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Examination of platelet adhesion by Streptococcus sanguinis

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EXAMINATION OF PLATELET ADHESION BY *STREPTOCOCCUS SANGUINIS*

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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Acknowledgement

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Abstract

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Streptococcus sanguinis is a leading cause of infective endocarditis. Bacterial adhesion to platelets is likely important in the pathogenesis of infective endocarditis. Bacterial cell wall-anchored (Cwa) proteins may mediate this adhesion. To begin to test this hypothesis, *S. sanguinis* adhesion to platelets was examined *in vitro*. The requirement of 12 Cwa proteins for *S. sanguinis*-platelet adhesion was individually assessed, measuring adhesion of purified platelets to polystyrene wells coated with *S. sanguinis* strain SK36 or 12 isogenic Cwa protein mutants. Significantly fewer platelets adhered to wells coated with one mutant strain, VT1614. However, results of a whole-cell enzyme-linked immunosorbent assay (ELISA) showed that 8 mutants, including VT1614, adhered in significantly lower numbers to wells than did SK36. After accounting for unequal bacterial numbers, we determined there was no significant difference in platelet adhesion among the strains. This suggests that none of the Cwa proteins examined were required for *S. sanguinis*-platelet adhesion.

Introduction

Streptococcus sanguinis

Streptococcus sanguinis, formerly referred to as *Streptococcus sanguis*, is a gram-positive, facultative anaerobe belonging to the viridans group of streptococci. Other members of this group include *Streptococcus gordonii* and *Streptococcus parasanguinis*. Members of the viridans streptococci, including *S. sanguinis*, are initial bacterial colonizers of the oral cavity (1, 2). While oral streptococci, which constitute ~20% of the normal human oral flora, are abundant at most oral sites, *S. sanguinis* preferentially colonizes the tooth surface (3). The dental enamel of a tooth is composed of hydroxyapatite crystals. On the surface of the tooth, the hydroxyapatite crystals are covered in salivary proteins (the salivary pellicle), which provides a wealth of binding sites used by oral streptococci. *S. sanguinis* has been shown to bind specific salivary proteins, including salivary IgA and α-amylase, on the tooth surface (2, 4). In the oral cavity, *S. sanguinis* is recognized as having a benign, or even advantageous, role by competing with other oral bacteria that cause dental caries and periodontitis (5, 6). Outside of its native niche, however, *S. sanguinis* is one of the foremost causative agents of bacterial infective endocarditis (IE), a life-threatening endothelial infection.

Infective endocarditis

      Infective endocarditis is a rare—but serious—infection characterized by the formation of septic masses of platelets and fibrin, otherwise referred to as vegetations, on the surfaces of the cardiac endothelium. Streptococcal species are the causative agents in approximately 42% of bacterial IE cases (7, 8). Furthermore, of all the oral streptococci, *S. sanguinis* is most often implicated as the cause of native-valve IE (9-12). The mean incidence of IE has been reported to be 3.6 cases per 100,000 per year (13), and appears to increase with age. The reported incidence of fewer than 5 cases per 100,000 in individuals less than 50 years of age increases to more than 15 cases per 100,000 in individuals over 65 years of age (13). Although there have been improvements in diagnosis and medical treatment over the years, IE is still associated with high morbidity and mortality, which has been reported to range from 11-30% (13, 14). Death can be attributed to congestive heart failure or embolization of vegetation components to major organs (14, 15). Such high morbidity and mortality could be due to the fact that prophylaxis, diagnosis, and treatment of IE are still a major challenge in clinical practice (14).

      Early diagnosis of IE relies heavily on blood cultures, which test for the presence of IE-causing microorganisms, and echocardiograms, which may indicate the presence of a vegetation (16). Later diagnosis may be made through bacterial culture from, or histological examination of, an excised vegetation found during open heart surgery or at autopsy (16). Other minor criteria for IE diagnosis include predisposing cardiac conditions and intravenous drug use, which is a risk factor for IE in some populations (16). If left untreated, IE is uniformly fatal. Treatment may involve the use of antimicrobial therapy, surgical intervention, and monitoring by echocardiography (16). Effective treatment often requires collaboration among physicians from multiple disciplines (14).

Pathogenesis of infective endocarditis

Much is known about the overall pathogenesis of IE. In order to cause IE, a pathogenic organism must first be introduced into the bloodstream. Bacteria in the mouth can enter the bloodstream, causing a transient bacteremia, in a number of ways. Examples include dental procedures such as tooth extractions, oral surgery, periodontal treatment, and cleanings (17-20). More often though, transient bacteremias occur as the result of everyday activities, such as brushing, flossing, and chewing(17-20).

Bacteria in the bloodstream cause IE primarily at sites of pre-existing damage to the cardiac valves or endothelium. Therefore, individuals with prosthetic cardiac valves, congenital heart disease (CHD), heart transplantation, previous case of IE, or rheumatic heart disease may be predisposed to develop IE from a transient bacteremia (15). Following trauma to the cardiac endothelium, exposure of the sub-endothelial matrix and its extracellular matrix (ECM) proteins prompts the normal deposition of platelets and fibrin, forming a clot at the site of injury (21). This is referred to as nonbacterial thrombotic endocarditis (NBTE). At this point, the clot, or sterile vegetation, provides a surface to which IE-causing bacteria in the bloodstream may adhere. Once adhered to the vegetation, the bacteria stimulate the deposition of more platelets and fibrin. This sequesters them within a vegetation of increasing size where they can proliferate, resulting in IE.

Prophylaxis for infective endocarditis

As no vaccine for prevention of IE exists, other methods of prevention are required. It was previously thought that dental procedures were a main cause of IE. Consequently, over the past 50 years the American Heart Association (AHA) has published guidelines recommending

antimicrobial prophylaxis for at-risk patients prior to invasive dental procedures (22). These guidelines have been repeatedly updated. The most recent update dramatically reduced the number of pre-existing conditions for which antimicrobial prophylaxis is recommended (16). These new guidelines take into account more current studies, which suggest that IE cases caused by oral bacteria frequently result from transient bacteremias caused by routine daily activities (23-28). Furthermore, there is a lack of evidence as to the efficacy of prophylactic antibiotics in preventing IE. Even if antibiotic prophylaxis before dental procedures were 100% effective, it would likely only prevent an extremely small number of IE cases (15). It is not reasonable for individuals at risk for developing IE to use antibiotics on a daily basis. Therefore, a reduction in the frequency of daily bacteremias through maintenance of good oral hygiene and prevention of dental disease has been emphasized (29-33). The high morbidity and mortality associated with IE along with the lack of effective prophylaxis warrants further research into other prevention methods, such as a vaccine.

Platelets and thrombosis

Human blood platelets play an important role in hemostasis and thrombosis. Platelets are the smallest (3.6 x 0.7 μm) and most numerous ($3x10^8 - 4x10^8$ mL^{-1} blood) cells in the blood (34, 35). They are metabolically active, anucleated cells formed by megakaryocytes in the bone marrow. In primary hemostasis, platelets recognize sites of injury by adhering to collagen and glycoproteins in the exposed sub-endothelial matrix. Under low-shear conditions, initial platelet adherence is mediated by two main collagen receptors on the platelet surface: glycoprotein (GP) VI and GPIa/IIa (36, 37). Under high-shear conditions, the von Willebrand factor (vWF) receptor, GPIb-IX-V, is primarily responsible for mediating platelet adhesion (36, 37).

Platelet adhesion through any of these receptors leads to platelet activation. Biochemical signal transduction in activated platelets causes shape change, granule secretion, and activation of receptors that promote adhesion and aggregation. The activated receptor GPIIb/IIIa binds fibrinogen, which cross-links activated platelets, forming a platelet aggregate (35). Activated platelets secrete granules containing platelet activating molecules, such as adenosine diphosphate (ADP), serotonin, and thromboxane A2 (TxA2) (35). These secondary agonists amplify platelet activation, attracting more platelets to the site of injury. During secondary hemostasis, thrombin is generated on cell membranes at the site of thrombus formation. Thrombin converts soluble fibrinogen into fibrin, which forms strands. These fibrin strands interweave throughout the developing platelet thrombus, resulting in a stable platelet-fibrin clot (35). In NBTE lesions, the platelet-fibrin clot is free of bacteria. However, in IE, bacteria have adhered to and colonized the platelet-fibrin surface. Therefore, bacterial adherence to this surface is thought to be an important event in the pathogenesis of IE.

Gram-positive bacterial surface exposed proteins are potential virulence factors in infective endocarditis

On the bacterial surface, there are specific molecules that may act as adhesins by interacting with host cell receptors or host extracellular matrix molecules. These include the cell wall-anchored (Cwa) proteins, which are the major surface-exposed protein class in gram-positive bacteria. This class of proteins is typified by covalent linkage to the peptidoglycan layer of the bacterial cell wall. Both adhesive and enzymatic functions have been credited to streptococcal Cwa proteins. Some Cwa proteins considered to be important in pathogenesis have

been grouped as MSCRAMMs, or microbial surface components recognizing adhesive matrix molecules of host tissue and host cells (38).

Several staphylococcal Cwa protein MSCRAMMs, such as clumping factor A (ClfA) and fibronectin-binding proteins A and B (FnbA, FnbB) of the gram-positive bacterium *Staphylococcus aureus*, have been demonstrated to be important for pathogenesis in experimental IE (21). FnbA and FnbB both bind fibronectin *in vitro* (39), and ClfA has fibrinogen-binding capacity *in vitro* (40, 41); both of these proteins are soluble in blood, and fibronectin is present in the extracellular matrix of host tissues (42). Furthermore, the *Staphylococcus aureus* proteins clumping factor A (ClfA) and FnbA, when expressed on the surface of *Lactococcus lactis*, a poorly pathogenic, food-grade bacterium, increased the adherence of *L. lactis* to both immobilized fibrinogen and fibronectin *in vitro* (43).

Multiple streptococcal Cwa protein MSCRAMMs have been identified as well. In *S. gordonii*, the high-molecular-mass cell surface polypeptides CshA and CshB have been suggested to act as multifunctional adhesins (44). Mutants of *Streptococcus gordonii,* in which the genes encoding CshA and/or CshB were inactivated, were deficient in binding to immobilized human fibronectin (44). Another *S. gordonii* adhesin, the sialic acid-binding protein, Hsa, has been shown to possess fibronectin-binding capacity *in vitro* and to contribute to infectivity under competitive conditions in an animal model of IE (45).

Bacterial cell surface proteins as vaccine candidates

Bacterial cell surface proteins, including Cwa proteins, are of interest not only for their roles in disease, but also as potential targets for vaccine therapy. Localized on the cell surface, they have the potential to be accessible to the host immune response. In *S. aureus,* cell wall-

anchored surface proteins have been tested as vaccinogens in a murine model of abscess formation. It was determined that immunization with four surface proteins generated significant protective immunity that correlated with the induction of opsonophagocytic antibodies (46). If adhesive Cwa proteins are important mediators of pathogenesis, antibodies directed against them could prevent infection by either of two separate mechanisms: (*i*) opsonization, resulting in phagocytosis and killing of the bacteria; or (*ii*) prevention of bacterial adherence by interference with adhesin function.

No investigation has yet examined the effects of immunization with *S. sanguinis* surface proteins. Perhaps this is because there is a lack of information regarding their potential role in *S. sanguinis*-IE. Studies that examine the role of various *S. sanguinis* Cwa proteins in IE may provide an indication of their relative potential as vaccine candidates. Since IE carries such high morbidity and mortality, primary prevention is very important and the development of an effective IE vaccine would be of great significance.

Platelet interactions with gram-positive bacteria

Bacterial-platelet adherence has been suggested to be an important early event in the pathogenesis of IE (47). Initial bacterial colonization of sites of endovascular damage may be mediated by adhesion to platelets present in the sterile vegetation. Platelet interactions by several IE-causing gram-positive bacteria (Figure 1) have been reported. Previous studies have shown that the *S. aureus* proteins clumping factor A, clumping factor B, protein A and fibronectin-binding protein (FnbA) all bind to platelets (48-51). More recently, a high-molecular-mass protein called SraP was shown to promote binding of *S. aureus* cells to platelets and to enhance

Figure 1. Platelet interactions with surface proteins on gram-positive bacteria. Reported interactions between bacterial surface proteins and platelet receptors are shown for three gram-positive infective endocarditis-causing bacteria; *Streptococcus gordonii, Streptococcus sanguinis,* and *Staphylococcus aureus*. Question marks indicate as yet, unidentified platelet receptors. Bacterial adhesion to or aggregation of platelets resulting from the interactions is indicated next to each association.

Figure 1.
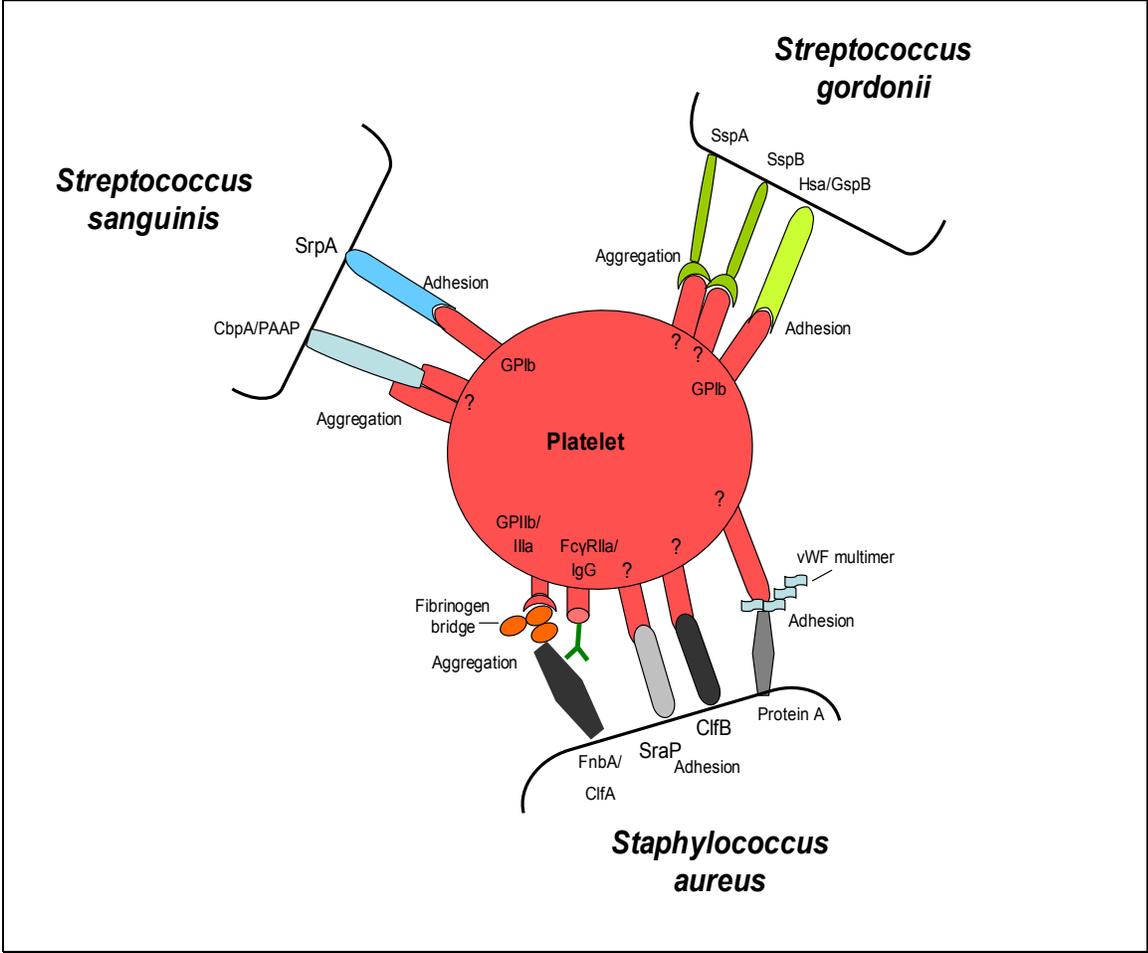
virulence in a rabbit model of endocarditis (52). Furthermore, reduced platelet binding *in vitro* by *S. aureus* has been associated with reduced virulence in a rabbit model of IE (53). This was an important finding because it suggests a link between a platelet-binding phenotype *in vitro* and virulence in an animal model of endocarditis. Since endocarditis is characterized by platelet vegetations, bacterial activation and resulting aggregation of platelets has been suggested to be a virulence factor (54).The *S. aureus* Cwa proteins ClfA and ClfB, as well as the serine-aspartate repeat protein SdrE, are capable of inducing platelet aggregation when expressed in non-aggregating *L. lactis* (50). The *S. aureus* protein SraP is a homolog of the *S. gordonii* Cwa protein GspB. The cell-surface glycoproteins GspB and Hsa of *S. gordonii* have been reported to mediate platelet adherence by directly interacting with platelet receptor GPIb (55, 56).

Platelet interactions with *Streptococcus sanguinis*

The investigation of potential *S. sanguinis* virulence factors for IE has included examination of adhesion and aggregation of platelets. Some studies have examined *S. sanguinis*-platelet adhesion, although these studies have not directly examined this property in relation to endocarditis virulence. Scheld et al. previously reported that *S. sanguinis* strain M-5 adheres to platelet-fibrin matrixes *in vitro*, utilizing bacterial colonies grown on the surface of platelet-fibrin matrixes as a measure of adherence (57). They also reported that the presence of platelets in a fibrin matrix increased adherence of *S. sanguinis* over that observed with fibrin alone, which suggested a possible bacteria-platelet interaction (57). There have since been multiple reports of *S. sanguinis* binding platelets *in vitro* (56, 58-61). The results of a study examining *S. sanguinis*-platelet binding by flow cytometry demonstrated that streptococcus-platelet binding is rapid, reversible, and saturable, which suggests a specific receptor-ligand interaction (61). Given the

adhesive properties of many Cwa proteins, some of which have been shown to bind platelets in related species, it is plausible that these proteins could contribute to platelet adhesion in *S. sanguinis* via such an interaction.

A number of studies support this suggestion. A *S. sanguinis* cell-surface protein related to the cell surface hydrophobicity (Csh) protein A of *S. gordonii,* a reported adhesin, has been implicated in platelet adhesion and aggregation of platelets (62). It was reported that a CshA mutant derived from *S. sanguinis* strain 133-79, which is a strain that is positive for both platelet adhesion and aggregation phenotypes (Adh$^+$, Agg$^+$), showed a loss of both phenotypes (62). Additionally, a *S. sanguinis* Cwa protein has been reported to mediate adhesion to platelets via a specific receptor on the platelet surface. A serine-rich protein, designated SrpA, was shown to bind glycocalicin (GC), the extracellular domain of the platelet vWF receptor glycoprotein Ibα (GPIbα) (56). Furthermore, a *S. sanguinis* SrpA$^-$ mutant strain reportedly exhibited reduced adherence to platelets, suggesting this Cwa protein may contribute to platelet binding by *S. sanguinis* (56).

Other studies have also examined *S. sanguinis*-platelet aggregation. In *S. sanguinis*, a cell wall platelet aggregation-associated protein (PAAP), more recently identified as CbpA, has been reported to interact with a receptor on platelets to induce platelet aggregation (62-65). Additionally, *S. sanguinis*-induced platelet aggregation has been linked to vegetation enlargement and virulence in experimental IE. In a rabbit model of IE, inoculation with a strain of *Streptococcus sanguinis* (133-79), which induced rabbit platelets to aggregate *in vitro* (Agg$^+$ phenotype), consistently caused endocarditis with a more severe clinical course than inoculation with an Agg$^-$ strain (L50) or the Agg+ strain pretreated with an antibody against its platelet aggregation-associated protein (PAAP; class II) (54). This suggested that the PAAP may be an

important virulence factor. Herzberg et al. (59) reported that selective adhesion of *S. sanguinis* to platelets was apparently required for aggregation, suggesting a link between the two phenotypes. Unfortunately, however, this experiment has not been repeated with a defined PAAP mutant, suggesting caution in its interpretation.

A study by Plummer et al. (56) was the first to examine the potential contribution of a Cwa protein to *S. sanguinis*-platelet adhesion in the strain SK36. This is an important strain for several reasons. First, although SK36 is an oral isolate, it has been previously demonstrated in our lab to be virulent in experimental animal models of IE (66, 67). Second, it possesses a platelet aggregation phenotype. Finally, its genome was recently sequenced (68), which has allowed for the identification of genes encoding putative adhesins, including Cwa proteins. SK36 has 33 predicted Cwa proteins (67) and mutants of 12 of these proteins were tested in this study for their ability to adhere to platelets.

Project goal

Platelets are a main component of the vegetation colonized by bacteria in IE. It has been suggested that *S. sanguinis* adherence to platelets is an important early step in the pathogenesis of IE, and cell-surface proteins, such as Cwa proteins, are thought to mediate this adherence. For this study, we hypothesized that certain Cwa proteins contribute to *S. sanguinis*-platelet adhesion and set out to test that hypothesis. There have been few previous investigations of this topic reported. If multiple Cwa proteins were discovered to contribute to *S. sanguinis*-platelet binding it could lead to new methods of IE prevention by interference with *S. sanguinis*-platelet interaction, as well as further understanding of the processes of bacterial adhesion and colonization in IE.

Materials and Methods

Bacterial strains

Bacterial strains used in the following experiments are listed in Table 1. *Streptococcus sanguinis* strain SK36 is an oral isolate originally obtained from the University of Aarhus, Denmark (68). All *S. sanguinis* mutant strains were derived from SK36 (Table 1). *S. sanguinis* strain VT1614, a *srpA* mutant, was obtained from Dr. Hui Wu, University of Alabama (56). All other *S. sanguinis* mutants (Table 1) were created using a directed signature-tagged mutagenesis approach and have been described previously (67).

Bacterial cultivation and cell preparation

All liquid cultures of *S. sanguinis* strains were grown in brain-heart infusion (BHI) broth (Difco) for 16 hours at 37°C under microaerobic conditions (6% 0_2, 80% N_2, 7% CO_2, 7% H2). Liquid cultures of *E. coli* strain DH10B were grown aerobically in LB broth (BD) for 16 hours at 37°C. Liquid cultures of *L. lactis* strain MG1363 were grown aerobically in M17 growth media (Difco) + 0.5% (w/v) glucose for 16 hours at 30°C. For each strain, a 16-hour liquid culture was combined with sterile glycerol (70% culture + 30% glycerol), aliquoted, and frozen at -75°C. In all experiments, bacterial overnight cultures were inoculated using individual, thawed aliquots originating from a single culture maintained at -75°C.

Table 1. Bacterial strains used

Bacteria/Strain	Description	Reference
Escherichia coli		
DH10B	Laboratory strain	(69)
Lactococcus lactis		
MG1363	Food-grade lactic acid bacteria	(70)
Streptococcus sanguinis		
SK36	Human plaque isolate	(71)
VT1614	Δ*srpA*, derived from SK36	(56)
Cwa 15	SSA_1023::*magellan2*	(67)
Cwa 25	SSA_1063::*magellan2*	(67)
Cwa 7	SSA_1633::*magellan2*	(67)
Cwa 6	SSA_1634::*magellan2*	(67)
Cwa 3	SSA_1663::*magellan2*	(67)
Cwa 13	SSA_0303::*magellan2*	(67)
Cwa 33	SSA_0904::*magellan2*	(67)
Cwa 9	SSA_0956::*magellan2*	(67)
Cwa 8	SSA_1632::*magellan2*	(67)
SSA_1635 mutant	SSA_1635::*aad9*	(67)
Cwa 30	SSA_1666::*magellan2*	(67)

Bacterial cells were intrinsically labeled with [³H]thymidine by adding 25 μL (25 μCi) of [³H]thymidine (GE Healthcare) to a plastic culture tube, containing 5 μL of cells and 5 mL BHI liquid broth, and incubating overnight.

All cells were harvested by centrifugation at 5k x *g* for 10 minutes at 4°C. Cells were then washed with 5 mL PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4; pH 7.3) and harvested as before. Cells were washed again with 1.5 mL PBS, centrifuged at 14,000 rpm, for 2 minutes at room temperature, and then resuspended in 1 mL PBS. Non-labeled cells of each strain were serially diluted 10-fold and plated in triplicate on growth media + agarose (Difco). SK36 was grown on plates containing BHI + agar and incubated for two days at 37°C under microaerobic conditions (6% O_2). DH10B was grown on plates containing LB + agar and incubated overnight under conditions described for liquid culture. MG1363 was grown on plates containing M17 + agar + 0.5% (w/v) glucose and incubated for two days under conditions described for liquid culture. Colony counts were used to determine the number of colony forming units (CFUs) mL^{-1} in the cell preparations for each strain.

For the *S. sanguinis*-platelet adhesion assay, cultured cells were harvested by centrifugation at 5k x g for 10 minutes at 4°C. Bacteria were washed in 5 mL PBS, centrifuged again as described above, and finally suspended in 1 mL PBS. The optical densities (660 nm) of the cell suspensions were adjusted to 1.8 (~2.0×10^9 CFU mL^{-1}) using a Helios Gamma spectrophotometer (Unicam). For each strain, 1 mL of concentrated cells was sonicated for 1 minute 30 seconds at 50% power using an ultrasonic homogenizer (BioLogics, Inc.).

Preparation of platelets

Blood collection and platelet preparation techniques were based on those described previously (57, 60, 72). For examination of *S. sanguinis* adherence to platelet-fibrin matrixes via colony counting, platelet-rich plasma was prepared as follows. Eleven mL of blood bank whole anti-coagulated blood (Virginia Blood Donor Services) was centrifuged at 500 x g for 4 minutes at room temperature in a 15 mL polystyrene tube. The uppermost layer was removed and designated platelet-rich plasma (PRP). For all other experiments involving platelets, fresh blood was drawn from the antecubital vein of healthy, adult volunteer donors using a 21-gauge butterfly needle. Donors had not taken any aspirin, ibuprofen or anticoagulant medications in the previous two weeks. All experiments involving donors were performed with Institutional Review Board approval and complied with all university regulations.

For experiments measuring adherence of ^{3}H-labeled bacteria to platelet-fibrin matrixes, whole blood was collected in 4.5 mL-draw tubes (BD Vacutainers) containing 3.2% sodium citrate (citrate solution, 0.5 mL; sodium citrate, 12.35 mg; citric acid, 2.21 mg) at a blood:sodium citrate ratio of 9:1. Anticoagulated whole blood was centrifuged at 500 x g for 4 minutes at room temperature. The upper layer of platelet-rich plasma (PRP) was aspirated into a separate polypropylene tube.

For experiments using gel-filtered platelets, whole blood was collected in 6 mL-draw BD Vacutainers containing ACD solution B (trisodium citrate, 13.2 g L^{-1}; citric acid, 4.8 g L^{-1}; and dextrose, 14.7 g L^{-1}; 1.0 mL) at a blood: ACD ratio of 6:1. Anticoagulated whole blood was centrifuged at 150 x g for 10 minutes at room temperature. The upper layer of PRP was aspirated into a separate polypropylene tube.

Platelet separation from plasma proteins by gel filtration was based on methods previously described by Kerrigan, et al 2002 (60). The pH of collected PRP was adjusted to 6.5 with ACD. Prostaglandin-E1 was added to a final concentration of 2 μmol L^{-1} in order to minimize platelet clumping by inhibiting activation. The plasma was centrifuged at 630 x g for ten minutes at room temperature. The supernatant, platelet-poor plasma (PPP), was removed and the remaining platelet pellet was gently re-suspended in 2 mL JNL buffer (6 mM dextrose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM Na Citrate, 10 mM Tris Base, 3 mM KCl, 900 μM $MgCl_2$, 2 mM HEPES). The suspended platelets were added to a 20 mL disposable chromatography column (Bio-Rad) containing 5 mL Sepharose 2B-300 (Sigma-Aldrich) at room temperature. The column was previously equilibrated with 20 mL JNL buffer. Eluted platelet fractions were collected in 0.5 mL volumes and manually counted under a phase-contrast microscope using a C-Chip DHC-N01-2 disposable hemocytometer (INCYTO). Platelet fractions were pooled and the concentration adjusted to approximately 2×10^7 platelets mL^{-1}.

Treatment of SK36 with a tissue solubilizer for microscopic examination

SK36 cells from an overnight culture were washed and concentrated in PBS, as before. TS-2 (0.5 mL) and SK36 cells (0.25 mL) were combined in a 1.5 mL plastic tube. Control SK36 cells (0.25 mL) were combined with PBS (0.50 mL) in a separate tube. Cells in both tubes were mixed by vortexing and incubated under a step-wise increase in temperature. The tubes were incubated at room temperature for 30 minutes and then at 37°C for 45 minutes. Tubes were vortexed again, and further incubated at 37°C for 1 hour and 30 minutes. Tubes were then placed in a 45°C water bath for 2 more hours. After a total incubation period of 4 hours and 15 minutes, the tubes were vortexed and cells examined microscopically. Five μL of each cell suspension

was placed on a glass microscope slide, covered with a coverslip and examined under a phase contrast microscope (Olympus BH-2; 40 X objective).

Bacterial adhesion to platelet-fibrin matrixes

Preparation of the platelet-fibrin matrixes is based on the methods previously described by Scheld et al. (57). For examination of *S. sanguinis* adherence to platelet-fibrin matrixes via colony counting, platelet-fibrin matrixes were prepared as follows. In each well of a 24-well polystyrene plate (Costar), the following were combined: 70 μL PRP, 30 μL bovine thrombin (Dade Behring; 100 units ml^{-1}), and 30 μL of 0.2 M CaCl$_2$. The mixtures were incubated for 30 minutes at 37°C, which was followed by inoculation with 350 μL prepared SK36 cells. The plate was incubated for 15 minutes at 37°C in a shaking incubator (120 cycles min.$^{-1}$). After incubation, wells were washed three times, sequentially, with 350 μL PBS on a rotary shaker for 5 minutes per wash. After removing the wash buffer, the surface of each well was overlaid with 640 μL BHI + low melting point agarose (Promega), which cooled for 20 minutes at room temperature. The plate was incubated for 48 hours at 37°C and colonies were counted.

For examination of ^{3}H-labeled *S. sanguinis* adherence to platelet-fibrin matrixes by liquid scintillation counting, the matrixes were prepared as follows. In each well of a 96-well filter plate (Millipore), the following were combined: 50 μL of PRP, 40 μL of a 1:1 solution of bovine thrombin (Dade Behring; 100 units ml^{-1}) and 0.2 M CaCl$_2$. The mixtures were incubated for 30 minutes at 37°C. The wells were gently washed twice with 100 μL PBS containing 1% BSA and then incubated with 100 μL of the BSA solution for 60 minutes at room temperature. The BSA solution was removed and 75 μL of ^{3}H-labeled cells were added to each well and incubated at 37°C for varying durations. The wells were washed three times with 100 μL PBS. The plate was

then placed in a -75°C freezer for 25 minutes to solidify the gel-like platelet-fibrin matrixes. A Millipore Multiple Punch System was used to transfer the contents of each well into an individual scintillation vial containing 0.75 mL TS-2 tissue solubilizer (RPI). For use in creating a standard curve for each strain, 75 μL of ^{3}H-labeled cells were added in triplicate to scintillation vials containing 0.75 mL TS-2 and serially diluted two-fold. All vials were placed in a shaking incubator for 4 hours at 50°C and 100 cycles/minute, per TS-2 manufacturer recommendations. Following incubation, the vials were allowed to cool to room temperature overnight. The following morning, 200 μL of 10% glacial acetic acid were added to each vial to neutralize the strongly basic TS-2 and help prevent chemiluminescence. Each vial was filled with 5 mL of BioSafe II liquid scintillation cocktail and mixed thoroughly. Finally, all vials were wiped with an anti-static cloth, loaded in a liquid scintillation counter and their contents measured in triplicate for the presence of ^{3}H using the ^{3}H-detection program on the scintillation counter.

Initial measurements of radioactivity were expressed in counts per minute (CPM). To obtain bacterial adherence values, the triplicate measurements of each vial were averaged. Next, the average values of all replicate vials were calculated. The average of three background vials, each containing no radioactivity, was then subtracted. A standard curve was developed in order to determine the relationship between the measured CPM and the corresponding number of adherent CFUs. Using the standard curve, all CPM values were converted to the number of adherent CFUs. Finally, bacterial adherence to the platelet-fibrin matrix was expressed as a percentage of the total number of bacterial CFUs originally added to the platelet-fibrin matrix. Significance was determined by a One-way Analysis of Variance (ANOVA) and a Dunnett multiple-comparisons post test, with a P value < 0.05 representing a significant difference.

S. sanguinis-platelet adhesion assay

A platelet adhesion assay was used to measure adherence of platelets to *S. sanguinis* immobilized on a polystyrene surface. This assay is based on the methods previously described by Kerrigan et al. (60) and makes use of the intracellular platelet enzyme, acid phosphatase, for quantification. One hundred μL of *S. sanguinis* (~2.0 × 10^9 cfu mL^{-1}) cells suspended in PBS were added to six wells/strain in a 96-well polystyrene plate (Greiner Bio-One). Next, 100 μL of 2.5% (w/v) BSA in PBS were added to six wells of the same plate, which was then incubated for 2 hours at 37°C. The wells were washed twice with 100 μL of the BSA solution and then incubated in 100μL of the same blocking solution for 1 hour at 37°C. The wells were washed three times in JNL buffer to remove any unbound protein. 50 μL of freshly gel-filtered platelets (~2 ×10^7 platelets mL^{-1}) were added to three wells/strain and the platelets were allowed to adhere for 30 minutes at 37°C. Unused gel-filtered platelets were set aside. All wells were gently washed three times with 100 μL of JNL buffer to remove nonadherent platelets. For use in creating a standard curve, 50 μL of unused gel-filtered platelets were added in quadruplicate to untreated wells on the plate and serially diluted two-fold. Next, 100 μL of lysis buffer containing a substrate for acid phosphatase [0.1 M Na acetate (pH 5.5), 0.1% Triton X-100, 10 mM p-nitrophenol phosphate] were added to each well and the plate was incubated for 2 hours at 37°C. The reaction was stopped with 50 μL of 1 M NaOH. 50 μL of JNL buffer were added to non-standard curve wells in order to equalize volumes. Light absorbance was measured on a FLUOstar microtiter plate reader (BMG) at 410 nm. Initial absorbance values were too high to detect differences between strains so all wells were diluted 1:4 in JNL buffer and absorbance was re-measured.

Data was expressed by first subtracting the average values of three wells/strain not treated with platelets from those that were treated with platelets. Using the standard curve, all absorbance values were converted to number of adherent platelets. Next, the average number of adherent platelets to the three BSA treated wells was subtracted from the number adherent to bacteria treated wells. Finally, the average number of adherent platelets to all strains was expressed as a percentage of the average number adherent to SK36. Significance was determined by a One-way Analysis of Variance (ANOVA) and a Dunnett multiple-comparisons post test, with a P value of <0.05 representing a significant difference.

Chemical fixation of cells by glutaraldehyde treatment

Treatment of *S. sanguinis* cells with glutaraldehyde was used in an attempt to chemically fix the cells to polystyrene wells and tested in the *S. sanguinis*-platelet adhesion assay. One-hundred μL of SK36 cells (adjusted to 3 x 10^9 CFU ml^{-1}) or BSA (2%) (w/v) were added in triplicate to a 96-well polystyrene plate (Greiner Bio-One) and incubated for 2 hours at 37°C. Without washing, 100 μL of 0.25% (w/v) glutaraldehyde in sterile dH_2O were added to each well. The plate was incubated for 30 minutes at 37°C. Wells were washed, blocked with BSA and otherwise treated as in the platelet adhesion assay (described above).

Whole-cell enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to measure bacterial adherence to a polystyrene plate surface. The assay was performed based on methods previously described (67, 73, 74). For each strain, 100 μL prepared cells were added in triplicate to a 96-well polystyrene plate (Greiner Bio-One). Three background wells were filled with 100 μL of 2.5% (w/v) BSA in PBS. For use in creating

a standard curve, 100 µL of prepared SK36 cells were added in triplicate and serially diluted 2-fold. The plate was incubated for 2 hours at 37°C. Contents of all wells were removed and the wells were washed twice in 0.1 mL of TBST-B blocking buffer [10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.1% (v/v) Tween-20, 2.5% (w/v) BSA]. Wells were then incubated with 0.1mL of TBST-B for 1 hour at 37°C. Contents of the wells were removed and replaced with 0.05 mL of anti-SK36 polyclonal antiserum (67) diluted 1:25,000 in TBST-B blocking buffer. The plate was incubated at room temperature for 2.5 hours and then washed three times with 0.1mL of TBST [10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.1% (v/v) Tween-20]. Next, 0.05 mL of alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (diluted 1:2,000 in TBST) was added as a secondary antibody. The plate was incubated and washed as before. Bound secondary antibody was detected by addition of 0.1 mL of a *p*- nitrophenylphosphate (*p*-NPP) substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. After ten minutes, a dark straw color developed and the reaction was stopped with 100 µL of 5% EDTA. Light absorbance at 410 nm was measured using a FLUOstar microtiter plate reader (BMG). Resulting absorbance values were adjusted by subtracting the average of three background wells. Significance was determined by a One-way Analysis of Variance (ANOVA) and a Dunnett multiple-comparisons post test, with a P value of <0.05 representing a significant difference.

Combining platelet adhesion and ELISA data

The whole-cell ELISA was run simultaneously alongside platelet adhesion experiments. The same set of prepared cells was added in both the ELISA and platelet adhesion assay. Data from each pair of experiments was combined using the following steps: first, the mean bacterial

adherence of each strain to the polystyrene plate surface as a percentage of SK36 adherence was calculated from the results of three separate ELISA experiments (Mean 1). In the same way, the mean platelet adherence to each strain as a percentage of platelet adherence to SK36 was calculated from the results of three separate *S. sanguinis*-platelet adhesion experiments (Mean 2). Next, Mean 1 was divided into Mean 2 and multiplied by 100, yielding a single, adjusted mean value of platelet adherence to each strain (Mean 3). This final value of platelet adherence to each strain is expressed as a percentage of platelet adherence to SK36.

After dividing one mean into another, the following equation was used to calculate the adjusted standard deviation:

$$S3 = \sqrt{\left(\frac{S1}{M1}\right)^2 + \left(\frac{S2}{M2}\right)^2} \times M3$$

Where M1 = Mean 1 with Standard Deviation (S1), M2 = Mean 2 with Standard Deviation (S2), and M3 = Mean 3 with Standard Deviation (S3).

Platelet adherence (Mean 3) was compared between strains. Significance was determined by a One-way Analysis of Variance (ANOVA) and a Dunnett multiple-comparisons post test, with $P < 0.05$ representing a significant difference.

Results

Examination of *S. sanguinis* adherence to platelet-fibrin matrixes via colony counting

We wanted to determine the contribution of various SK36 Cwa proteins to platelet

adhesion. To do this, we first needed to establish that SK36 adheres to platelets in an *in vitro*

assay and that we could measure this adhesion. As an indicator of potential platelet adherence,

we initially tried to measure SK36 adhesion to platelet-fibrin matrixes. Our first approach to

measuring SK36 adherence to a platelet-fibrin matrix was based on the methods of Scheld, et al.

(57) and used bacterial colony formation on the surface of a platelet-fibrin matrix as an indicator

for bacterial adherence. Log-phase SK36 cells were added to wells of a 24-well microtiter dish

containing freshly prepared platelet-fibrin matrixes and incubated for 15 minutes at 37°C in a

shaking incubator (120 cycles/min.). Non-adherent cells were washed away and the surface was

overlaid with BHI + low melting point agarose. After incubation, colonies were counted. Figure

2 shows the number of SK36 colonies that were observed in the presence (rows A and B) and

absence (rows C and D) of platelet-fibrin matrixes. Colonies were very small and difficult to

count when more than about 20 were present in a well. Countable colonies came from wells

inoculated with bacterial concentrations under 10^2 CFU mL^{-1}. Similar numbers of colonies were

counted in wells with and without platelet-fibrin matrixes. This result was not as discouraging as

might be imagined, as SK36 has been shown to bind well to microtiter plates. However, colonies

were non-uniform in size and partially obscured by the platelet-fibrin matrix, making counting

difficult. Moreover, it could be expected that a colony might arise in different instances from

Figure 2. SK36 colonies in the presence and absence of platelet-fibrin matrixes. Platelet-fibrin matrixes with adherent SK36 were overlaid with BHI + low melting point agarose and incubated at 37°C for two days to allow for colony formation. (A) Concentration of SK36 added to each well and the number of colonies counted. (B) Actual image of the plate at the time colonies were counted. Wells in rows A and B contained matrixes while wells in rows C and D did not. * = empty/unused wells. Results shown are from a single experiment.

Figure 2.

A.

Bacteria Added (CFU/mL)						
	1	**2**	**3**	**4**	**5**	**6**
A	5×10^7	5E+06	5E+05	5E+04	5E+03	5E+02
B	5E+01	0E+00	*	*	*	*
C	5E+07	5E+06	5E+05	5E+04	5E+03	5E+02
D	5E+01	0E+00	*	*	*	*

Colonies Counted						
	1	**2**	**3**	**4**	**5**	**6**
A	TNTC	TNTC	TNTC	TNTC	TNTC	~35
B	10	2	*	*	*	*
C	TNTC	TNTC	TNTC	TNTC	TNTC	15
D	~25	0	*	*	*	*

B.
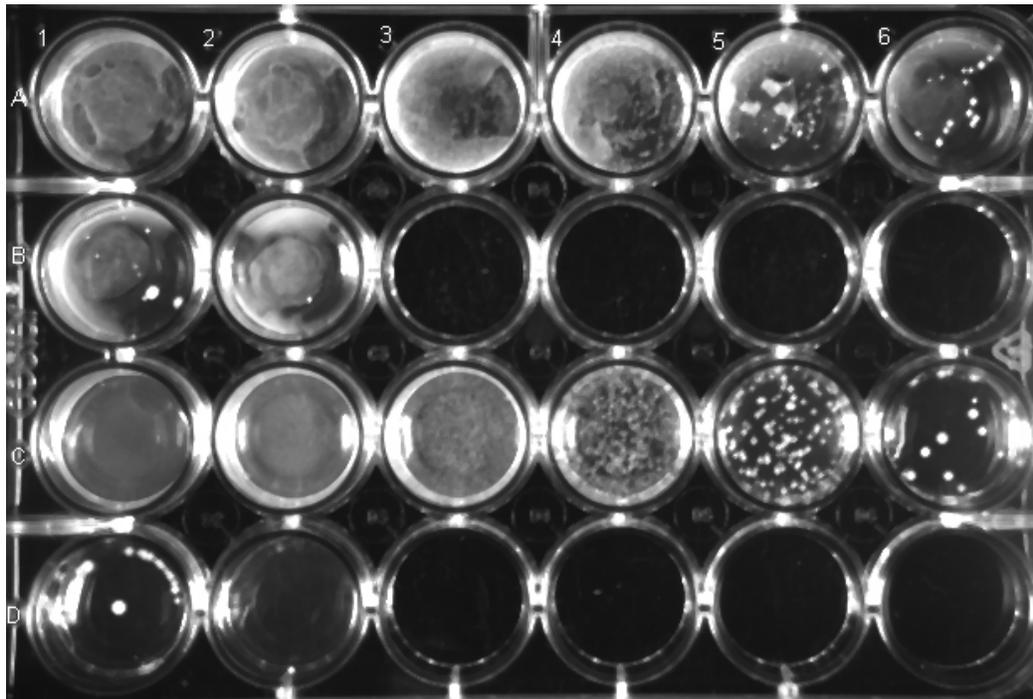
different numbers of adherent cells, making quantification difficult. A different approach was therefore taken.

Examination of *S. sanguinis* adherence to platelet-fibrin matrixes via detection of radioactively labeled cells—optimization of sample preparation for scintillation counting

Because measurement of bacterial adhesion to platelet-fibrin matrixes by colony counting proved unsatisfactory, we next decided to measure adherence of radioactively labeled cells by scintillation counting, as has been done previously (75). Early optimization experiments using the scintillation counting technique for detection of ^{3}H-labeled bacteria were plagued by inconsistency and increasing CPM in samples over time. Figure 3 shows the results of an experiment in which ^{3}H-labeled SK36 cells were added to five vials of liquid scintillation cocktail, which were examined by scintillation counting over a period of seven days. With each later measurement, the CPM was significantly larger than the measurement that came before it ($P<0.001$, ANOVA). This was unexpected because no additional ^{3}H had been added to the samples after day one. Examination of the literature concerning the chemistry and physics of scintillation counting revealed that efficient and reproducible counting requires that samples be homogeneous not only at the macroscopic level but also at the molecular level (76). This suggested the possibility that *S. sanguinis* cells were continuing to lyse over the course of several days' incubation in the scintillation fluid, which was increasing the efficiency of liquid scintillation counting. A biological tissue solubilizer (TS-2) was therefore used to attempt to more thoroughly homogenize the samples.

Figure 3. Increasing detection of ^{3}H-labeled SK36 cells by scintillation counting over time. ^{3}H-labeled SK36 cells were added to vials of liquid scintillation cocktail. Using a liquid scintillation counter, the amount of ^{3}H in the sample was measured over a period of seven days. Values shown (mean + standard deviation) are from five replicate samples.
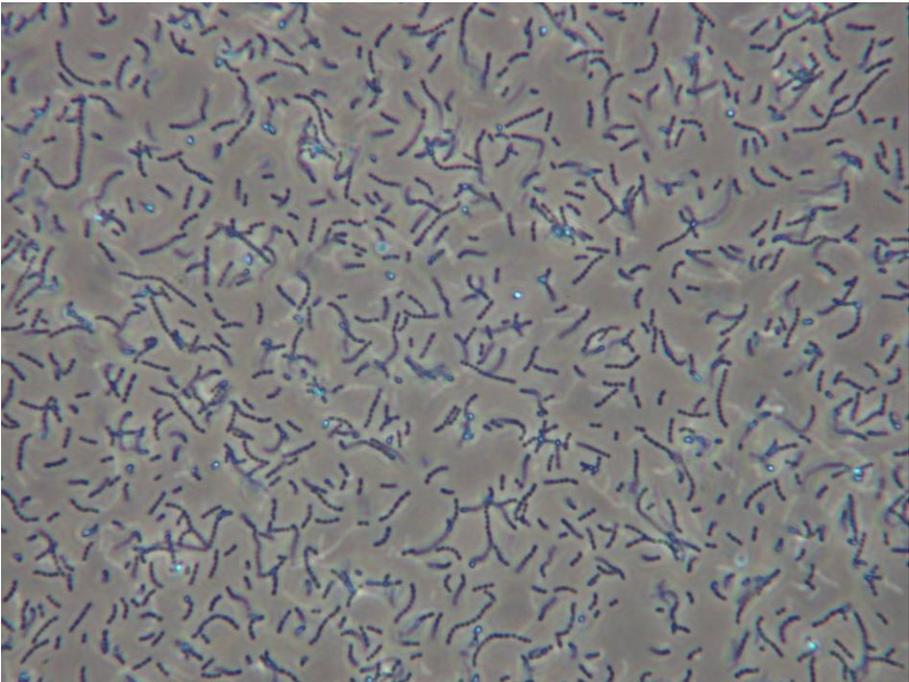
Figure 3.
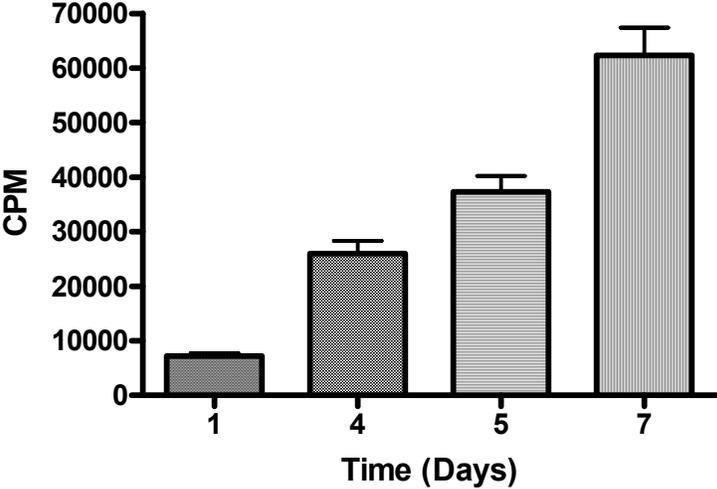
We first examined the effect of TS-2 on non-radiolabeled SK36 microscopically. SK36 cells from an overnight culture were washed and concentrated in PBS. TS-2 and suspended cells were combined at a ratio of 2:1, while control SK36 cells were diluted with PBS at the same ratio. All cells were incubated under a step-wise increase in temperature for 4 hours and 15 minutes with periodic mixing. This protocol was improvised due to a lack of manufacturer recommendations at the time. Later experiments utilizing the tissue solubilizer followed manufacturer recommendations. After incubation, cells were examined microscopically (Figure 4). In the control sample, cells were plentiful and arranged mostly in chains. In the sample treated with TS-2, there were far fewer cells. Instead, several small clumps, which may or may not have contained whole cells, were visible. We next examined the effect of TS-2 on ^{3}H-labeled SK36 cells by liquid scintillation counting. TS-2 and ^{3}H-labeled SK36 cells were combined at a ratio of ~7:1 and incubated for 4 hours at 50°C in a shaking incubator (100 cycles/min.). Samples were examined by liquid scintillation counting over a period of four days (Figure 5). Comparison of the CPM on all days determined that there was no significant difference (ANOVA).

After testing the effect of TS-2 on ^{3}H-labeled *S. sanguinis* alone, the solubilizer treatment was used to dissolve whole platelet-fibrin matrixes with adherent cells. ^{3}H-labeled SK36 cells were added to wells of a 96-well microtiter filter plate containing freshly prepared platelet-fibrin matrixes and incubated for 30 minutes at 37°C. The matrixes were washed to remove non-adherent cells, treated with TS-2, and then measured in a liquid scintillation counter for the presence of ^{3}H. In one such experiment, the mean CPM from samples treated with TS-2 (8481 ± 853, mean ± SD) was significantly different from that of samples not treated with the solubilizer

Figure 4. Effect of a biological tissue solubilizer (TS-2) on the microscopic appearance of SK36 cells. (A.) Untreated SK36. (B.) SK36 cells treated with TS-2. Images were taken under a phase contrast microscope (40X objective).

Figure 4.

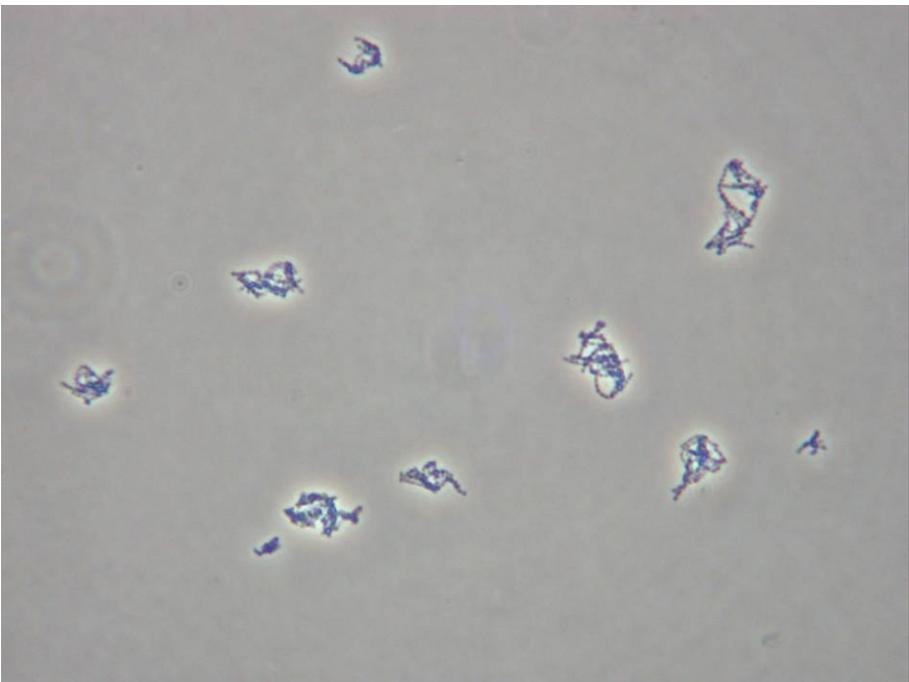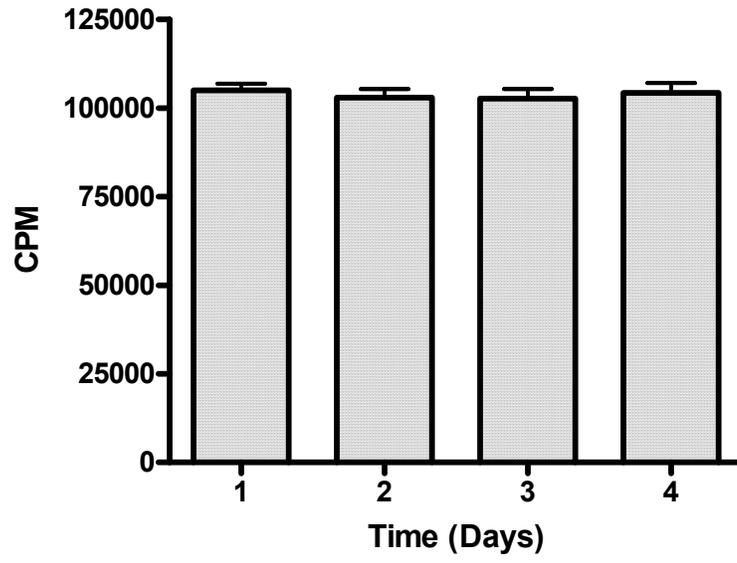**A.**

B.

Figure 5. Effect of a biological tissue solubilizer (TS-2) on the detection of ^{3}H-labeled SK36 cells by scintillation counting over time. ^{3}H-labeled SK36 cells were treated with TS-2 and the amount of ^{3}H was measured in CPM by liquid scintillation counting over a period of four days. The values (mean + SD) are from four replicates in one experiment.

Figure 5.

(4182.7 ± 1743.9, mean ± SD) (P<0.05, Unpaired t test). Based on these results, it appeared that treatment of ^{3}H-labeled SK36, both in the presence and absence of platelet-fibrin matrixes, increased the reproducibility and sensitivity of ^{3}H detection. The tissue solubilization step was therefore included in all subsequent experiments with the bacterial – platelet-fibrin matrix adhesion assay.

Bacterial adhesion to platelet-fibrin matrixes

Using a bacterial – platelet-fibrin matrix adhesion assay, we measured adherence of radioactively labeled cells to a platelet-fibrin matrix by scintillation counting. One culture of cells was grown in the presence of [^{3}H]thymidine and was split into two parts for use in the adhesion assay and for development of a standard curve. For the former, ^{3}H-labeled cells were added to wells of a 96-well microtiter filter plate containing freshly prepared platelet-fibrin matrixes and incubated at 37°C. The matrixes were washed to remove non-adherent cells, treated with TS-2, and then measured in a liquid scintillation counter for the presence of ^{3}H. For the standard curve, ^{3}H-labeled cells were treated with TS-2 and measured by scintillation counting. A replicate overnight culture, grown in the absence of [^{3}H]thymidine, was plated on growth media and incubated for measurement of CFUs via colony count. Figure 6 shows a standard curve from a single bacteria – platelet-fibrin matrix adhesion experiment and shows the CPM detected in samples to which increasing numbers of bacteria were added directly to scintillation vials. The standard curve allowed conversion of measured CPM to percent of added bacteria that adhered to the matrix.

The standard curve was necessary because we determined that different bacteria incorporated different amounts of [^{3}H]thymidine into their cells. In one experiment, a culture of

Figure 6. Standard curve from a single bacteria – platelet-fibrin matrix adherence experiment. 3H-labeled SK36 cells, from the same culture used to inoculate platelet-fibrin matrixes, were added in increasing concentrations to scintillation vials, treated with TS-2, and counted in a liquid scintillation counter. A replicate culture was used for the plating and counting of bacterial colonies.

Figure 6.

SK36 Standard Curve

$y = 0.0013x - 2840.4$

$R^2 = 0.9981$
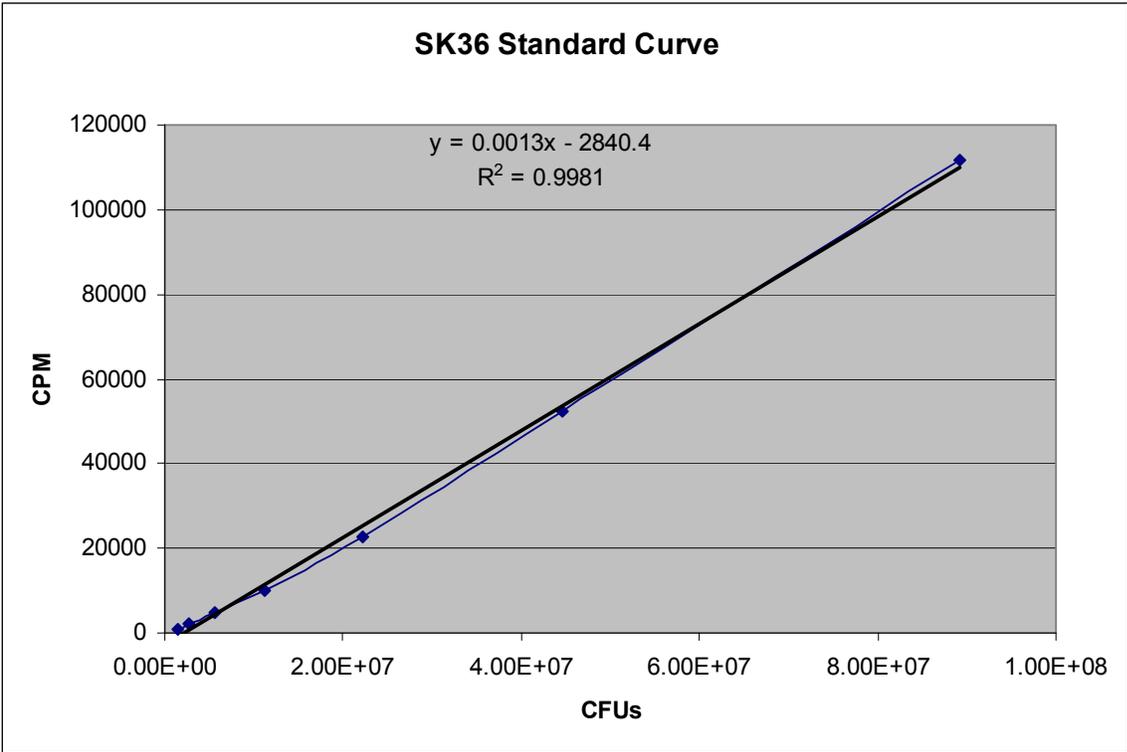
^{3}H-labeled MG1363, containing ~1.0 x 10^8 CFU mL^{-1}, produced 3.96 x 10^4 CPM. In the same experiment, a culture of ^{3}H-labeled SK36, containing the same concentration of CFU mL^{-1}, produced 2.36 x 10^5 CPM. A trendline inserted in each standard curve provided an equation for converting CPM into bacterial CFUs. Subsequently, bacterial adherence to the platelet-fibrin matrix was expressed as the percentage of adherent CFUs divided by the total number of CFUs added to the matrix. Three different bacterial species were tested for their ability to adhere to a platelet-fibrin matrix *in vitro*. The *S. sanguinis* strain SK36 has been reported to bind platelets (56), but had not yet been shown to adhere to a platelet-fibrin matrix. *Lactococcus lactis* strain MG1363 is a food-grade, lactic acid-producing bacteria. It was chosen for testing as a possible negative control because it is non-pathogenic, does not colonize mammals, and it possesses a GRAS (generally regarded as safe) status (75, 77, 78). DH10B is a genetically engineered laboratory strain of *E. coli*. It also was chosen for testing as a possible negative control because it was thought to be unlikely to specifically bind a platelet-fibrin matrix.

Percent bacterial adherence to a platelet-fibrin matrix is shown (Figure 7) as a function of increasing adhesion time (time in which matrixes were incubated in the presence of ^{3}H-labeled bacteria before being washed to remove non-adherent cells). At each time point except for one, comparison of percent adherence of DH10B and MG1363 with that of SK36, which has been reported to adhere to platelets (56), showed no significant difference. At 30 minutes, adherence of DH10B was significantly greater (P<0.01, ANOVA) than that of SK36. For both SK36 and MG1363, all time points showed a significantly higher adherence at 120 minutes than at 15 minutes (P<0.001, ANOVA), suggesting that more cells of each strain were adhering over time. The same comparison for DH10B determined that there was no significant difference between time points, suggesting that the level of adherence remained constant over time. The results show

Figure 7. Bacterial adherence of ^{3}H-labeled cells to platelet-fibrin matrixes. Adherence is expressed as a percentage of the total number of bacterial CFUs added to the matrix. SK36 data were derived from three experiments. DH10B and MG1363 data came from one experiment each. Mean % adherence + SD is shown.

Figure 7.
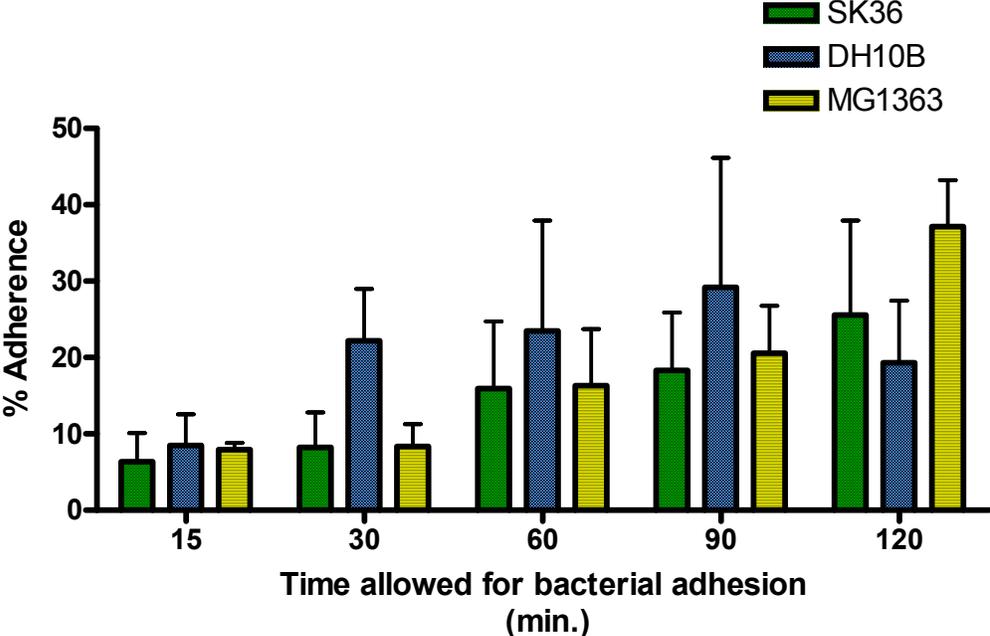
that DH10B and MG1363 adherence to platelet-fibrin matrixes is equal to or greater than that of SK36 in this assay.

S. sanguinis-platelet adhesion

Because we were unable to demonstrate greater binding of SK36 compared to that of negative control strains by measuring adherence of radioactively labeled cells to a platelet-fibrin matrix, we next decided to measure adhesion of purified platelets to bacteria immobilized on polystyrene wells, as has been done previously (56, 60). For this assay, platelets were purified from freshly collected, whole blood by gel filtration. They were then added to microtiter plates which had been previously coated with SK36 or another bacterial strain. After washing, the relative number of adherent platelets in each sample were determined by measurement of the intracellular platelet enzyme, acid phosphatase. Adherent platelets were lysed with a solution containing a substrate for acid phosphatase, which develops a soluble yellow product when it reacts with the enzyme. Absorbance of the resulting color in the wells was read at 410nm with a plate reader and used as a measure of platelet adherence. Using this assay, it was previously reported that SK36 adhered well to platelets and VT1614 did not (56). Therefore, we tested SK36 and VT1614 in the platelet-adhesion assay. Figure 8 shows platelet adherence to polystyrene wells coated with different bacterial strains. Platelets adhered significantly less well to wells coated with VT1614 than to wells coated with SK36, as expected. Since these results were consistent with previous findings, we continued to use the assay.

As with the bacterial – platelet-fibrin matrix adhesion assay described above, a standard curve was developed. This was done to determine the relationship between a measured absorbance value and the corresponding number of adherent platelets, thus providing a more

Figure 8. Platelet adhesion to polystyrene wells coated with immobilized bacteria. Adherent platelets were lysed with a solution containing a substrate for the platelet enzyme, acid phosphatase, resulting in a color change within the wells. Contents of the wells were diluted 1:4 and light absorbance was read at 410nm on a plate reader. Values shown (mean absorbance + SD) are from 3 replicates in one experiment. Asterisk indicates an absorbance significantly different from SK36 (P<0.05).

Figure 8.
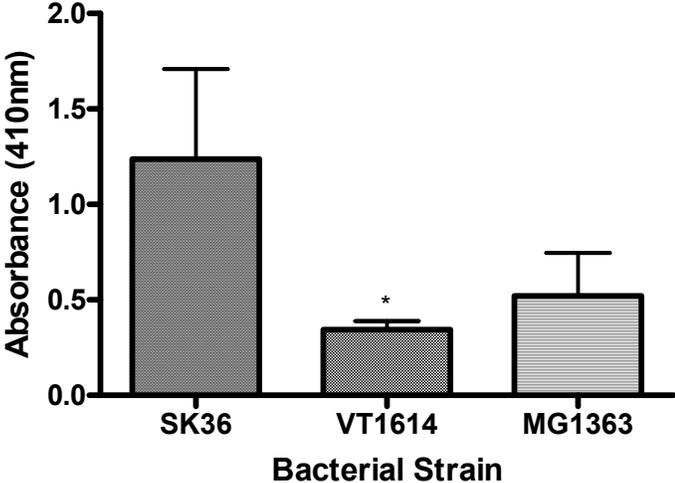
quantitative measure of platelet adherence. First, the number of platelets in the original gel-filtrate were manually counted under a phase contrast microscope (40X objective). Next, the gel-filtered platelets were serially diluted in the polystyrene wells and combined with the platelet-lysing/acid phosphatase substrate solution. Finally, the absorbance of the resulting color was measured as before. Figure 9 is a standard curve from a single *S. sanguinis*-platelet-adhesion experiment and shows the measured absorbancies from wells with increasing concentrations of platelets. A trendline was inserted into the graph. The equation from the trendline was used in a corresponding *S. sanguinis*-platelet-adhesion experiment to convert measured absorbance to number of platelets adherent to bacteria-coated wells. Subsequently, platelet adherence to the *S. sanguinis*-coated polystyrene wells was expressed as the percentage of adherent platelets divided by the total number of platelets added.

After establishing that platelets adhered to SK36 in this assay and that we could measure their adhesion, we examined platelet adhesion to 12 different Cwa protein mutants, each missing a single Cwa protein (Tables 1 and 2). SK36 has 33 predicted Cwa proteins and the 12 individual Cwa protein mutant strains tested in this study were all derived from SK36. Table 2 shows the *Streptococcus sanguinis* mutants used in this study, and provides descriptions of their functions or the functions of their orthologs in other bacterial species. All of the mutants, with the exception of VT1614 (56), were created previously in our lab (67).

Tested strains were separated into two groups because it was too cumbersome to run experiments with all of them at once. SK36 was tested alongside each of the two groups. Percent platelet adherence to immobilized *S. sanguinis* is shown in Figure 10. Polystyrene wells coated with VT1614 had a significantly lower percentage of adherent platelets than did wells coated with SK36 ($P<0.01$, ANOVA). This was the only strain that differed significantly from SK36.

Figure 9. Standard curve from a single *S. sanguinis*-platelet-adhesion experiment. Serially diluted gel-filtered platelets were combined with a platelet-lysing solution containing the chromogenic acid phosphatase substrate, p-nitrophenol phosphate. The absorbance of the resulting colored solution was measured at 410nm. The concentration (platelets mL^{-1}) of added platelets was determined prior to the reaction by manual counting under a phase contrast microscope.

Figure 9.
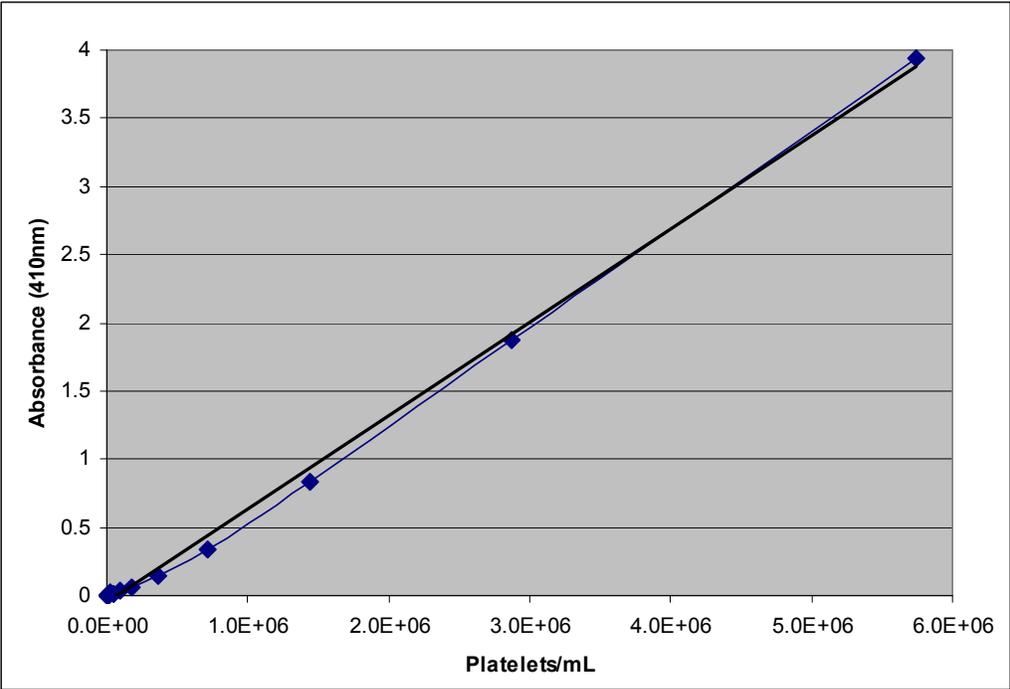
Table 2. *Streptococcus sanguinis* **cell-wall anchored protein mutants used and descriptions of predicted** *S. sanguinis* **cell wall-anchored proteins**

Cwa Mutant	Missing Protein	Gene ID	Gene	Ortholog	Bacterium	Description of *S. sanguinis* missing protein or orthologous protein
VT1614	SrpA	SSA_0829	*srpA*		*S. sanguinis*	Platelet binding & aggregation (56)
				hsa	*S. gordonii*	Platelet binding (55), endocarditis virulence (45), binding to gp340 and HEP-2 epithelial cells (79)
13	SspC	SSA_0303	*sspC*	*sspA, sspB*	*S. gordonii*	Antigen I/II, promotes binding to collagen (80); binding to gp340 (79)
				spaP, pac	*S. mutans*	Adherence to fibronectin, collagen and fibrinogen (81); adherence to gp340 (82)
33	CrpA	SSA_0904	*crpA*	*cshA*	*S. gordonii*	Platelet adhesion (62); adherence to immobilized fibronectin (44)
9	SspD	SSA_0956	*sspD*	*sspA, sspB*	*S. gordonii*	Antigen I/II, promotes binding to collagen, dentinal tubule invasion (80); binding to gp340 (79)
15		SSA_1023		*vwb1*	*S. lugdunensis*	Von Willebrand factor (vWF) binding protein (83)
25		SSA_1063				Contains Von Willebrand factor type A domain, cd00198 (84)
8		SSA_1632		*rrgA, rrgB*	*S. pneumoniae*	Surface pilus locus; Pili linked to in vitro adherence to A549 epithelial cells, colonization and virulence in vivo (85)
7		SSA_1633				
6		SSA_1634				
		SSA_1635				
3	CbpA	SSA_1663	*cbpA*		*S. sanguinis*	Platelet aggregation (62); vegetation enlargement (54)/collagen binding domain, pfam05737 (84)
30		SSA_1666			*S. sanguinis*	Contains collagen binding domain, pfam05737 (84)

This table was adapted from (67).

Figure 10. Platelet adhesion to *S. sanguinis*-coated polystyrene surface. Platelet adherence to *S. sanguinis* mutants in group 1 (A.) and group 2 (B.) is expressed as a percentage relative to SK36. The values (mean + SD) are from three replicates each in three independent experiments. Asterisks indicate percent adherence significantly different from SK36 (** P<0.01).

Figure 10.
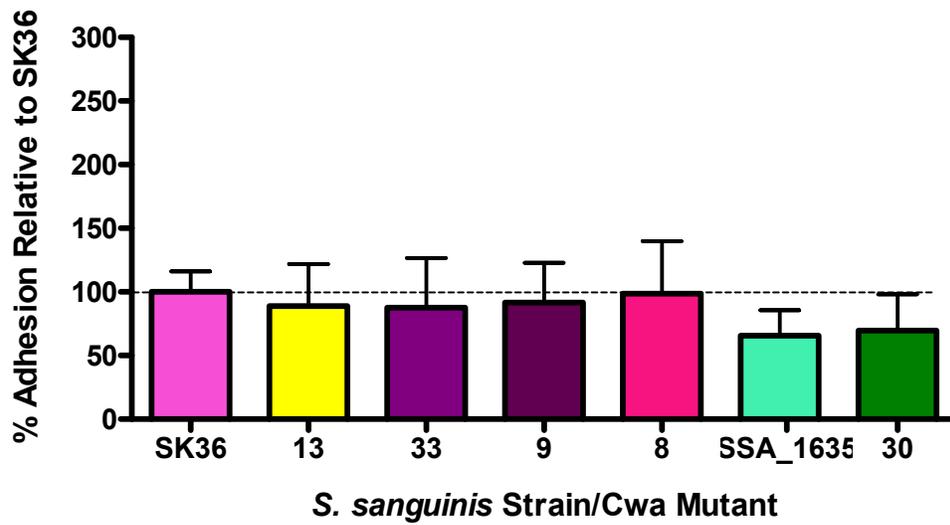
A.
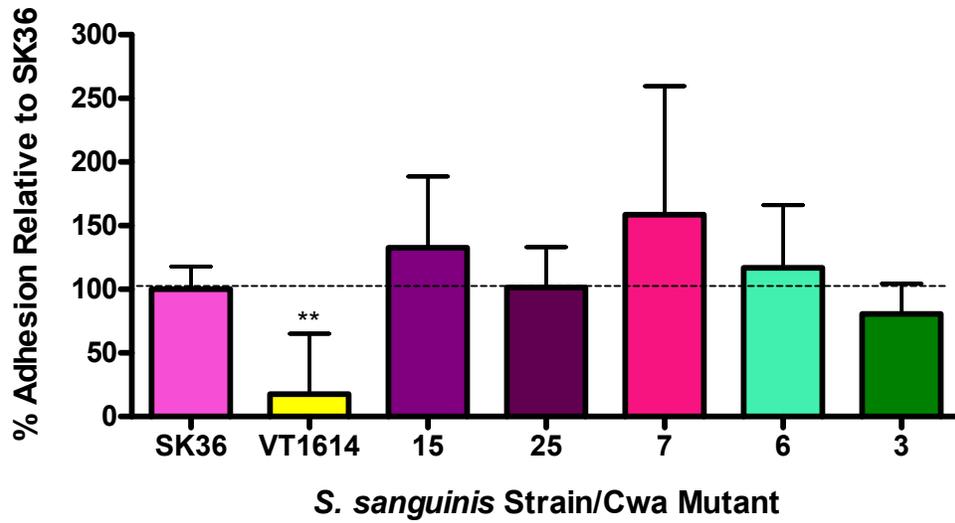
B.

This result suggested that gel-filtered platelets adhere less well to VT1614 than SK36 but equally well to the other mutant strains.

S. sanguinis adherence to a polystyrene surface

The first step in the *S. sanguinis*-platelet-adhesion assay is immobilization of bacterial cells on a polystyrene surface. It was previously observed that VT1614 attached poorly to polystyrene plates following overnight incubation (67). This suggested an alternative explanation for our observation of poor platelet binding to strain VT1614. Perhaps the cells of this strain bound platelets just as efficiently as SK36, but because fewer cells were binding to the plate, fewer platelets were binding. Therefore, in order to determine the amount of bacterial adherence to the polystyrene wells, a whole-cell bacterial ELISA was performed. *S. sanguinis* cells were prepared and allowed to adhere to polystyrene wells, as in the *S. sanguinis*-platelet adhesion assay. Adherent cells were treated with an anti-SK36 polyclonal antiserum (67), followed by alkaline phosphatase-conjugated anti-rabbit immunoglobulin G as a secondary antibody. Bound antibody was detected by addition of *p*-nitrophenyl phosphate substrate, which produced a soluble yellow product upon reaction with alkaline phosphatase. Absorbance at 410nm of the resulting colored solution was measured on a plate reader.

Rather than using absorbance as a measure of bacterial adherence, a standard curve was developed in each ELISA. This was done in order to determine the relationship between a measured absorbance value and the corresponding number of bacterial CFUs adherent to a polystyrene well, as well as provide a more quantitative measure of bacterial adherence. A SK36 culture of known concentration (CFU mL^{-1}) was serially diluted. The diluted cells were allowed to adhere to the polystyrene wells. Immunodetection of adherent cells was performed as

described in Materials and Methods. Figure 11 shows a standard curve from a single whole-cell bacterial ELISA and indicates the absorbance from wells with increasing concentrations of added bacteria. A trendline was inserted into the graph. The equation from the trendline was used to convert measured absorbance to number of bacterial CFUs adherent to the polystyrene wells. Subsequently, bacterial adherence to polystyrene wells was expressed as the percentage of adherent CFUs out of the total number of CFUs originally added.

Twelve different *S. sanguinis* CWA mutants (Table 2) were tested alongside SK36 for their ability to adhere to polystyrene wells. Percent adherence is shown (Figure 12). Eight of the 12 mutants, including VT1614, adhered significantly less well to the polystyrene surface than did SK36 (ANOVA). This reproduced the reduced-adhesion phenotype previously reported for VT1614 (67). Since an equal number of adherent cells of each strain in the polystyrene wells is important for the *S. sanguinis*-platelet-adhesion assay, these results presented a potential problem. We therefore attempted to fix cells chemically to the polystyrene surface in equal numbers between strains. This was based on the previous finding from our lab that VT1614 cells could be fixed to the plate surface in numbers equal to SK36 by glutaraldehyde treatment (67). However, incorporation of this treatment into the platelet-adhesion assay resulted in a significant increase (P<0.05, Unpaired t test) in the amount of platelets adherent to background wells coated with 2% BSA (w/v) (measured by absorbance at 410nm). This suggested the glutaraldehyde was affecting platelet adherence to the polystyrene wells. Moreover, glutaraldehyde treatment reduced binding of platelets to SK36. This suggested the treatment was eliminating specific binding of platelets by SK36. For these reasons, the treatment was abandoned and another approach was used to account for differences in the number of cells adherent to the plate surface.

Figure 11. Standard curve from a single whole-cell bacterial ELISA. Serially diluted SK36 cells from a culture of known concentration (CFU ml^{-1}) were added to polystyrene wells. Immunodetection of adherent cells was measured via absorbance at 410nm.

Figure 11.

y = 0.3851Ln(x) - 5.8817

Absorbance 410nm

Added SK36 (CFU/mL)
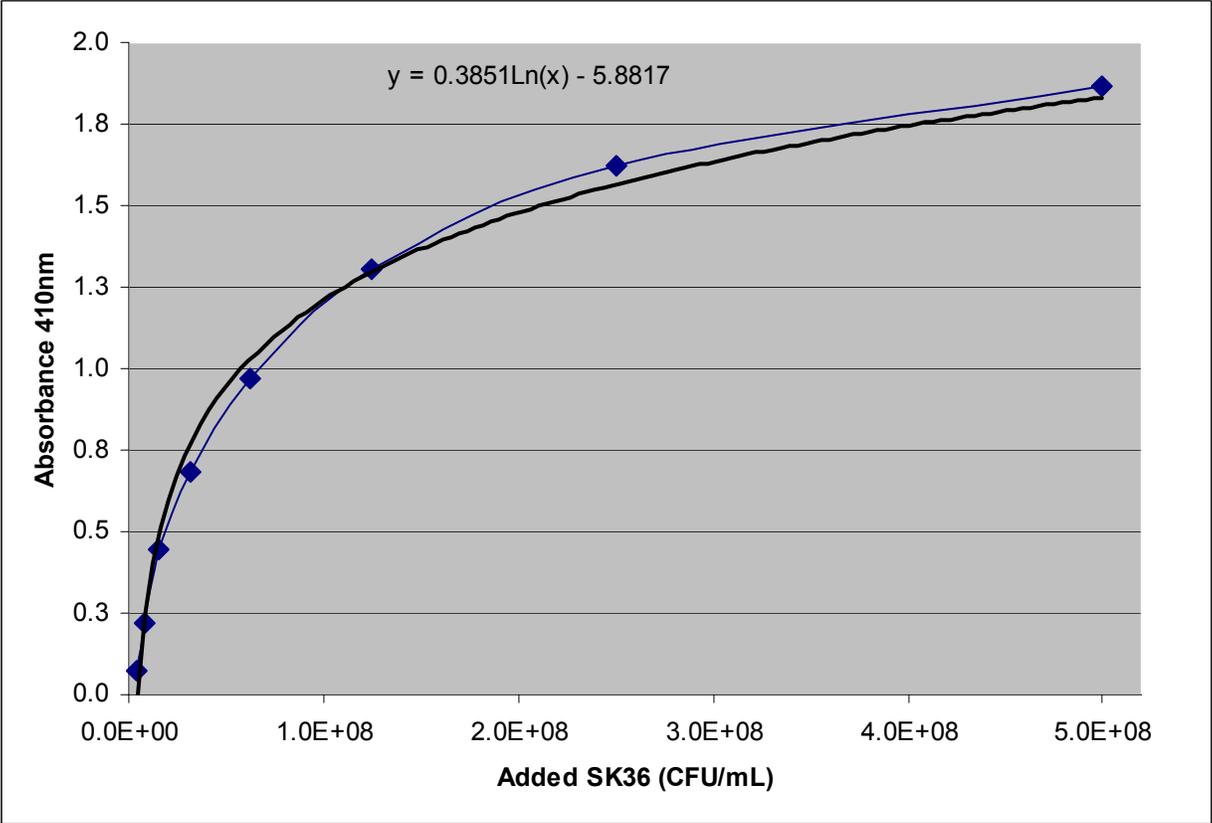
Figure 12. *S. sanguinis* adhesion to a polystyrene surface. A whole-cell ELISA was performed on *S. sanguinis* adherent to a polystyrene surface. Bacterial adherence of *S. sanguinis* Cwa protein mutants in group 1 (A.) and group 2 (B.) is expressed as a percentage relative to SK36 adherence. The values (mean + SD) are from three replicates in three independent experiments. Asterisks indicate percent adherence significantly different from SK36 (* $P<0.05$; ** $P<0.01$)

Figure 12.

A.
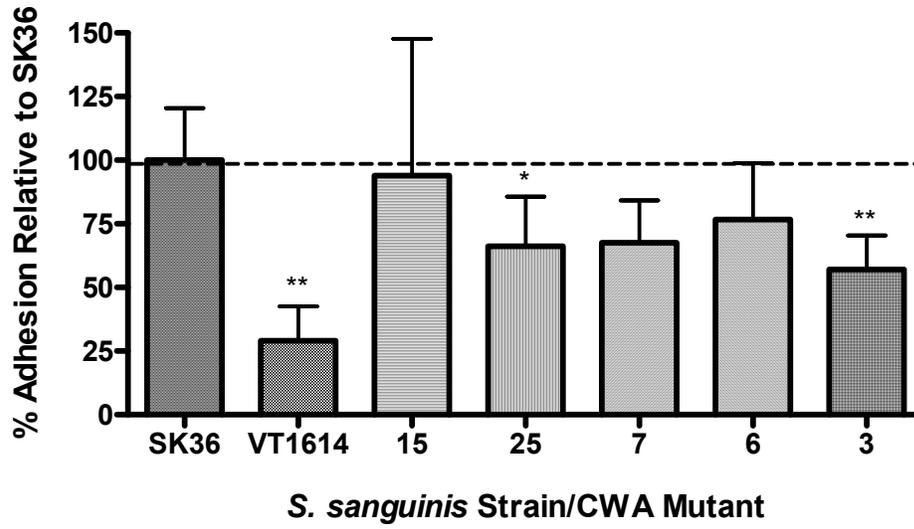
B.
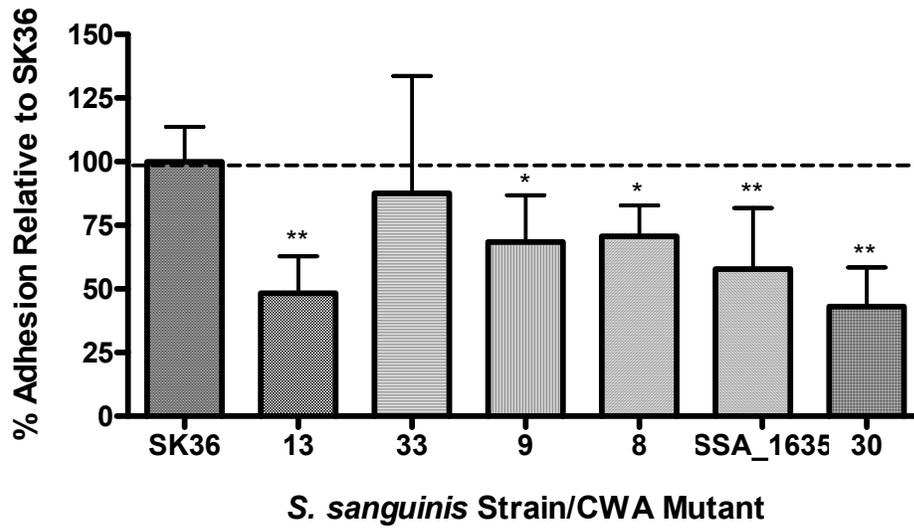
Combining platelet adherence and ELISA data

Because we could not ensure equal adherence of different strains to the plate surface, we decided to measure adherence of each strain and then adjust for these differences when measuring bound platelets. The whole-cell bacterial ELISA was therefore performed simultaneously with the *S. sanguinis*-platelet-adhesion assay. The results of both sets of experiments, described above, are shown side by side in Figure 13. To account for the variation between mutant strains in the number of bacterial CFUs adherent to the polystyrene surface, adhesion percentages from the ELISA experiments were divided into adhesion percentages from the *S. sanguinis*-platelet-adhesion experiments, as was described in Materials and Methods. The resulting, adjusted values for platelet adherence are shown (Figure 14). None of the tested mutants differed significantly from SK36 in their ability to bind platelets.

Figure 13. Platelet adhesion to *S. sanguinis*-coated polystyrene surface*/S. sanguinis* adhesion to a polystyrene surface. The results of the two separate sets of experiments are shown together. Two different types of adhesion are shown here: platelet-bacteria adhesion, and bacteria-plate adhesion.

Figure 13.

A.
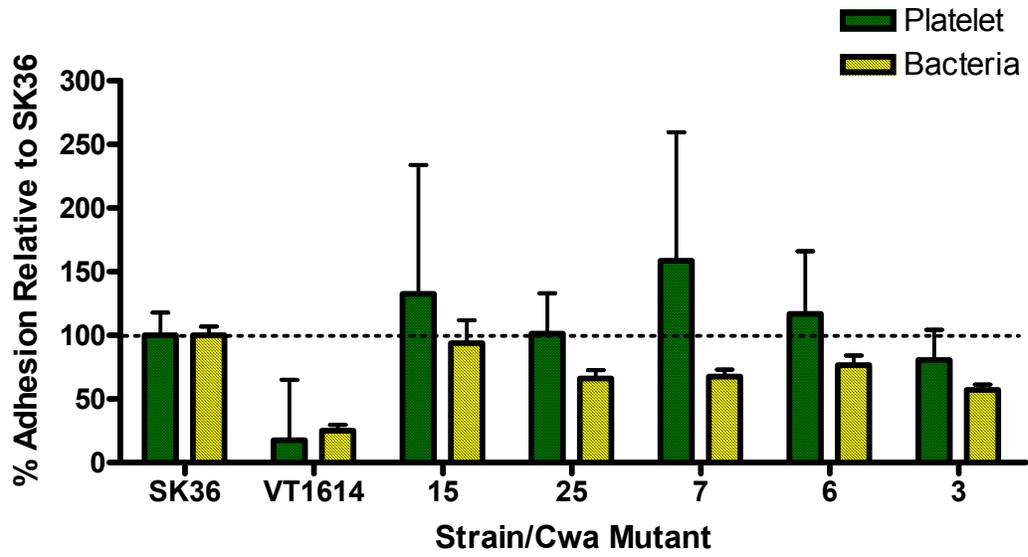
B.
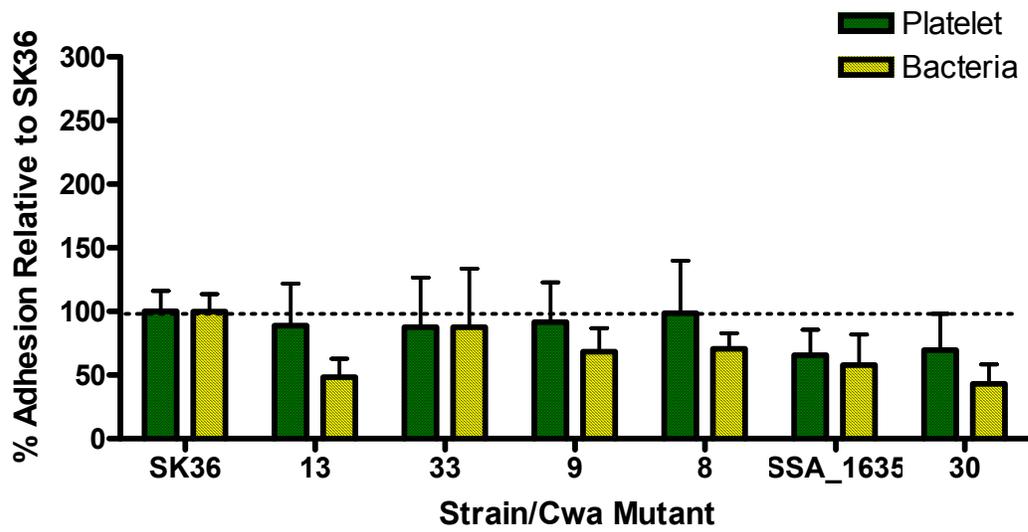
Figure 14. Adjusted values for *S. sanguinis*-platelet adhesion. Platelet adherence to *S. sanguinis* mutants in group 1 (A.) and group 2 (B.) is expressed as a percentage relative to platelet-SK36 adherence. The values (mean + SD) were obtained by dividing data from the whole cell ELISA experiments into data from *S. sanguinis*-platelet adhesion experiments.

Figure 14.

A.
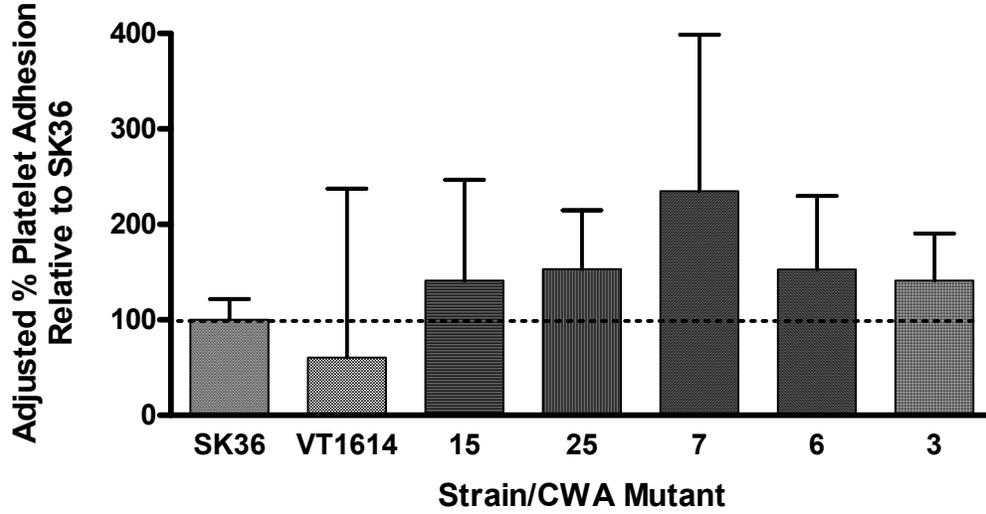
B.
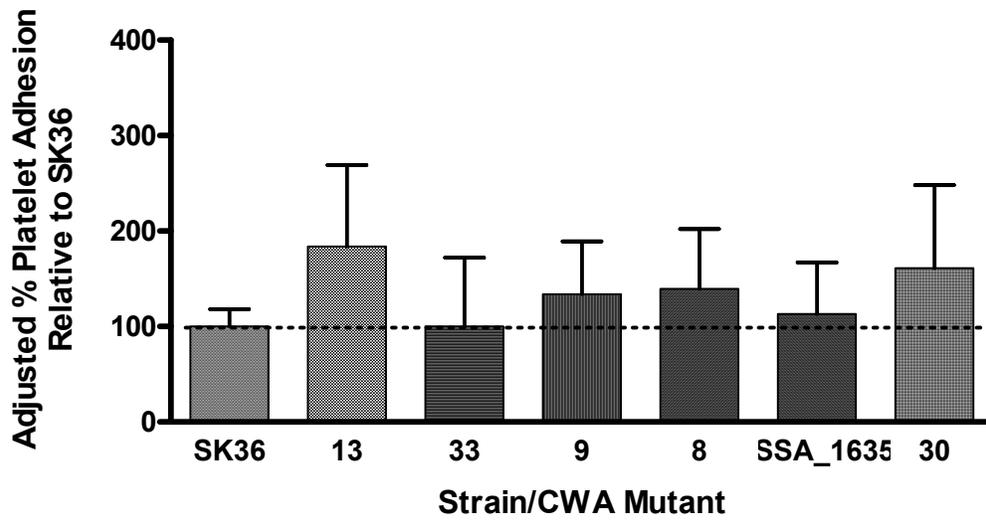
Discussion

Infective endocarditis remains a disease with high morbidity and mortality. Bacterial-platelet adherence has been suggested to be an important early event in the pathogenesis of IE (47). Furthermore, there are multiple reports of *S. sanguinis* binding platelets *in vitro* (56, 58-61). Some bacterial Cwa proteins have been reported to contribute to platelet binding by gram-positive bacteria (48-52, 55, 56, 62). Only two of these studies involved *S. sanguinis* Cwa proteins. (56, 62). Therefore, we set out to further this research and determine if multiple Cwa proteins contribute to *S. sanguinis*-platelet adhesion. To that end, we attempted to measure both bacterial adhesion to platelet-fibrin matrixes and platelet adhesion to polystyrene wells coated with immobilized *S. sanguinis*.

It is unclear why an oral commensal bacteria, such as *S. sanguinis*, would play a benign or even beneficial role in the oral cavity, and a pathogenic role on the heart endothelium. Moreover, given that oral bacteria do not frequently encounter platelets in the oral cavity, it initially seems inexplicable why they would be able to interact with them at all, let alone colonize a platelet vegetation. It has previously been reported that the platelet membrane sialoglycoprotein GPIbα acts as the receptor for two strains of *S. sanguinis* (60), and that a serine-rich glycoprotein on the bacteria (SrpA) mediates this adhesion in a sialic-acid-dependent manner (56). In *S. gordonii*, the Hsa and GspB proteins have been shown to interact with certain sialylated salivary molecules, including the low-molecular-weight mucin, MG2 (86). A

relationship between the capability of oral viridans streptococci to interact with the platelet receptor, GPIbα, which they rarely encounter and their ability to interact with salivary proteins, which they routinely encounter has been reported. It was previously shown that platelet adhesive strains of *S. sanguinis* and *S. gordonii* that adhered to platelets in a GPIbα-dependant manner also bound to the low-molecular-weight submandibular salivary protein, MG2, and this interaction was sialic acid-dependent (87). Based on these collective results, it was suggested that *S. sanguinis* and *S. gordonii* may be efficient colonizers of platelet vegetations because of their adaptation to recognize sialylated salivary mucins (87). In the oral cavity, Fim A of S. parasanguinis is a recognized oral adhesin, which mediates adherence to the salivary pellicle. However, it is also reported to be a major virulence determinant in S. parasanguis endocarditis due to a potential role in initial colonization of damaged heart tissue (88). Reports such as these suggest that the naturally occurring adhesins of oral bacteria, which mediate bacterial adhesion in their normal niche, may be inadvertently binding to surfaces in the heart and contributing to the pathogenesis of IE.

To examine bacterial adhesion to a platelet-fibrin matrix, our first approach utilized bacterial colony formation on the surface of a platelet-fibrin matrix as an indicator of bacterial adherence, as has been done before (57). We examined the presence of SK36 colonies in wells with and without platelet-fibrin matrixes and found similar numbers in both. Although only one experiment was performed, it appeared as though SK36 adhered equally well to the matrix and plate surface. The presence of SK36 colonies in the wells without matrixes was not surprising, as the ability of SK36 to adhere to a polystyrene surface has been used in assays by us and others (67). What stood out about this approach was how difficult it was to count the colonies. Countable numbers of colonies were observed in wells that were inoculated with fewer than 100

CFU mL^{-1}. In our assay, the platelet-fibrin matrixes were prepared in 24-well polystyrene plates, with a well diameter of 16mm, rather than much larger 60mm diameter tissue culture dishes, previously used (57). We decided to do this for two reasons. Logistically, multiple matrixes on a single plate would have allowed for easier screening of a large number of Cwa mutant strains than would the larger, individual Petri dishes. Furthermore, the smaller wells allowed for the creation of smaller platelet-fibrin matrixes, which in turn, required less platelet-rich plasma. Because it was readily available, we had utilized PRP obtained from blood bank whole-blood for initial testing in this assay. Had we continued to use this assay, we would have used fresh PRP obtained from volunteer blood donors, which is a more commonly used source for *in vitro* bacterial adherence assays. Since we wanted to take as little as necessary from our potential blood donors, the amount of PRP used in our assay became important. However, the smaller size of the matrixes forced a smaller countable number of colonies.

There is also a theoretical problem with using colony formation as an indicator for adherence. A bacterial colony is not an exact indicator of the number of adherent cells. In the case of *S. sanguinis*, which forms chains of cells, a single colony can arise from a single chain, a clump of chains, or even multiple unlinked chains present in close proximity. This approach only provides a qualitative measure of bacterial adherence. It does not measure the actual number of bacterial cells adhering to the platelet-fibrin matrix.

Our next approach to examine bacterial adhesion to a platelet-fibrin matrix measured adherence of radioactively labeled cells by scintillation counting, as has been done previously (75). We determined that treatment of ^{3}H-labeled SK36 with TS-2 tissue solubilizer, both in the presence and absence of platelet-fibrin matrixes, increased the sensitivity and reproducibility of ^{3}H detection in these samples. Based on microscopic examination, we believe this is due to

dispersing chains of cells as well as lysing whole cells, leading to a more homogeneous mixture in the scintillation fluid. This finding could be important for future studies measuring ^{3}H-labeled *S. sanguinis* or other species by liquid scintillation counting. Examination of the literature revealed several studies which measured ^{3}H-labeled bacteria via liquid scintillation counting that did not report a solubilization treatment in their methods (80, 89). These studies examined oral streptococcal species as well. Although this seems unlikely, it is possible that those species may not have required solubilization for stable and efficient ^{3}H counting. In our own study, we did not examine the effect of TS-2 on detection of ^{3}H-labeled MG1363 or DH10B. These strains are gram-positive diplococci and gram-negative rods, respectively, which don't form chains and also may not have required solubilization for stable and efficient ^{3}H counting.

Upon examination of bacterial adherence to the platelet-fibrin matrixes, we were unable to find another bacterial species that adhered poorly compared to SK36. It is unclear why DH10B and MG1363 adherence was equal to or greater than that of SK36. Perhaps, not all of the measured CPM were the result of adherent ^{3}H-labeled bacteria. It is possible that some cells could have lysed when incubated with the matrixes, allowing free [^{3}H]thymidine or [^{3}H]thymidine-containing nucleic acid to adhere to the matrix. Such a situation could have provided a false positive result. Another possibility is that the matrix surface is "sticky" and it may not have been possible to wash away our tested negative control species without also washing away the matrix. Finally, it is unlikely that our matrixes consisted of purely platelets and fibrin. Although they were washed, the matrixes were formed in the presence of platelet rich plasma, and their surfaces were likely covered in many plasma proteins that could potentially have been contributing to the proposed "stickiness."

SK36 adherence to the matrixes varied considerably between experiments. Some of this variation could potentially be due to variability in matrix formation between experiments caused by different platelet counts in blood donors, as the platelet concentration of the PRP was not determined. It was previously suggested that platelet donor-specific polymorphisms may affect the potential for platelet aggregating strains of *S. sanguinis* to act as thrombogenic agents (62). Perhaps platelet donor-specific differences affected matrix formation and or bacterial adherence in our experiments. However, this would not explain the intra-experimental variation in adherence exhibited by our negative control strains, which were compared side-by-side with matrixes formed by PRP from the same donor. Alternatively, variation in SK36 adherence could have been due to bacterial clumping on the matrix surface. It has previously been observed in our lab that SK36 is capable of forming large clumps of cells. If SK36 did form clumps prior to or during incubation with the matrixes, it is possible that this could have contributed to varying adherence. Sonication of cells prior to incubation with the matrixes may be one way to deter clump formation.

The *S. sanguinis*-platelet adhesion assay was intended to employ equal numbers of cells adherent to the plate surface for each strain tested. By observing that not all *S. sanguinis* Cwa mutants adhere equally well to a polystyrene surface, our study has elucidated a potential problem with this previously used method for measuring platelet adherence to immobilized *S. sanguinis* (56). Because we were unable to obtain equal adherence of the strains to the matrixes, we attempted to correct for these differences when comparing platelet adherence. The correction may not have been entirely accurate, because it is possible that the relationship between cell number and platelet binding might not be linear. To illustrate this point, we do not know, for

example, whether a surface completely covered with cells will bind twice the number of platelets bound by a surface that is only half covered with cells.

Nevertheless, using this approach, we saw no significant difference in platelet adherence among strains. This appeared to conflict with results previously published by Plummer et al. who reported that VT1614 adhered very poorly to platelets in the same platelet adhesion assay we used (56). However, before accounting for differences in bacterial adhesion to the plate, our initial observation that platelets adhered significantly less well to VT1614 than SK36 was consistent with the previous finding by Plummer et al. (56). The most obvious explanation for this is that the previously mentioned study did not account for reduced VT1614 adherence to the plate surface, since this issue was not reported (56). If this was the case, the observed difference in platelet adherence between VT1614 and SK36 may be partially or entirely due to fewer VT1614 cells available on the plate surface to bind platelets.

Our results show that none of the Cwa proteins missing in our tested mutants are individually required for platelet adhesion. This suggests that either the mutated Cwa proteins are not required for *S. sanguinis*-platelet adhesion or that functional redundancy masks the individual contributions of the Cwa proteins, as was previously suggested (67). In this case, an example of possible functional redundancy would be multiple Cwa proteins conferring the same platelet adhesion phenotype upon *S. sanguinis*. If a single one of the proteins is missing, others are still there to perform its function.

If functional redundancy is occurring, one way to possibly reveal it would be to perform an experiment measuring platelet adhesion to a Cwa mutant deficient in multiple Cwa proteins. Another member of our lab has created several such mutants, the most notable being a *srtA* mutant (ref). The *srtA* gene in *S. sanguinis* encodes a putative cell membrane-bound enzyme

called sortase A (SrtA), which is the sortase responsible for anchoring the majority of Cwa proteins to the peptidoglycan layer of the bacterial cell wall via a trans-peptidation reaction (90-92). If screening of the *srtA* mutant in a platelet adherence assay resulted in decreased platelet adhesion compared to the individual Cwa protein mutants, it would support the functional redundancy hypothesis. However, a different assay from ours would be required, given the problems with Cwa protein mutants adhering to a polystyrene surface.

An alternative approach in which platelet-bacterial adhesion occurs in suspension, rather than on a surface has been previously reported (59), although this particular assay provides only a qualitative measure of adherence. As another option, qualitative analyses of staphylococcal and streptococcal-platelet adhesion using flow cytometry have been reported (61, 93). Approaches to measure bacterial-platelet binding such as these would circumvent the issue of mutant strains adhering less well to a polystyrene surface. Yet another approach would be to employ heterologous expression of Cwa proteins in MG1363, described previously (43, 50, 75), to screen for increased platelet adherence vs. the wild type.

In our study, we examined mutants of 12 Cwa proteins with putative adhesion capability to different molecules (Table 2). Three of these Cwa proteins, SrpA, CbpA, and CrpA have previously been reported to interact with platelets, which made them obvious choices for further investigation in this study (56, 62). While the other nine Cwa proteins had not been reported to interact with platelets, what we did know about them (Table 2) suggested they might be relevant to platelet binding.

The Cwa protein missing from Cwa mutant 25 shares homology with the vWF type-A domain, which has been reported to bind the platelet receptor glycoprotein Ib (GPIb) (94) and collagen (95). The predicted homolog for the missing protein in Cwa mutant 15 is the vWF

binding protein, vWf1, of *Streptococcus lugdunensis* (83). If this missing Cwa protein is, in fact, capable of binding vWF, perhaps a vWF multimer could act as a molecular bridge between the Cwa protein and the platelet receptor GPIb, which is a proposed model for the interaction of *S. aureus* protein A with platelets (34). Platelet granules contain many secretion products, including vWF, which are released upon activation. Although care was taken in our preparation of platelets for use in assay to avoid activation, and subsequent clumping, it is likely some did activate. Therefore, vWF could have been present to contribute to a potential bacteria-platelet interaction.

An ortholog of the mutated SspC protein in Cwa mutant 13 promotes adherence to both fibronectin and the plasma protein fibrinogen (81). If our homologous Cwa protein can bind these host proteins, they may be able to act as a bridge between the Cwa protein and a platelet receptor, similar to the platelet interactions of the *S. aureus* proteins, clumping factor A (ClfA) and fibronectin binding protein A (FnbA) (34, 96). Although our gel filtered platelet technique (described in Materials and Methods) attempted to isolate platelets from plasma proteins, it probably did not eliminate all of these proteins. Furthermore the surface of the filtered platelets may have been covered in plasma proteins. Therefore, it is possible that fibrinogen may have been present to participate in such a bridging mechanism.

The mutated Cwa proteins in mutants 13 and 9 are SspC and SspD, respectively. These proteins share sequence identity with the well characterized antigen I/II proteins of *S. gordonii*, SspA and SspB, which have been shown to promote binding to collagen (80) and glycoproteins in the oral cavity (79). Furthermore, the Cwa proteins missing in Cwa mutants 30 and 3 may also interact with collagen, as they share homology with a conserved collagen-binding domain. Perhaps these putative collagen adhesins can interact with platelets by mechanisms similar to that of platelet-collagen interactions (36, 37).

Finally, the mutated genes in Cwa mutants 6 through 8 and the SSA_1635 mutant are thought to encode proteins involved in the formation of a bacterial surface pilus. A pilus in another streptococcal species is an important adhesin. Expression of the previously characterized gram-positive surface pilus in *Streptococcus pneumoniae* was associated with colonization of A549 lung epithelial cells *in vitro* and a strong inflammatory response *in vivo*, suggesting the pilus contributes to adherence and virulence (85). Perhaps a *S. sanguinis* surface pilus would contribute to platelet adhesion.

The previously mentioned study by Turner et al. (67) was conducted by other members of our lab at the same time our study was being conducted. Turner et al. evaluated the role of *S. sanguinis* Cwa proteins in the development of IE. Virulence screening of thirty-three predicted cell wall-anchored protein mutants, including the 12 screened in our study, and three putative sortase mutants was performed in a rabbit model of IE. It was found that no single Cwa protein was essential for the development of early IE and that individual sortases of *S. sanguinis* play a minimal role in competitive colonization at the onset of IE (67). These very recent findings relate to our current study because they suggest that even if Cwa proteins were found to contribute to platelet binding by *S. sanguinis*, strategies designed to interfere with this interaction would not be effective for disease prevention. While we had previously been hoping that future prophylaxis strategies might interfere with *S. sanguinis* IE by blocking Cwa protein-mediated adhesion to platelets, it appears this isn't a viable option. However, opsonization of bacteria by host protein-Cwa protein interactions may still be a promising strategy.

While we were unable to show that the Cwa proteins missing in our tested mutants contribute to *S. sanguinis*-platelet adhesion, our study has elucidated a potential problem with a previously used method for measuring platelet adherence to immobilized *S. sanguinis*. If future

studies cannot find a way to ensure equal adherence of tested strains to a polystyrene surface, a different adhesion assay should be used. Also, our study has shown that treatment of ^{3}H-labeled *S. sanguinis* with a chemical solubilizer is important to the efficiency and stability of tritium detection by liquid scintillation counting. With respect to *S. sanguinis*-platelet adhesion, further study is required to determine the mechanism(s) by which *S. sanguinis* adheres to platelets.

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Vita

Brian Christopher Mahoney was born on June 18, 1983, in Newport, Rhode Island, and is an American citizen. He graduated from Catholic High School, in Virginia Beach, Virginia, in 2001. He received his Bachelor of Science in Biology from George Mason University, Fairfax, Virginia, in 2006 and subsequently began pursuing a career in dentistry. Toward that end, he completed more than 375 hours of volunteer community service helping to provide much-needed dental care to underserved Virginians. In April 2007, he was honored with the Dental Programs Volunteer of the Year award by the American Red Cross of Southeastern Virginia. He decided to further his science education by matriculating as a graduate student at Virginia Commonwealth University, Richmond, Virginia, in August 2007. During his studies, he earned two awards for graduate achievement: the Poland Award and the C. C. Clayton Award in May 2008 and 2009, respectively. In March 2009, he was accepted into the VCU School of Dentistry's D.5 Program and, upon successful completion of his D.5 dental coursework, is expected to receive acceptance into the graduating dental class of 2014.