was made 50 µg/ml in L-(tosylamido-2-phenyl)ethyl chloromethyl ketone–trypsin (Organon Teknika, Malvern, Pa.). After 3 min of incubation, digestion was stopped by addition of 50 µg of lima bean trypsin inhibitor (Organon Teknika) per ml and ice immersion.

Preparation and purification of MAbs. MAbs were produced by the methods of Kohler and Milstein (17). BALB/c mice (Charles River Breeding Laboratories, Inc., Boston, Mass.) were immunized by intraperitoneal injection of live *S. sanguis* I 133-79 (8 × 10⁷ bacterial cells per mouse). Spleens were removed, and cells were dispersed and fused to myeloma line NS-1. The resulting hybridomas were screened by ELISA for antibodies against either homologous bacterial cells (6) grown in chemically defined synthetic medium (32, 33) or a minimal (3-min) tryptic digest of *S. sanguis* cells containing surface proteins. MAbs were isolated from hybridoma supernatants by sequential ammonium sulfate precipitation at 50 and 35% saturation at 4°C (7). Additional purification of selected MAbs was performed by chromatography on either DEAE–Affi-gel Blue (Bio-Rad) (3) or an anti-mouse immunoglobulin G (IgG) affinity column (Sigma Chemical Co., St. Louis, Mo.).

Fixation of whole bacteria to microtiter plates. Ninety-six-well flat-bottom microtiter plates (Costar, Cambridge, Mass.) were prepared as described previously (6), with slight modifications. Briefly, 100 µl of a standard suspension of formalinized bacteria was added to each well. Plates were centrifuged at 250 × *g* for 10 min at 4°C. A 200-µl volume of 95% ethanol was added to each well, immediately aspirated, and followed by 100 µl of methanol per well. After incubation for 5 min at room temperature, the methanol was removed. Plates were dried at 37°C for 30 min and stored at 4°C. Immediately before use in ELISA, the wells were washed three times with a wash solution of 0.9% NaCl with 0.05% Tween 20. To determine the number of cells bound to each well, the difference between the starting suspension and supernatant was measured by spectrophotometry (A₆₂₀). Approximately 3.8 × 10⁸ cells bound per well.

ELISA (indirect binding) (6, 30). After being washed, microtiter plates coated with whole cells were incubated with 100 µl of PBS (pH 7.6)–0.5% bovine serum albumin per well for 90 min at 37°C and washed three more times with wash solution. MAbs (100 µl per well) were diluted in PBS–0.5% bovine serum albumin with 0.05% Tween 20, added to the wells, and then incubated for 90 min at 37°C. After three additional washes with wash solution, a 1:1,000 dilution of reporter (second) antibodies was added at 100 µl

TABLE 1. Characteristics of the *S. sanguis* strains used

Strain	Biotype ^a	Fermentation of ^b :			Hydrolysis of:		% Adhesion	Time (min) to aggregation	Phenotype ^c	Colony morphology ^d	GTF activity (mU/mg)
		Inulin	Raffinose	Mannose	Arginine	Esculin					
133-79	I	+	—	—	+	+	58	2.5	Adh ⁺ Agg ⁺	O	64
S1219	I	+	—	—	+	+	76	5.4	Adh ⁺ Agg ⁺	O	66
L14	I	+	+	—	+	+	53	5.0	Adh ⁺ Agg ⁺	O	1.2
L52	I	+	+	—	+	+	48	3.9	Adh ⁺ Agg ⁺	O	
L4123	I	+	—	—	—	+	52	4.0	Adh ⁺ Agg ⁺		
L4124	I	+	+	+	+	+	62	1.8	Adh ⁺ Agg ⁺		11.8
10558	I	+	—	—	+	+	78	12.8	Adh ⁺ Agg [±]		
L22	I	+	+	—	+	+	52	14.0	Adh ⁺ Agg [±]	G	
L59	I	+	—	—	+	+	41	9.9	Adh ⁺ Agg [±]	G	
NR133	I	+	±	±	+	—	16	12.0	Adh [±] Agg [±]	G	2.3
L13	II	—	+	—	—	—	27	>25	Adh ⁺ Agg [—]	G	2.8
L74	I	+	+	—	+	+	43	>25	Adh ⁺ Agg [—]	G	
E1219	I	+	—	—	+	+	1	14.8	Adh [—] Agg [±]	G	4.6
FW213	II	—	—	+	—	+	4	11.8	Adh [—] Agg [±]		0.2
L31	I	+	—	—	+	+	6	>25	Adh [—] Agg [—]	O	11.3
10556	I	+	—	—	+	+	3	>25	Adh [—] Agg [—]		
M5	I	+	—	—	+	+	2	>25	Adh [—] Agg [—]		
L50	II	—	+	—	+	—	0	>25	Adh [—] Agg [—]	G	0.6

^a Biotype I: positive for glucose and inulin fermentation and arginine and esculin hydrolysis, positive or negative for raffinose fermentation, and negative for mannose fermentation. Biotype II: positive for glucose and raffinose fermentation, positive or negative for arginine hydrolysis, and negative for inulin and mannose fermentation and esculin hydrolysis.

^b All strains were positive for glucose fermentation.

^c Phenotype and % adhesion or time to aggregation: Adh[—], <10%; Adh[±], 10 to 24%; Adh⁺, ≥25%; Agg[—], >20 min; Agg[±], 10 to 20 min; Agg⁺, <10 min.

^d Strains showed opaque (O) or glossy (G) colony morphology after growth on sucrose-containing nutrient agar as described in Materials and Methods.

per well (goat antimurine IgG conjugated with alkaline phosphatase [Bio-Rad]). After 90 min of incubation at 37°C, the wells were washed three more times. Next, the substrate *p*-nitrophenylphosphate (0.2 mg/ml) was added at 100 µl per well as specified by Bio-Rad. The plates were allowed to stand at room temperature for 60 min and the plates were read on an enzyme immunoassay reader (model 2550; Bio-Rad) at a λ of 405 nm. Reactions of a reporter antibody with PBS–0.5% bovine serum albumin alone, coated wells, and uncoated wells were performed on each plate as controls. Absorbance values for these controls were negligible.

RESULTS

A detailed survey (Table 1) of 18 strains in our collection that were biotypeable as *S. sanguis* showed that 15 of the strains were biotype I (Table 1; footnote *a*). Variants of strain 133-79 were selected for resistance to erythromycin (E1219), streptomycin (S1219), and reduced adhesion to platelets (NR133). The antibiotic-resistant strains were biotypically identical to the parental 133-79 strain, while NR133 lost the ability to hydrolyze esculin. Strain 133-79 is prototypic of many recent isolates obtained from positive blood cultures from patients with BE (14, 16, 31), while all of the L-designated strains are recent isolates from dental plaque (31). With 95% confidence intervals, both BE and dental plaque isolates have shown very reproducible differences across strains in the ability to induce platelet aggregation (31). Strains were groupable into Agg[—], Agg[±], and Agg⁺ phenotypes. Ranges of adhesion to platelets across strains of *S. sanguis* were estimated to suggest Adh[—], Adh[±], and Adh⁺ phenotypes (Table 1, footnote *b*). The strains surveyed were grouped into six platelet-interactivity phenotypes based on Adh and Agg. While 7 of the 18 strains were typed as Adh[—] or Adh[±], 6 were Agg[—]. Of the Agg⁺ strains, all were Adh⁺. However, three Adh⁺ strains were Agg[±] and two Adh⁺ strains were Agg[—].

When plated on blood agar or mitis salivarius agar, each strain exhibited a characteristic colony morphology which did not seem to distinguish platelet-interactive and noninteractive strains. Similarly, colony morphology on THB–5% sucrose plates (Table 1) failed to segregate completely on the basis of the platelet interactivity phenotype. Opaque colonies were always biotype I, and with the exception of strain L31, these strains were Adh⁺ and Agg⁺. Glossy strains tended to show less platelet interactivity. While half of the glossy colonies did not induce platelet aggregation within 25 min, each Adh phenotype was represented in this group. Therefore, colony morphology on THB–5% sucrose may be linked with the Agg phenotype but not the Adh phenotype.

To confirm that opaque and glossy colonies were associated with synthesis of extracellular polysaccharides, extracellular GTF (Table 1) and fructosyltransferase FTF activities and percentages of water-insoluble glucans from representative strains were measured. Four of the five opaque strains analyzed showed relatively high GTF activities, while low activity was noted in each of the five glossy strains tested. All strains showed small amounts of FTF activity, with values ranging from 0.17 to 1.35 mU/mg. Strain 133-79 produced 75.4% insoluble glucans. The proportion of insoluble glucans produced by strains L31 (35.4%), S1219 (7.7%), and L4124 (7.0%) distinguished these from all other strains, which failed to produce detectable amounts. Note that Adh⁺ Agg⁺ strain L14 produced little extracellular polysaccharide.

To better establish the identity of the sucrose-metabolizing enzymes in the representative strains, culture fluids were concentrated and analyzed by silver-stained (Fig. 1A) and glucan synthesis activity (Fig. 1B) SDS-PAGE. Strains 133-79, S1219, L14, L31, and L4124, with moderate to high levels of GTF activity, showed pairs of silver-stained bands in the expected *M_r* range for GTFs at about 170,000 to 145,000, respectively. While the pattern in this region was identical for 133-79 and its streptomycin-resistant variant

FIG. 1. SDS-PAGE of culture supernatants of *S. sanguis* strains stained with metachromatic silver (A) and periodic acid-Schiff (B) to localize enzymes that synthesize extracellular polysaccharides as described in Materials and Methods. Panels A and B contain identical bacterial strain samples of approximately 3.5 μ g of protein. Lanes: 1, strain E1219; 2, strain L14; 3, strain NR133-79; 4, strain L50; 5, strain FW213; 6, low- M_r standards; 7, high- M_r standards; 8, strain S1219; 9, strain 133-79; 10, strain L4124; 11, strain L13; 12, strain L31; 13, uninoculated medium concentrated eightfold. The numbers to the left are M_r s (10^3).

S1219, the distribution of proteins in this range differed among the other strains. Strain L14 showed prominent bands, while FW213 and L13, with modest platelet interactivity, did not show bands in this region. Upon disclosure with the periodic acid-Schiff reagent (Fig. 1B), strains 133-79, S1219, and L4124 showed intense staining for polysaccharide at an M_r of about 170,000. Similar staining for GTF was noted for weakly interactive strains E1219 and NR133. Adh⁺ Agg⁺ strain L14 showed little extracellular GTF activity and stained weakly at an M_r of 170,000. While strain L13 did not show stained materials in this gel, non-platelet-

interactive strain L31 did stain at an M_r of 170,000. These differences in extracellular GTF were not associated with rebinding of the enzyme to bacterial cell surfaces. Surface-associated GTF activity did not exceed 2.45 nM glucose incorporated per ml of cells per min for any of the 10 strains tested. Therefore, the platelet interactivity phenotype must be associated with other substances.

The veracity of the Adh Agg phenotyping scheme was tested with a new panel of 38 fresh isolates of *S. sanguis*. While many of these isolates were obtained from children who received antibiotics, there was no relationship between the patterns of antibiotic resistance and platelet aggregation (P. R. Erickson, M. C. Herzberg, R. Anderson, and R. Karasov, J. Dent. Res. 66 (special issue), abstr. no. 1962, p. 352, 1987). Note that the frequency of each fresh isolate meeting the criteria for phenotype assignment was similar among these strains (Table 2) to that of the strains reported in Table 1. Of the nine possible phenotypes, Adh⁺ Agg⁻ was the only one in which none of the 56 total strains could be assigned. Generally, the mean percent adhesion and mean lag time of the isolates in this panel approximated the middle of the range of the phenotyping criteria (Table 2, footnote b), suggesting that the categories represented a useful classification of biotyped strains of *S. sanguis*.

To learn whether expression of platelet-interactive determinants might distinguish phenotypic variants of *S. sanguis*, anti-*S. sanguis* 133-79 murine MAbs were prepared. MAbs harvested from culture media of four anti-*S. sanguis* 133-79 hybridomas were reacted in an ELISA with homologous cells (Table 3). Immobilized cells were grown in chemically defined synthetic medium to rule out anomalous cross-reactions with epitopes on acquired antigens from the THB growth medium of the cells used for immunization. Clones 4C3, 4B3, and 1C5 were selected because they reacted well with the homologous Adh⁺ Agg⁺ strain and streptomycin-resistant Adh⁺ Agg⁺ variant S1219 but modestly with the Adh⁻ Agg⁺ E1219 and Adh⁺ Agg⁺ NR133 variants of strain 133. Apparently recognizing a common epitope across strains, clone 1D1 reacted consistently at levels well in excess of the binding of nonspecific normal mouse IgG.

MAbs which marked platelet interactivity phenotypes also may react with *S. sanguis* determinants associated with platelet interactions. *S. sanguis* 133-79 and its three variants were preincubated with each of the MAbs in the panel and then tested for adhesion to platelets and induction of aggregation (Table 4). MAbs 4C3, 4B3, and 1C5 inhibited both adhesion and aggregation upon preincubation with strain 133-79; primarily aggregation was inhibited for strain S1219. While 4C3 also inhibited the low-level adhesion of strain NR133 to platelets, the panel of MAbs generally promoted adhesion of Adh⁻ Agg⁺ E1219 to platelets. Adhesion of strain S1219 to platelets was essentially unaffected by the MAbs. Mab 1D1 reacted with a common determinant on each strain. That MAbs often promoted adhesion of strain NR133 and E1219 to platelets was probably due to nonspecific effects, since this was also noted with nonimmune mouse IgG. The MAbs and nonimmune IgG did not change the relative inability of strains NR133 and E1219 to induce platelet-rich plasma aggregation.

These data confirm that determinants available on the cell surface of *S. sanguis* may confer the ability of these organisms to adhere to and induce platelets to aggregate. While the availability of these determinants may serve as appropriate Adh Agg phenotypic markers, it must be learned whether platelet-interactive determinants are available in cryptic form on noninteractive strains. To address this question,

TABLE 2. Frequency of isolation of platelet interactivity phenotypes of *S. sanguis* from dental plaque

Phenotype	% of total isolates	Mean % adhesion (range) ^a	Mean lag time (min) (range) ^a
Adh ⁺ Agg ⁺	34.2 (33.3) ^b	58.9 (27.6 ± 0.9–7.8 ± 2.6)	5.8 (2.5 ± 0.1–9.4 ± 0.1)
Adh ⁺ Agg [±]	2.6 (16.7)	60.7 ± 1.8	10.3 ± 0.4
Adh ⁺ Agg ⁻	0 (11.1)		
Adh [±] Agg ⁺	5.2 (0)	20.9 (18.2 ± 1.1–23.5 ± 0.9)	7.7 (3.0 ± 0.1–9.4 ± 0.2)
Adh [±] Agg [±]	2.6 (5.6)	18.1 ± 1.1	14.0 ± 0.3
Adh [±] Agg ⁻	0 (0)		
Adh ⁻ Agg ⁺	21.1 (0)	4.3 (1.8 ± 1.0–8.2 ± 1.6)	6.4 (3.1 ± 0.1–9.2 ± 0.1)
Adh ⁻ Agg [±]	13.2 (11.1)	3.7 (1.0 ± 0.2–7.3 ± 1.1)	11.9 (10.6 ± 0.4–14.6 ± 0.4)
Adh ⁻ Agg ⁻	21.1 (22.2)	3.0 (0–7.5 ± 0.7)	>20

^a The mean percent adhesion and lag time to the onset of platelet aggregation of all isolates in this panel of 38 strains are shown for each phenotype. Each isolate was tested three times, and the range shows the mean ± the standard deviation of the extremes within each phenotypic category. The criteria for each phenotype are described in Table 1, footnote b.

^b The percentage of the total number of strains described in Table 1 that had each phenotype is reported in parentheses.

minimal tryptic digests of cells representing platelet-interactive and noninteractive strains were prepared and tested for inhibition of adhesion and induction of aggregation of pre-treated platelets by *S. sanguis* 133-79 (Table 5). Tryptic digests in equal concentrations from Adh⁺ Agg⁺ strains, as expected, were generally effective in inhibiting adhesion to and induction of aggregation of platelets by strain 133-79. Comparably effective were the digests from the Adh[±] Agg[±] and Adh⁻ Agg[±] variants derived from strains 133-79, NR133, and E1219, respectively. Therefore, the phenotypic variants derived from strain 133-79 possessed similar platelet-interactive determinants, which were not expressed at the cell surface. Indeed, preparations from most strains were able to inhibit platelet-*S. sanguis* 133-79 interactions. Interactions were not apparently affected by digests from Adh⁻ Agg⁻ strains L50 and 10556. No consistent differences among digests of these strains could be discerned by analysis on 5 to 10% gradient SDS-PAGE (data not shown).

DISCUSSION

These studies compare strains of *S. sanguis* bityped by the scheme of Facklam (9) for several traits that may be associated with their virulence in the etiology of BE. Accurate phenotyping and genotyping (13) schemes for these and other alpha-hemolytic (viridans) streptococci have been under development for many years. We sought to learn whether a typing scheme based on the highly reproducible abilities of dental plaque and BE isolates to adhere to (14) and induce platelets in plasma to aggregate (14, 31) might yield associations with other characteristics of these microorganisms.

Strains in this panel were assigned Adh Agg phenotypes based on their interactions with platelets in standard assays. These traits may be associated with virulence in experimental endocarditis. New evidence (M. C. Herzberg et al., J. Dent. Res. 68 (special issue), abstr. no. 1189, p. 1015, 1989; M. C. Herzberg and M. W. Meyer, manuscript in preparation) suggests that after bacterial infusion in the rabbit model of experimental endocarditis, Adh⁺ Agg⁺ strain 133-79 induces platelet vegetations of significantly greater mass than does an Adh⁻ Agg⁻ strain. On this basis, the distribution of Adh Agg phenotypes among strains was compared with dextran synthesis, another putative virulence factor in BE (23, 25, 29). GTF and FTF activities, percentages of water-insoluble glucans, and colony morphology on sucrose-containing agar failed to show consistent relationships with platelet interactivity phenotypes, although trends were noted. If the GTF system contained multifunctional proteins conferring Adh⁺ and Agg⁺, it would be difficult to explain the existence of strains such as L31, which produce dextrans but are essentially noninteractive with platelets. Analysis of a second panel of 38 fresh dental plaque isolates showed that *S. sanguis* distributes in a reproducible manner into eight of the nine possible phenotypic groups.

Since surface determinants in *S. sanguis* have been implicated in platelet interactions (8, 14, 15), we raised MAbs against the Adh⁺ Agg⁺ strain to develop possible phenotypic markers. The selected MAbs reacted only with cells of the two Adh⁺ Agg⁺ strains tested, failing to react appreciably with variants showing reduced platelet interactions. The MAbs distinguished platelet-interactive and noninteractive variants. MAb 1C5 appeared to mark the Agg⁺ phenotype

TABLE 3. Reactions of MAbs to cells of *S. sanguis* 133-79 and variant strains

MAb ^a	IgG subclass ^b	Mean A ₄₀₅ ± SD of strain ^c :			
		133-79	S1219	NR133	E1219
4C3	IgG1	0.160 ± 0.019	0.157 ± 0.018	0.028 ± 0.006	0.053 ± 0.008
4B3	IgG2b	0.123 ± 0.011	0.117 ± 0.014	0.036 ± 0.005	0.054 ± 0.009
1C5	IgG2a	0.119 ± 0.006	0.146 ± 0.023	0.037 ± 0.014	0.050 ± 0.012
1D1		0.097 ± 0.023	0.117 ± 0.010	0.107 ± 0.011	0.092 ± 0.019
Mouse IgG ^d		0.080 ± 0.017	0.089 ± 0.010	0.044 ± 0.015	0.054 ± 0.012

^a MAbs were prepared as ammonium sulfate fractions of hybridoma supernatants. Final protein concentration, 100 µg/ml (10 µg per well).

^b MAbs were isolated from hybridoma supernatants by ammonium sulfate precipitation and purified by chromatography in DEAE-Affi-gel Blue. IgG subclasses were tested by double diffusion in Ouchterlony plates. Final MAb IgG concentration, 1.5 mg/ml. Clone 1D1 did not show precipitation with any of the IgG subclass antisera or with anti-murine IgG, IgA, or IgM, but it did react in an ELISA with goat antimurine IgG.

^c Eight determinations were made for each MAb-strain pair with a whole-cell ELISA (6). Immobilized cells were grown in FMC medium (31, 33).

^d Commercial preparations of mouse IgG.

TABLE 4. Percent change in interactions with platelets upon preincubation of *S. sanguis* cells with MAbs to *S. sanguis* 133-79

MAb ^a	% Inhibition of adhesion ^b (aggregation) ^c of strain:			
	133-79	S1219	NR133	E1219
4C3	-29 (-40)	-8 (-41)	-71 (0)	+105 (0)
4B3	-20 (-93)	-4 (-25)	-15 (0)	+58 (0)
1C5	-27 (-146)	-5 (-38)	+12 (0)	+53 (0)
1D1	-7 (0)	+2 (0)	+47 (0)	+10 (0)
Mouse IgG	+2 (+11)	+6 (+6)	+12 (0)	+15 (0)

^a MAbs were tested as ammonium sulfate fractions of hybridoma supernatants. Final protein concentration, 150 µg/ml. MAbs (100 µl) were preincubated for 10 min at 37°C with bacteria (A_{620} , 2.0) and remained present during the assay of platelet interaction.

^b Inhibition of adhesion was calculated with the following equation: % change = [(adhesion of untreated cells - adhesion of treated cells)/adhesion of untreated cells] × 100.

^c Change in aggregation was calculated with the following equation: % change = [(lag time of control - lag time of test strain)/lag time of control] × 100. Note that 100% inhibition (-100) corresponds to a doubling of the lag time to onset. When MAbs were inhibitory, the lag time test exceeded the control, and change is indicated by a minus. Strains NR133 and E1219 did not induce aggregation with this platelet donor. From a solution of 150 µg/ml, 100 µl containing 15 µg of protein was incubated with bacteria at an A_{620} of 2.0.

preferentially, while 4C3 may indicate Adh⁺. These data could not be explained on the basis of nonspecific interactions between MAbs and *S. sanguis* or platelets, since MAb 1D1 and mouse IgG were largely without effect. While the distinguishing epitopes have not been identified, these data do support the potential of a MAb-based scheme to classify Adh Agg phenotypes. Additional strains and new clones will be screened for this purpose.

An Adh Agg phenotyping scheme will not, however, lead directly to a genotyping strategy, since platelet-interactive determinants may actually be cryptic, even on Adh⁻ Agg⁻ strains. Minimal tryptic digests from cells of strains in each

TABLE 5. Inhibition of interactions of *S. sanguis* 133-79 with platelets after pretreatment with cell surface digests of other strains^a

Strain (phenotype)	% Inhibition ^b of:	
	Adhesion	Aggregation
133-79 (Adh ⁺ Agg ⁺) (homologous)	49	175
S1219 (Adh ⁺ Agg ⁺)	90	>400
L14 (Adh ⁺ Agg ⁺)	46	38
L52 (Adh ⁺ Agg ⁺)	30	88
L22 (Adh ⁺ Agg [±])	29	62
L59 (Adh ⁺ Agg [±])	18	121
NR133 (Adh [±] Agg [±])	94	>400
L13 (Adh ⁺ Agg ⁻)	73	21
L74 (Adh ⁺ Agg ⁻)	31	52
E1219 (Adh ⁻ Agg [±])	43	188
L31 (Adh ⁻ Agg ⁻)	41	5
L50 (Adh ⁻ Agg ⁻)	2	0
10556 (Adh ⁻ Agg ⁻)	9	0

^a A tryptic digest of each strain (final protein concentration, 500 µg/ml for the adhesion assay and 100 µg/ml for the aggregation assay) was preincubated with platelet preparations for 10 min at 37°C and then removed by washing before incubation with cells of strain 133-79.

^b Percent inhibition = [(control - test strain)/control] × 100. Note that percent inhibition is expressed as a positive number, in contrast to percent change in Table 3.

platelet interactivity phenotype were able to inhibit platelet interactions with strain 133-79. Conversely, equal amounts of a tryptic digest from Adh⁻ Agg⁻ strains L50 and 10556 failed to inhibit platelet interactions. As expected, rabbit anti-*S. sanguis* 133-79 serum monospecific for the collagen-like antigen associated with Agg⁺ was readily neutralized by digests from Agg⁺ strains L14 and 133-79 and Agg[±] strains L22 and L59 but not by strain L31 (data not shown). Hence, Agg⁺ antigens, largely cryptic on the cell wall, were available to platelets in the digests. Since the determinant implicated in the Agg⁺ phenotype is a collagenlike protein (8), a genotyping scheme might focus on probes that identify controls of surface expression in addition to biosynthesis. Viridans streptococci, however, can clearly be subjugated to a functional and antigenic taxonomy based on their stable interactions with platelets. The clinical significance of the Adh Agg characteristics would be another advantage of this scheme.

Previous reports (14, 31) have shown that a typical BE isolate of *S. sanguis* would be platelet interactive. It is now clear that most, but not all, dental plaque strains express similar phenotypes. Each of these sources yields strains that may be able to synthesize dextran and other extracellular polysaccharides. If dextran production contributes to virulence, it is likely to be that carried as a cell-associated polysaccharide from dental plaque. A sucrose substrate, although available in foodstuffs that enter the oral cavity, lacks plasma. Hence, the synthesis of new extracellular dextran by virulent microorganisms at the site of the vegetation is unlikely to occur. In addition, the level of surface-associated GTF activity expressed in the absence of sucrose was low for all strains in comparison with the extracellular enzyme. Since interactions with platelets in vitro were independent of growth in sucrose or added dextrans (14, 16), virulence in bacterial endocarditis may be more strongly associated with Adh Agg phenotypes. Indeed, an Adh⁺ Agg⁺ strain derived from the oral cavity could participate in the latter three of the four stages implicated by Freedman (10) in the initiation of bacterial endocarditis. After damage to the endothelium and deposition of an NBTv, the Adh⁺ Agg⁺ strain could enter the circulation from an oral site, adhere to platelets in the NBTv, induce deposition of additional platelets and fibrin, and subsequently colonize the platelet-fibrin vegetation. Hence, the virulence of *S. sanguis* in BE may be associated most strongly with the frequently recovered Adh⁺ Agg⁺ phenotype.

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