Platelets Enhance Biofilm Formation and Resistance of Endocarditis-Inducing Streptococci on the Injured Heart Valve

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Infective endocarditis is a typical biofilm-associated infectious disease frequently caused by commensal streptococci, but the contribution of host factors in biofilm formation is unclear. We found that platelets are essential for in vitro biofilm formation by Streptococcus mutans or Streptococcus gordonii grown in human plasma. The biofilms were composed of bacterial flocs embedded with platelet aggregates in layers, and a similar architecture was also detected in situ on the injured valves of a rat model of experimental endocarditis. Similar to planktonic cells, the streptococci in biofilms were also able to induce platelet aggregation, which facilitates multilayer biofilm formation. Entrapping of platelets directly enhances the resistance of streptococcal biofilms to clindamycin. Prophylactic antibiotics or aspirin can reduce but not prevent or abolish biofilm formation on injured heart valves. Therefore, the platelet is a host factor for commensal streptococci in the circulation to consolidate biofilm formation and protect bacteria against antibiotics.

Infective endocarditis is a typical biofilm-associated infectious disease frequently caused by commensal streptococci, but the contribution of host factors in biofilm formation is unclear. We found that platelets are essential for in vitro biofilm formation by Streptococcus mutans or Streptococcus gordonii grown in human plasma. The biofilms were composed of bacterial flocs embedded with platelet aggregates in layers, and a similar architecture was also detected in situ on the injured valves of a rat model of experimental endocarditis. Similar to planktonic cells, the streptococci in biofilms were also able to induce platelet aggregation, which facilitates multilayer biofilm formation. Entrapping of platelets directly enhances the resistance of streptococcal biofilms to clindamycin. Prophylactic antibiotics or aspirin can reduce but not prevent or abolish biofilm formation on injured heart valves. Therefore, the platelet is a host factor for commensal streptococci in the circulation to consolidate biofilm formation and protect bacteria against antibiotics.

Commensal or pathogenic bacteria can colonize and accumulate on the interface to form biofilms, which are characterized by polymicrobial aggregates in the form of mats or flocs and are typically encased in an extracellular matrix that is mostly produced by the organisms themselves [1, 2]. The most severe clinical problems caused by bacterial biofilms are the persistence of infection and the resistance to conventional antibiotic therapy [3, 4]. It is estimated that 65% of current infections in the developed world are the result of drug-resistant biofilms [5]. A typical example of biofilm-associated disease is found in infective endocarditis (IE), which is induced most frequently by staphylococci or streptococci [1, 6].

The pathogenesis of IE is mainly attributed to the formation of septic vegetations, which are fibrin-platelet complexes embedded with bacteria on heart valves. Bacteria are found in complex with the platelets, as observed by electron microscopic analysis [7]. The persistent nature of biofilms can also induce inflammation and contribute directly to chronic bacteremia and thromboembolic events, which are serious complications associated with IE [8, 9]. A recent population-based cohort study indicated that viridans streptococci continue to outnumber Staphylococcus aureus as the most common causative organism of IE in the general population [10, 11]. The most common streptococci isolated from patients with IE include Streptococcus sanguinis, Streptococcus gordonii, Streptococcus bovis, Streptococcus mutans, and Streptococcus mitis [12, 13]. Interestingly, S. aureus or different species of oral streptococci can interact or induce platelet aggregation, and such activity is hypothesized to be directly linked to the proliferation of the thrombotic vegetation [14–16]. However, the dynamic or sequential events of bacteria-platelet interactions that occur in the bacterial colonization and biofilm formation on heart valves are unclear.
In contrast to *S. aureus*, which is considered to be a potential pathogen that harbors various virulent properties and toxins [17], viridans streptococci are oral commensals that are well tolerated by the host and can evade host immune surveillance efficiently [18]. We reported recently that host factors, such as calcium ions in plasma, are sensed by *S. mutans* in the bloodstream to enhance fibronectin binding activity and to escape phagocytosis [18]. In addition, it has been shown that a cell wall component in soluble form is as efficient as the entire *S. mutans* cell in inducing human platelet aggregation [19]. These results suggest that *S. mutans* and other commensal streptococci can adapt quickly through genetic regulation when entering the bloodstream and adjust their ability to interact with or hijack surrounding host factors to promote survival and protect against hazards. One strategy for bacteria to survive in the circulation is to form a biofilm at the damaged heart valve. In this study, we demonstrated that platelets contribute directly to oral streptococci colonization and biofilm formation in the experimental IE rat model. In addition, the biofilm formation was refractory to antibiotic prophylaxis in vivo.

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmid**

*Streptococcus mutans* GS5 and *S. gordonii* DL1 wild-type strains were grown and maintained in brain-heart infusion (BHI) broth (Difco Laboratories). To generate green fluorescent protein (GFP)–tagged bacteria, these strains were transformed with the GFPuv sequence containing the shuttle plasmid pPDGFPUv, which was described in our previous study [18].

**Experimental Streptococcal Endocarditis Rat Model**

Approval for animal use was obtained from the National Taiwan University Institutional Animal Care and Use Committee prior to initiation of experiments. A modified rat model of experimental streptococcal endocarditis was performed as previously described [20]. Detailed procedures are available in the Supplementary Data. For each experiment, 6 rats were tested, and each rat was considered an independent experiment using independent bacterial cultures. For evaluating the prophylaxis effect of penicillin, 600 000 units/kg were injected into the rats 30 minutes before the inoculation of the bacteria at 1 × 10⁶ colony-forming units (CFU). To analyze the effect of aspirin, 25 mg/kg of aspirin (Sigma-Aldrich) was injected into the rats 30 minutes before the inoculation of the bacteria, and 2 more injections were performed at 30 minutes and 6 hours after the infection, respectively. At 24 hours after the infection, the vegetation was harvested. The effect of penicillin or aspirin was evaluated by detecting the size of vegetation and the colonized bacteria density in the vegetation [20]. For the confocal laser scanning microscopic (CLSM) analysis, the vegetation harvested from the rat was fixed on the glass slide and stained with a hamster antirat CD42d antibody (1:50 dilution; eBioscience) followed by a rhodamine-labeled antihamster immunoglobulin G (IgG) antibody (1:200 dilution; Jackson ImmunoResearch Labs). The bacterial biofilm was then observed on a confocal microscope (Leica TCS SP5).

**Preparation of Human Platelets**

Human platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [19]. In brief, whole blood was collected from healthy human volunteers, and 9 vol of blood was added to 1 vol of 3.2% sodium citrate. PRP was prepared by centrifugation of whole blood at room temperature at 150g for 10 minutes. The blood that remained after removing the PRP was centrifuged at 630g for 10 minutes at room temperature to yield PPP. The washed platelet suspension (PS) was prepared according to the previously described protocol [19, 21]. The platelets were washed twice with Tyrode solution (136.8 mM sodium chloride, 2.8 mM potassium chloride, 11.9 mM sodium bicarbonate, 1.1 mM magnesium chloride, 0.33 mM sodium phosphate, 1.0 mM calcium chloride, 11.2 mM glucose, and 3.5 mg bovine serum albumin per milliliter) and finally resuspended in Tyrode solution at a concentration of 3 × 10⁹ to 5 × 10⁸ platelets/mL.

**Platelet Aggregation**

Platelet aggregation was analyzed using a turbidimetric method with a Lumi-Aggregometer (Payton Scientific) [22]. Bacteria for the test were cultured in the BHI medium overnight and collected by centrifugation. After washing 3 times with phosphate-buffered saline (PBS), the bacterial suspension was adjusted to 10¹⁰ CFU/mL. PPP was used to adjust the baseline of the minimal light transmission. PRP was prewarmed for 3 minutes prior to the addition of bacteria, and all of the procedures were carried out at 37°C with shaking at 900 rpm. The extent of platelet aggregation was continually monitored and expressed as an increase in light transmission after the addition of approximate 10⁸ CFU of bacteria. To analyze the inhibition effect of the platelet activation inhibitors, aspirin (500 µg/mL; Sigma-Aldrich) was added 5 minutes before the addition of the bacteria.

**Biofilm Formation Assay**

For quantification, 96-well polystyrene microtiter plates were used for the growth of biofilms. The bacterial biofilm growth was initiated by inoculating individual wells of a 96-well microtiter plate with approximately 10⁶ CFU of bacteria in 200 µL culture medium, including PPP, PRP, Roswell Park Memorial Institute medium, or BHI broth supplemented with 1% (w/v) glucose. If the culture medium contained platelets (PRP or PS), the platelets were adjusted to a final concentration of 3 × 10⁶ to 5 × 10⁸ platelets/mL. The pure fibrinogen (Fg) was purchased...
from Sigma-Aldrich, and the human IgG was prepared using the Montage Antibody Purification Kit (Millipore) according to the manufacturer’s instructions. After a 16-hour incubation at 37°C, the liquid medium was removed and the wells were rinsed gently 3 times with sterile distilled water. The plates were air dried, stained with 0.1% crystal violet for 10 minutes, rinsed with distilled water 3 times, and then air dried for 10 minutes. Each assay was performed in triplicate, and the wells without biofilms were used as blank controls after crystal violet staining. The biofilms were quantified by measuring the absorbance of stained biofilms at 550 nm using a MicroELISA reader (Dynatech Corp).

For observations by confocal microscopy, the bacterial biofilms were cultured in a 24-well plate with a round glass coverslip in individual wells. After the 16-hour incubation, the biofilm that had formed on the glass coverslip was gently washed with PBS 3 times and then fixed with 2% paraformadehyde for 15 minutes. After the fixation, the biofilm was incubated in 0.5% Triton X-100 for 15 minutes and then stained with rhodamine-conjugated phalloidin (1:200 dilution; Invitrogen). After washing with PBS 3 times, the coverslips were transferred to a slide and observed with a confocal microscope (Leica TCS SP5).

Antibiotics Susceptibility Testing Methods

Minimal bactericidal concentration for planktonic growing bacteria or biofilm populations were determined with a microdilution assay according to the Clinical and Laboratory Standards Institute guidelines with some modification [23–25]. The tested antibiotics were prepared at final concentrations (serial 2-fold dilutions) of 1024–0.03 µg/mL. The lowest concentration showing no visible growth on agar subculture was taken as the minimal bactericidal concentration value. All experiments were performed as 3 independent assays in triplicate and summarized in Table 1. More detailed procedures are available in the Supplementary Data.

### RESULTS

**Platelets Are Involved in Biofilm Formation In Situ**

Two common endocarditis-inducing viridans streptococci, *S. mutans* GS5 and *S. gordonii* DL1, were selected for this study; they have similar ID95 values for inducing experimental endocarditis (approximately 10^6 to 10^7 CFU) in a well-established rat model [20, 26]. To monitor the colonization and biofilm formation in situ by these streptococci on the injured cardiac valves, GFP-tagged *S. mutans* GS5 or *S. gordonii* DL1 was constructed for intravenous infection [18]. By using CLSM analysis, the biofilm architecture in experimental endocarditis was demonstrated for the first time for both *S. mutans* GS5 and *S. gordonii* DL1 (Figure 1A and 1B). The vertically projected images of CLSM showed that *S. mutans* GS5 or *S. gordonii* DL1 formed a multilayered architecture of biofilms on the injured valve (Figure 1A and 1B, bottom panels). Detection of platelets by staining with a CD42d-specific antibody suggested that the biofilms were composed of bacterial flocs embedded with aggregates of platelets (Figure 1C and 1D). Therefore, both *S. mutans* and *S. gordonii* can colonize and form multilayer biofilms containing platelet aggregates on the injured heart valves.

**Platelets Are Required for Biofilm Formation in Plasma In Vitro**

To test the hypothesis that platelets are involved in biofilm formation when streptococci are grown in the presence of plasma, an in vitro assay was established by monitoring biofilm formation in PRP or PPP. Our previous results indicated that either *S. mutans* GS5 or *S. gordonii* DL1 could grow efficiently in PPP or PRP due to the presence of glucose and other metabolites in plasma [18]. GFP-tagged *S. mutans* GS5 readily formed biofilms when grown in the glucose-containing nutrient medium as previously reported, but in contrast, *S. gordonii* DL1 could not (Figure 2A) [27]. Interestingly, *S. mutans* GS5 and *S. gordonii* DL1 both acquired the ability to form biofilms in the presence of platelets when grown in PRP,

### Table 1. Antibiotic Susceptibility of *Streptococcus mutans* GS5 and *Streptococcus gordonii* DL1 as a Planktonic or a Biofilm Population

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>BHI</th>
<th>BHI + 1% PS Extract</th>
<th>BHI</th>
<th>PRP</th>
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<tr>
<td></td>
<td>MBC-P (µg/mL)</td>
<td>MBC-P (µg/mL)</td>
<td>MBC-B (µg/mL)</td>
<td>MBC-B (µg/mL)</td>
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<tr>
<td><em>S. mutans</em></td>
<td></td>
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</tr>
<tr>
<td>GS5</td>
<td>0.03</td>
<td>0.03</td>
<td>8</td>
<td>8</td>
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<tr>
<td><em>S. gordonii</em></td>
<td></td>
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<td>8</td>
<td>8</td>
</tr>
<tr>
<td>DL1</td>
<td>0.03</td>
<td>0.03</td>
<td>8</td>
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</table>

Abbreviations: BHI, brain-heart infusion; MBC-B, minimal bactericidal concentration of biofilm-grown cells; MBC-P, minimal bactericidal concentration of planktonically grown cells; PRP, platelet-rich plasma; PS, platelet suspension.

a Values are based on results from at least 3 separate experiments.

b *Streptococcus gordonii* DL1 could not form the biofilm in the 1% glucose-containing BHI medium.
but not in PPP, suggesting that platelets are required for bacterial biofilm formation when grown in plasma (Figure 2A–D). The essential role of platelets in biofilm formation by GFP-tagged \textit{S. mutans} GS5 was further confirmed by the restoration of biofilm formation when bacteria were grown in PPP replenished with platelets (Figure 2E). Together, these results indicated that commensal streptococci may acquire distinct mechanisms for biofilm formation upon encountering host-factor platelets.

**Platelet Aggregation Enhances \textit{S. mutans} and \textit{S. gordonii} Biofilm Formation**

Planktonic cells of \textit{S. mutans} GS5 or \textit{S. gordonii} DL1 readily induced platelet aggregation (Figure 3A and 3B). It is likely that bacteria-induced platelet aggregation may contribute directly to biofilm formation. \textit{Streptococcus mutans} GS5–induced platelet aggregation or biofilm formation in the presence of PRP was inhibited by aspirin (Figure 3A and 3C), and was dependent on anti–\textit{S. mutans} IgG and Fg (Figure 3E and 3F) [19]. In the absence of platelets, IgG actually inhibited \textit{S. mutans} biofilm formation (Figure 3G), suggesting the unique role of IgG-dependent platelet aggregation in \textit{S. mutans} biofilm formation. In contrast to \textit{S. mutans}, \textit{S. gordonii} DL1–induced platelet aggregation was not inhibited by aspirin (Figure 3A). Consistently, \textit{S. gordonii} biofilm formation in the presence of PRP was not inhibited by aspirin, and the surrounding or embedded platelet aggregates were still present with a similar architecture (Figure 3D). Similar results were also demonstrated.

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**Figure 1.** \textit{S. mutans} GS5 (A and C) or \textit{S. gordonii} DL1 (B and D) biofilms on the injured heart valve were observed by confocal laser scanning microscopic (CLSM) analysis. Catheterized Wistar rats were intervenously infected with green fluorescent protein–tagged \textit{S. mutans} GS5 or \textit{S. gordonii} DL1 at an inoculum of 10^9 colony-forming units, and the vegetations were then harvested. A and B, Extended focus images (upper panels) and the vertically projected images (lower panels) from CLSM images of the multilayer biofilm formation inside the vegetation (magnification ×630). C and D, Orthogonal views of z stacks of the biofilms (magnification ×630), which were composed of bacterial flocs embedded with aggregates of platelets and stained with an anti-CD42d antibody (1:50 dilution) followed by a rhodamine-conjugated secondary antibody (1:200 dilution). A yellow appearance is observed where \textit{S. mutans} and the platelets are both present (arrow).
when 4 clinical isolates were tested, 2 each of *S. mutans* and *S. gordonii*, which were isolated from patients with endocarditis (data not shown). These results suggested that platelet aggregation directly enhances the biofilm formation by IE-inducing streptococci despite the distinct mechanisms used by these bacteria to interact with or activate platelets.

**Platelets Sustain Streptococci Adherence for Biofilm Formation**

In this experimental endocarditis model, the platelet-fibrin clot formation is detectable before bacteria challenge [20]. Therefore, we hypothesized that platelets may provide 2 advantages for bacteria in the circulation: first, bacteria can colonize on the preformed platelet-fibrin clots, and second, platelets allow for vegetation formation through bacteria-induced platelet aggregation. To test if the platelets may serve as an early step of streptococcal colonization and to determine whether bacteria in biofilms can induce subsequent platelet aggregation, adenosine diphosphate (ADP)-activated platelet aggregates were initially fixed on coverslips by centrifugation, and then the ability of *S. mutans* to adhere to the platelets, form biofilms, and subsequently induce platelet aggregation was monitored (Figure 4A). *Streptococcus mutans* readily adhered onto the ADP-activated platelet layer and formed a biofilm (Figure 4B, left panel). The addition of platelet aggregation inhibitors, such as aspirin, did not affect the binding or biofilm formation by *S. mutans* on the platelet-coated surface (Figure 4B, right panel). Furthermore, when PRP was added together with GFP-tagged *S. mutans* GS5, a biofilm structure consisting of layers of bacterial floes embedded with aggregates of platelets was observed, similar to that seen in situ (Figure 4C, left panel). Aspirin reduced the thickness of the biofilm but did not abolish the formation (Figure 4C and 4D). Consistent with this in vitro model, administration of aspirin reduced the thickness but could not prevent *S. mutans* biofilm formation on the injured valve (Figure 4E). Repeated experiments on all tested animals indicated that aspirin could only reduce but not abolish...
S. mutans GS5 colonization and vegetation formation on the injured valve in the experimental IE model (Figure 5A and 5B). Together, these results indicated that activated platelets serve as the niche for the adherence of IE-inducing streptococci and the formation of biofilms in vitro and in vivo. Furthermore, the adhered bacteria induced additional platelets to adhere and aggregate, which enhanced the overall biofilm formation.

Platelets Enhance the Bacterial Resistance to Antibiotics
Bacterial biofilm formation is thought to be responsible for the antibiotic resistance observed with IE treatment, but the contribution of host factors to such resistance is unknown [1, 2]. Therefore, the role of platelets in the bacterial biofilm resistance to antibiotics was analyzed. Planktonic cells of S. mutans GS5 or S. gordonii DL1 were susceptible to all tested antibiotics, whereas S. mutans exhibited increased resistance to...
penicillin, vancomycin, and gentamicin by forming a biofilm in the glucose-containing BHI medium or in PRP. Inclusion of platelets in the biofilm further enhanced the resistance of *S. mutans* to clindamycin (Table 1). A similar enhancement of antibiotic resistance was also observed in *S. gordonii* biofilm formation in PRP. The addition of penicillin or other tested antibiotics completely blocked biofilm formation in BHI medium (data not shown) but failed to inhibit *S. mutans* GS5 or *S. gordonii* DL1 biofilm formation with unaltered architectures in PRP (Figure 5C and 5D). Interestingly, results from a pilot study conducted on groups of 6 rats each revealed that prophylactic use of penicillin could not prevent *S. mutans* from forming biofilms or vegetation in situ on the injured heart valves of this experimental endocarditis model (Figure 5A, 5B and 5E).

Taken together, these results showed that platelets not only facilitate the formation of the bacterial biofilm, exhibiting high resistance to antibiotic treatment, but also directly enhance bacterial resistance to antibiotics, such as clindamycin.

**DISCUSSION**

Oral commensals such as *S. mutans* are well known for their ability to form biofilms (dental plaque) on the surfaces of teeth and cause dental caries [27]. In this study, we demonstrated that
the oral commensals *S. mutans* and *S. gordonii* could attach to the injured heart valves in a rat model of experimental endocarditis and form biofilms through interactions with platelets. Results obtained in this report suggest that platelets enhance biofilm formation of IE-inducing streptococci in situ on the injured heart valves through 2 underlying mechanisms (Figure 6). First, the fibrin-platelet clots that exist on the predamaged heart valves may serve as niches for the initial adherence of circulating bacteria or bacteria-platelet aggregates in the bloodstream. After attachment, the bacteria may sustain their growth and biofilm formation through the acquisition of nutrients from plasma, such as glucose. Subsequently, the bacteria...
in the biofilm may induce additional platelet adhesion or aggregation that surrounds the bacterial biofilm layer by layer and finally contribute to the formation of vegetation, which is characterized by platelet-fibrin clots that contain bacteria biofilms buried inside [9]. In support of this model, we found that the administration of aspirin before intravenous infection with IE-inducing streptococci could reduce the thickness of the biofilm in situ by interfering with the platelet aggregation induced by the bacteria or their components in the biofilm. Aspirin is a routine prescription administered to patients in clinics who have a high risk of platelet thrombus formation in the circulation [28]. Therefore, aspirin may theoretically prevent the formation of platelet clots at injured heart valves and thereby reduce the niches for IE-inducing bacteria to establish their infection.

Commensal oral streptococci can gain access into the blood circulation during trauma caused by dental surgery, and these microorganisms may be well adapted evolutionally to the host factors in the bloodstream, such as platelets [15, 29]. Therefore, it is not surprising to find that the underlying mechanisms for the interactions between IE-inducing streptococci and platelets are complex and species dependent. In S. gordonii, the bacterial surface proteins Hsa or GspB bind to platelets through GPIb, and SspA or SspB (antigen I/antigen II family proteins) trigger the platelets to aggregate [30]. In contrast, there are no Hsa homologues in S. mutans, but S. mutans can hijack the plasma component IgG and secrete the soluble molecule to activate platelets [19]. Our results from in vitro and in vivo assays indicate that platelet aggregation contributes to the enhancement of biofilm formation by S. mutans or S. gordonii. The most important finding of this study was that adherence or biofilm formation of either S. mutans or S. gordonii on activated platelets is unaffected by the administration of aspirin in vitro or in vivo. These results suggest that administration of aspirin in patients who already have fibrin-clot formation (aseptic vegetation) at predamaged heart valves may be ineffective for preventing bacterial adherence or colonization. The inherent or adapted ability of the commensal oral streptococci to adhere to activated platelets may also partly account for the controversial results previously reported on the preventive effect of aspirin on the clinical incidence of IE [31, 32].

Streptococcus mutans is known for its ability to synthesize glucan (encoded by gtf), a biofilm matrix component that mediates the tight attachment of S. mutans to the tooth surface and promotes cell-cell aggregation to form dental plaque accounting for dental caries [27, 33]. Nevertheless, the Gtf-resistant deficient mutant strains are still able to cause the vegetation formation in the streptococcal IE model, suggesting a distinct matrix composition in the S. mutans biofilm on the heart valve [20]. The IE pathogens, such as S. mutans and S. gordonii, have been shown to bind plasma components, such as Fg or fibronectin [18, 34]. Therefore, the involvement of other host factors, including extracellular proteins, in bacterial biofilm formation on the injured valve cannot be excluded.

Interestingly, our results indicate that host factors, such as platelets, may contribute directly to the antibiotic resistance of these IE-inducing streptococci. Specifically, platelet entrapment in the bacterial biofilm may enhance the resistance of IE-inducing bacteria to clindamycin. Clindamycin is a frequent choice for the treatment of bacterial endocarditis in patients who are allergic to penicillin [35]. The inducible clindamycin resistance in S. aureus is due to translational control of the erm methylase [36]. However, no erm-homologous genes were found in S. mutans or S. gordonii [37, 38]. One possibility is that the platelet contents that are released could stimulate bacteria to alter their metabolism and reduce their sensitivity to antibiotics such as clindamycin (Table 1). In addition, the presence of antibiotics, such as penicillin, did not prevent bacterial biofilm formation in vitro in the presence of plasma components and platelets. Moreover, prophylaxis with penicillin or other antibiotics failed to prevent the colonization or biofilm formation in situ when tested in our rat model of endocarditis with predamaged valves. One explanation for the lack of efficacy of penicillin in controlling biofilm formation may be that the platelet itself can absorb penicillin and reduce...
its bactericidal activity [39]. Therefore, in addition to the inherent nature of endocarditis-inducing viridans streptococci, host factors like platelet are equally or more important for these bacteria to form biofilm and to resist antibiotics in vivo.

**Supplementary Data**

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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