Reduced Vancomycin Susceptibility in an In Vitro Catheter-Related Biofilm Model Correlates with Poor Therapeutic Outcomes in Experimental Endocarditis Due to Methicillin-Resistant Staphylococcus aureus

Wessam Abdelhady, Arnold S. Bayer, Kati Seidl, Cynthia C. Nast, Megan R. Kiedrowski, Alexander R. Horswill, Michael R. Yeaman, Yan Q. Xiong
Los Angeles Biomedical Research Institute at Harbor—UCLA Medical Center, Torrance, California, USA; David Geffen School of Medicine at UCLA, Los Angeles, California, USA; University Hospital Zurich, University of Zurich, Zurich, Switzerland; Cedars-Sinai Medical Center, Los Angeles, California, USA; Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

Staphylococcus aureus is the most common cause of endovascular infections, including catheter sepsis and infective endocarditis (IE). Vancomycin (VAN) is the primary choice for treatment of methicillin-resistant S. aureus (MRSA) infections. However, high rates of VAN treatment failure in MRSA infections caused by VAN-susceptible strains have been increasingly reported. Biofilm-associated MRSA infections are especially prone to clinical antibiotic failure. The present studies examined potential relationships between MRSA susceptibility to VAN in biofilms in vitro and nonsusceptibility to VAN in endovascular infection in vivo. Using 10 “VAN-susceptible” MRSA bloodstream isolates previously investigated for VAN responsiveness in experimental IE, we studied the mechanism(s) of such in vivo VAN resistance, including: (i) VAN binding to MRSA organisms; (ii) the impact of VAN on biofilm formation and biofilm composition; (iii) VAN efficacy in an in vitro catheter-related biofilm model; (iv) effects on cell wall thickness. As a group, the five strains previously categorized as VAN nonresponders (non-Rsp) in the experimental IE model differed from the five responders (Rsp) in terms of lower VAN binding, increased biofilm formation, higher survival in the presence of VAN within biofilms in the presence or absence of catheters, and greater biofilm reduction upon proteinase K treatment. Interestingly, sub-MICs of VAN significantly promoted biofilm formation only in the non-Rsp isolates. Cell wall thickness was similar among all MRSA strains. These results suggest that sublethal VAN levels that induce biofilm formation and reduce efficacy of VAN in the in vitro catheter-associated biofilms may contribute to suboptimal treatment outcomes for endovascular infections caused by “VAN-susceptible” MRSA strains.

Staphylococcus aureus is the leading cause of biofilm-associated infections, such as intravascular catheter-related sepsis and infective endocarditis (IE), which are associated with unacceptably high morbidity, mortality, and costs (1, 2). Two key factors have been linked with suboptimal outcomes in treating such invasive S. aureus infections: (i) the organism’s abilities to develop resistance to multiple antibiotics (e.g., meticillin-resistant [MRSA], vancomycin [VAN]-intermediate S. aureus [VISA], and VAN-resistant S. aureus [VRSA]) (3), and (ii) its ability to form biofilms on both native tissues and implanted biomaterials (2). It is well known that S. aureus cells within a complex biofilm matrix are refractory to both systemic antimicrobial agents and host immune responses (4–6).

VAN is the current standard treatment for invasive MRSA infections. However, the relatively high failure rate of VAN treatment against such syndromes is alarming (7–10), especially with infections caused by MRSA strains whose MICs fall within the susceptible range (MIC, ≤2 μg/ml) (11, 12). While the exact mechanisms for such reduced treatment successes remain unclear, reduced VAN accessibility to S. aureus cells within biofilms (13) and VAN induction of biofilm formation (14) are intriguing hypotheses.

In a recently published investigation, we evaluated the efficacy of VAN against 10 MRSA clinical bloodstream isolates in a catheter-induced IE model in rabbits (15). All 10 MRSA strains were VAN susceptible (MICs of 0.5 to 1.0 μg/ml) and nontolerant to this agent in vitro. However, VAN treatment outcomes in vivo for these strains were dramatically different (15). Thus, despite equivalent in vivo virulence profiles, five of these strains were highly susceptible to VAN therapy in this model (responders [Rsp]), while the remaining five isolates were substantially resistant to VAN therapy (nonresponders [non-Rsp]) (15). Thus, the current study was designed to evaluate the mechanism(s) that may contribute to these disparate VAN treatment outcomes previously observed in experimental IE, and we focused on biofilm-associated phenotypes and events.

MATERIALS AND METHODS

Bacterial strains. The 10 MRSA bloodstream study isolates are listed in Table 1; these strains have been described in detail previously (15–17). All 10 strains had VAN MICs within the susceptible range (0.5 to 1.0 μg/ml). The five Rsp strains were defined as isolates in which VAN treatment caused a ≥3-log10 CFU mean reduction per gram of vegetation and ≥3-log10 CFU mean reduction per gram of kidney or spleen in a rabbit IE model differing from the five responders (Rsp) in terms of lower VAN binding, increased biofilm formation, higher survival in the presence of VAN within biofilms in the presence or absence of catheters, and greater biofilm reduction upon proteinase K treatment. Interestingly, sub-MICs of VAN significantly promoted biofilm formation only in the non-Rsp isolates. Cell wall thickness was similar among all MRSA strains. These results suggest that sublethal VAN levels that induce biofilm formation and reduce efficacy of VAN in the in vitro catheter-associated biofilms may contribute to suboptimal treatment outcomes for endovascular infections caused by “VAN-susceptible” MRSA strains.

Received 10 October 2012 Returned for modification 21 November 2012 Accepted 1 January 2013 Published ahead of print 7 January 2013

Address correspondence to Yan Q. Xiong, yxiong@ucla.edu
Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.02073-12

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.02073-12

Downloaded from http://aac.asm.org/ on April 19, 2021 by guest
TABLE 1 Clinical methicillin-resistant Staphylococcus aureus strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>VAN MIC (µg/ml)</th>
<th>VAN response in IE model</th>
</tr>
</thead>
<tbody>
<tr>
<td>300-087</td>
<td>agel, SCCmeC IV, spa-NEW5, CC45</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>300-169</td>
<td>agel, SCCmeC IV, spa-NEW5, CC45</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>300-134</td>
<td>agel, SCCmeC IV, spa-NEW5, CC45</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>300-303</td>
<td>agel, SCCmeC IV, spa-NEW5, CC45</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>300-626</td>
<td>agel, SCCmeC I, spa-385, CC5</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>301-188</td>
<td>agel, SCCmeC IV, spa-NEW1, CC45</td>
<td>0.5</td>
<td>Nonresponder</td>
</tr>
<tr>
<td>010-016</td>
<td>agelII, SCCmeC II, spa-2, CC5</td>
<td>1.0</td>
<td>Responder</td>
</tr>
<tr>
<td>027-107</td>
<td>agelII, SCCmeC II, spa-2, CC5</td>
<td>1.0</td>
<td>Responder</td>
</tr>
<tr>
<td>088-180</td>
<td>agelII, SCCmeC II, spa-2, CC5</td>
<td>0.5</td>
<td>Responder</td>
</tr>
<tr>
<td>088-237</td>
<td>agelII, SCCmeC II, spa-2, CC5</td>
<td>0.5</td>
<td>Responder</td>
</tr>
</tbody>
</table>

a CC, clonal complex.

b Vancomycin response in an experimental endocarditis (IE) model, as previously described (15). See Materials and Methods for further descriptions.

---

model (15, 17). The five non-Rsp strains were defined as isolates in which <1.5-log, CFU mean reductions per gram of vegetation, kidney, and spleen were observed due to VAN treatment in the IE model (15, 17). To ensure that the phenotypic properties of these 10 strains were maintained upon long-term storage at -80°C, we restested a prototypic phenotype (delta-hemolysin production) (data not shown); this phenotype remained identical to those we previously described (15).

**Population analyses.** VAN population analyses of two S. aureus control strains and the 10 MRSA study strains were performed using standard protocols (18, 19). S. aureus strains ATCC 25923 (non-VISA) and MU50 (known VISA) (20) were used as negative- and positive-control isolates, respectively, for this assay. Briefly, an overnight culture of S. aureus cells was washed, adjusted to an optical density at 600 nm (OD600) of 1.000 (-10<sup>12</sup> CFU/ml), and diluted in saline to 10<sup>-6</sup> CFU/ml. Fifteen-microliter aliquots of the bacterial suspensions (no dilution to 10<sup>-6</sup>) were placed on Mueller-Hinton broth agar (MHBA) plates containing VAN at concentrations ranging from 0.125 to 16 µg/ml to encompass sublethal to lethal drug levels. Colony counts were performed after 48 h of incubation at 37°C, and the viable counts were plotted against VAN concentrations to create population analysis profiles (PAPs).

**In vitro time-kill curve for VAN.** To mimic human treatment scenarios in which high trough serum drug concentrations are targeted for severe MRSA infections, VAN at 15 µg/ml was used in time-kill analyses in cation-adjusted MHB. In these assays, a starting inoculum of 10<sup>6</sup> CFU/ml early-exponential-phase cells was used to mirror target tissue counts in IE (15); surviving bacterial colony counts were determined at 2, 4, 6, and 24 h of incubation at 37°C. Results are expressed as the Δlog<sub>10</sub> CFU/ml (± the standard deviation [SD]) compared to the initial inoculum.

**VAN binding to MRSA.** VAN binding to MRSA strains was measured using a modified boron dipyrromethene dihydrochloride-labeled VAN strategy (BodipyFL VAN; Invitrogen Corp., Carlsbad, CA) (21). The maximum excitation and emission spectra of BodipyFL VAN are 504 nm and 511 nm, respectively. Briefly, overnight-cultured MRSA cells (10<sup>6</sup> CFU/ml) were exposed to BodipyFL VAN at 15 µg/ml for 30 min at 37°C in cation-adjusted MHB. The binding of VAN was assayed by quantitative flow cytometry (FACScalibur; Becton, Dickinson [BD]) (22, 23). For each sample, 10,000 cells were acquired and analyzed, with data expressed as the proportion of cells exhibiting threshold levels of VAN binding (mean ± SD of fluorescent cells).

**Cell wall thickness.** Although the precise mechanisms are not clear, the degree of cell wall thickness of S. aureus is felt to play an important role in resistance to VAN killing (24). Therefore, MRSA strains were assessed for cell wall thickness by transmission electron microscopy (25, 26). For each MRSA strain, the cell wall thickness of 100 individual cells was measured at 190,000 magnification (model 100CX; JEOL, Tokyo, Japan), and data were analyzed to determine mean cell wall thickness (± SD). All cell wall measurements were performed by one of the authors (C. C. Nast), who was blinded as to the identity of the strains.

**Primary attachment assay.** The initial attachment to biologic surfaces is an important step in biofilm formation, as well as in the subsequent pathogenesis of S. aureus biofilm-associated infections. Therefore, we tested the capability of the 10 MRSA strains to attach to a polystyrene surface by using a method previously described (27, 28). Briefly, overnight S. aureus cultures were adjusted to an OD<sub>600</sub> of 0.5 (~10<sup>6</sup> CFU/ml) and diluted to 10<sup>-2</sup> CFU/ml. Aliquots (100 µl) of the suspension were spread on Primaria tissue culture polystyrene petri dishes (Falcon; catalog reference 353803; BD). After 30 min of incubation at 37°C, the petri dishes were gently rinsed three times with phosphate-buffered saline (PBS) and then covered with 15 ml of tryptic soy broth (TSB) agar. Primary attachment was expressed as the mean percentage of CFU (± SD) remaining on the petri dishes compared to the initial inoculum. Each experiment was repeated three times independently.

**Biofilm formation under static conditions.** The ability to form biofilms is believed to make microbes more resistant to antibiotics and the host defense system. In addition, some investigations have shown that sub-MIC levels of selected antibiotics can actually promote biofilm formation (29). In this regard, Hsu et al. (14) recently reported that sub-MIC levels of VAN can promote MRSA biofilm formation in vitro, a process that may have clinical significance. Thus, the degree of biofilm formation under static conditions was determined for our 10 MRSA strains in the presence or absence of 0.5× the MIC of VAN, as described previously (14, 16). Briefly, MRSA strains from fresh blood TSB agar plates were adjusted to a density of 0.5 McFarland standard and diluted 1:10 into brain heart infusion (BHI) medium supplemented with 0.5% glucose (BHIG; containing ~10<sup>7</sup> CFU/ml). Then, 200 µl of this suspension (with or without 0.5× the MIC of VAN) was transferred to flat-bottom 96-well Nunc polystyrene culture plates (Roskilde, Denmark) and incubated at 37°C for 18 h. After incubation, the plates were washed with PBS, air dried, stained with 0.1% safranin (Acros Organics), and then washed with distilled water. The adhering dye was dissolved in 30% acetic acid, and absorption was measured as the OD<sub>590</sub> to quantify biofilm formation.

**Biofilm formation under flow conditions.** To test biofilm formation under flow conditions, two randomly selected Rsp and non-Rsp strain pairs were assessed in an in vitro once-through flow cell model (30). Briefly, polycarbonate flow cells (channel dimensions, 5 by 35 by 1 mm) with acid-washed glass coverslips serving as a substratum were used. Sterile culture medium (2% BHI with 0.4% glucose) was pumped under laminar flow conditions (0.17 ml/min) through a bubble trap, a flow cell, and into a final collection device. The system was inoculated with 1 ml of overnight culture of each Rsp or non-Rsp strain diluted 1:20 in sterile water, and bacteria were allowed to attach for 1 h at 22°C before starting the flow. After 48 h, biofilms were poststained using the Syto 9 “live” and Topro-3 “dead” fluorescent stains (Molecular Probes) and then visualized by confocal microscopy using a Nikon Eclipse E600 microscope equipped with a Radiance 2100 imaging system (Bio-Rad). z-series of images were collected, and three-dimensional renderings of the confocal images were generated with Volocity software (Improvision). Average biomass and thickness of biofilms were determined by using the COMSTAT program (31).

**Biofilm composition determinations.** Biofilms formed by different bacteria have been shown to have distinctly different chemical compositions (i.e., carbohydrate, protein, or extracellular DNA is dominant) (2, 32). These compositional differences have been described to contribute functionally and structurally to the organization of the biofilm (2, 32). Therefore, relative differences in the stabilities of the biofilms formed by our MRSA study strains were determined as a function of specific biochemical components, using carbohydrate, protein, or DNA dispersal agents (16, 33). In addition, the impact of sub-MIC levels of VAN on such differences was assessed. In brief, the supernatants of 18-h-old biofilms generated by MRSA in the presence or absence of 0.5× the MIC of VAN were replaced by fresh medium supplemented with either (i) 10 mM so-
In independent experiments.

ical dispersal treatments, the biofilms were quantified as described before. Data are expressed as the Δlog_{10} CFU/ml (± SD) at 2, 4, 6, and 24 h of incubation versus the initial inoculum. Non-Rsp control, white; non-Rsp strains with VAN exposure, black; Rsp control, light gray; Rsp with VAN exposure, dark gray. * P < 0.05 between non-Rsp versus Rsp comparisons with VAN exposure.

FIG 1 (A) Population analyses of two control S. aureus strains, ATCC 25923 (○) and MU50 (■) upon exposure to a range of VAN concentrations (0 to 16 μg/ml). (B and C) VAN population analyses of 10 MRSA study strains, including nonresponders (B) and responders (C). Nonresponder strains: ○, 300-087; □, 300-169; ▲, 324-136; ×, 300-103; ○, 300-246. Responder strains: •, 301-188; □, 010-016; ▲, 077-107; ×, 088-180; ○, 088-237. These data represent the means (± SD) for two separate assays.

FIG 2 In vitro time-kill curve of VAN (15 μg/ml) versus 10 MRSA study strains at a high initial inoculum (~10^6 CFU/ml) of exponential-phase cells. The mean MRSA densities of the starting inocula in non-Rsp and Rsp groups were similar (7.87 ± 0.10 log_{10} CFU/ml and 7.84 ± 0.09 log_{10} CFU/ml, respectively). However, there was no significant correlation between the VAN susceptibility in vitro and VAN killing profiles described above and binding of VAN (data not shown).

Cell wall thickness. All 10 MRSA strains exhibited similar cell wall thickness profiles. The means (± SD) of cell wall thicknesses

susceptibility to VAN killing. Catheters covered with established biofilms after the initial overnight incubation as described above were rinsed in PBS, then transferred to fresh BHIG with or without VAN (15 μg/ml), and incubated with shaking (100 rpm) at 37°C for 3 days (chosen to mimic the therapy period in the experimental IE model) (15). At daily intervals, parallel catheter preparations were rinsed in PBS and transferred to fresh BHIG with or without VAN (15 μg/ml). At each time point, catheters from each group were recovered and were quantitatively cultured as described above.

Statistical analysis. Student’s t test was used to analyze experimental data and compare means. P values of <0.05 were considered statistically significant.

RESULTS

Population analyses profiles. VAN PAPs of the two control S. aureus and 10 MRSA strains are shown in Fig. 1. As expected, S. aureus ATCC 25923 had no subpopulations with a decreased VAN susceptibility. In contrast, the VISA MU50 had a notable rightward shift of the survival curve, with a large subpopulation that was resistant to VAN. In terms of the 10 study MRSA strains, 9/10 isolates had virtually identical PAP curves, without revealing any VAN-resistant subpopulations (Fig. 1). However, one of the non-Rsp strains, 300-103, had a small subpopulation that grew on the medium containing VAN at 2 μg/ml.

In vitro VAN time-kill curve. The mean MRSA densities of the starting inocula in non-Rsp and Rsp groups were similar (7.87 ± 0.10 log_{10} CFU/ml and 7.84 ± 0.09 log_{10} CFU/ml, respectively). Control cultures without VAN exposure increased ~2 log_{10} CFU/ml for both the non-Rsp and Rsp groups over the 24 h of incubation. As a group, the non-Rsp isolates had significantly smaller MRSA density reductions at all time points during the 24-h VAN exposures than did the Rsp strains (Fig. 2) (P < 0.05).

VAN binding. Overall, the non-Rsp strains demonstrated significantly less binding to VAN than the Rsp strains (Fig. 3) (75.6 ± 6.4% versus 82.3 ± 5.9%, respectively; P = 0.03). However, there was no significant correlation between the in vitro susceptibility in VAN killing profiles described above and binding of VAN (data not shown).

Cell wall thickness. All 10 MRSA strains exhibited similar cell wall thickness profiles. The means (± SD) of cell wall thicknesses
for the non-Rsp and Rsp groups were 24.30 ± 2.34 nm and 26.13 ± 2.83 nm, respectively.

**Primary attachment.** Primary attachment assays revealed no significant initial binding differences between the non-Rep and Rep groups to polystyrene surface (88.6 ± 12.9% and 83.6 ± 8.4% of the initial inoculum bound to the polystyrene petri dishes, respectively).

**Biofilm formation under static conditions.** The capacities of MRSA strains to form biofilms in the presence versus absence of sub-MIC levels of VAN are summarized in Fig. 4. The mean absorbance without VAN exposure for non-Rsp and Rsp strains was 1.14 ± 0.51 and 0.78 ± 0.27, respectively. However, this difference did not reach statistical significance. Interestingly, in the presence of 0.5× the MIC of VAN, four of the five non-Rsp MRSA strains exhibited significantly enhanced biofilm formation versus their respective controls (P < 0.05). In contrast, none of the Rsp strains showed significantly increased biofilm formation in the presence of sub-MIC VAN levels compared to their respective controls (Fig. 4).

**Biofilm formation under flow conditions.** To verify the static biofilm formation results, two non-Rsp and Rsp strain pairs were tested in a flow cell model system (Fig. 5). Overall, the biofilm growth of these selected pairs paralleled the static assay outcomes described above. For example, strain 300-169 formed a thick, confluent biofilm with an average biomass of 36 µm²/µm² and an average thickness of 44 µm among the strains tested (Fig. 5), supporting the high levels of biomass observed in the static assay. Similarly, both Rsp strains mirrored the static assay results, with poor biofilm capacities in the flow cell system. Strain 010-016 produced a biomass of just 0.0001 µm²/µm², and the second Rsp strain, 301-188, accumulated slightly more biomass than strain 010-016 (0.02 µm²/µm²) (Fig. 5).

**Biofilm compositions.** The percent reductions in biofilms for the 10 MRSA strains in the presence of sodium metaperiodate, proteinase K, and DNase I are shown in Fig. 6. Without exposure to sub-MIC levels of VAN, treatment with sodium metaperiodate or DNase I led to a similar reduction of biofilm mass between the non-Rsp and Rsp groups (Fig. 6A). However, the biofilms of the non-Rsp isolates were reduced by the treatment with proteinase K to a greater extent than the Rsp strains (mean reductions were 73.1 ± 17.3% versus 59.1 ± 10.4%, respectively; P < 0.05) (Fig. 6A). This result suggested that biofilm formation in the non-Rsp group was more dependent on protein content than was the Rsp group in the absence of VAN. Sub-MIC levels of VAN increased carbohydrate, protein, and DNA contents in the non-Rsp group (although these differences did not reach statistical significance), while causing no significant changes in biofilm composition in the Rsp group versus the group without VAN exposure (Fig. 6B).

**Activity of VAN within catheter-associated biofilms in vitro.** We further assessed the capacities of the 10 MRSA strains to form biofilms and the efficacy of VAN on preformed biofilms in the presence of catheters (Fig. 7). Without VAN exposure, similar MRSA density profiles were observed on catheters infected by the non-Rsp and Rsp MRSA strains, based on S. aureus counts within catheter biofilms. For instance, at 24 h of incubation, the numbers of viable MRSA recovered from catheters colonized with the non-Rsp and Rsp groups (before VAN exposure) were 7.85 ± 0.29 and 7.56 ± 0.15 log₁₀ CFU/catheter, respectively. By comparing the number of viable bacteria recovered from catheters infected with the strains over an additional 3-day experimental period without VAN exposure, a similar capacity to grow within a biofilm was also observed (Fig. 7). Taken together, these analyses indicated that the non-Rsp and Rsp strains had similar abilities to form catheter-associated biofilms (mirroring the similar intrinsic virulence in the non-Rsp and Rsp groups in the experimental IE model) (15).

In the presence of VAN, the numbers of viable MRSA cells recovered from catheters infected by all five Rsp isolates were markedly reduced during the 3 days of incubation (Fig. 7). In addition, this biofilm clearance effect of VAN in the Rsp group reached statistical significance for four of the five Rsp isolates compared to the respective untreated controls (Fig. 7) (P < 0.05). In contrast, for the non-Rsp group, VAN did not significantly decrease MRSA counts in any of the five isolates compared to their respective untreated controls over the same time period (Fig. 7).

**DISCUSSION**

VAN remains an important first-line antibiotic for the treatment of serious MRSA infections, such as endocarditis (IE). Although the majority of MRSA strains are susceptible to VAN in vitro, with MICs of ≤2 µg/ml (35), reports of clinical failures with VAN treatment are relatively frequent (36, 37). For instance, VAN treatment success rates of only 10 to 20% and 56 to 77% have been demonstrated for MRSA bacteremia caused by isolates with MICs of 1 to 2 µg/ml and ≥0.5 µg/ml, respectively (38, 39). The exact
mechanism(s) of VAN treatment failure among such “VAN-susceptible” MRSA isolates is not well defined. In this study, we investigated the relevant mechanism(s) of such “in vivo resistance” to VAN treatment in MRSA endovascular infection. Our results showed interesting parallels among (i) increased biofilm formation upon exposure to sub-MIC levels of VAN, (ii) reduced VAN activity in an in vitro catheter-associated biofilm model, and (iii) in vivo VAN treatment failure in the catheter-induced experimental IE model (15) for 10 MRSA clinical isolates that were all susceptible to VAN in vitro.

Several other notable findings emerged from this investigation. First, Rsp isolates were significantly more susceptible to VAN-induced killing than non-Rsp isolates with respect to VAN interactions with MRSA under in vitro conditions approximating key in vivo parameters, including the following: (i) utilizing VAN at 15 μg/ml to mirror targeted trough concentrations for severe MRSA infections (40); (ii) employing high initial MRSA counts (10^8 CFU/ml) to mirror bacterial densities in the major target tissues in the IE model (e.g., cardiac vegetations) (15). This difference in susceptibility was concordant with previous findings, in which a significant positive correlation with a VAN staphylocidal effect in vitro and VAN therapy success in MRSA bacteremia was demonstrated (39). In addition, we investigated several potential factors that may have contributed to these findings: (i) as a group, VAN bound significantly less to non-Rsp isolates than the Rsp strains; (ii) the non-Rsp strains displayed more extensive in vitro biofilm formation in the absence of catheters; (iii) biochemical analyses revealed significantly greater biofilm formation reduction with proteinase K treatment by non-Rsp than Rsp strains; (iv) sub-MIC VAN exposures significantly enhanced biofilm formation, and they substantially and globally increased biofilm composition only in the non-Rsp group; (v) a positive correlation was observed between VAN activity in the in vitro catheter-associated biofilm formation model and therapeutic outcomes previously observed in the experimental IE model (15).

Collectively, these in vitro differences described above between non-Rsp and Rsp strains provide substantial insights into different outcomes of VAN therapy in both clinical and experimental IE scenarios (15, 38, 39). For instance, the in vitro biofilm formation assay confirmed that the biofilm-forming capacities of the non-Rsp strains were significantly enhanced following sub-MIC VAN exposure, an outcome not observed in the Rsp group. This observation raises the notion that, in the IE model, VAN-enhanced biofilm formation may occur within cardiac vegetations, where some deeply embedded MRSA cells are likely to be exposed to sub-lethal VAN levels during the course of therapy in the IE.
model. This scenario may well contribute to VAN treatment failures in this model. However, the exact mechanisms involved in enhancing biofilm formation upon exposure to sub-MIC VAN levels in non-Rsp strains are not well known. In contrast to Staphylococcus epidermidis, few studies have examined the effects of sub-MIC antibiotics on S. aureus biofilm formation (14, 41–44). One recent study reported that sub-MIC VAN can enhance S. aureus cell lysis and extracellular DNA release and promote biofilm formation (14). We tested the effect of sub-MIC levels of VAN on autolysis, but no differences were found between the non-Rsp and Rsp groups (data not shown). In addition, Rachid et al. reported that subinhibitory concentrations of tetracycline and quinupristin-dalfopristin increased ica expression and subsequent biofilm formation in S. epidermidis (45). Taken together, it appears that biofilm formation can be triggered by certain antibiotic exposures in both coagulase-positive and coagulase-negative staphylococci, albeit by different mechanisms. Studies to further examine the phenotypic and genotypic impacts of sub-MIC VAN levels upon biofilm formation are in progress in our laboratory.

The data also demonstrated that biofilms formed by non-Rsp and Rsp isolates have different vulnerabilities to proteinase K and DNase I treatment (as quantified by the percentage of A490nm reduction versus controls). (A) Without VAN exposure; (B) 0.5× the MIC of VAN during overnight biofilm formation. White bars, non-Rsp group; black bars, Rsp group. *P < 0.05 versus non-Rsp group.

FIG 6 Biofilm stability of preformed biofilms by the 10 MRSA strains following sodium metaperiodate, proteinase K, or DNase I treatment (as quantified by the percentage of A490nm reduction versus controls). (A) Without VAN exposure; (B) 0.5× the MIC of VAN during overnight biofilm formation. White bars, non-Rsp group; black bars, Rsp group. *P < 0.05 versus non-Rsp group.

creased susceptibility of non-Rsp strains to proteinase K (Fig. 6). An alternate explanation could be that exposure to sub-MIC VAN levels may affect the expression of genes involved in biofilm production in a different manner across distinct MRSA strains.

The in vitro catheter-associated biofilm model provided a mean to assess VAN treatment in a setting akin to in vivo endovascular infections. Importantly, all 10 MRSA strains formed similar quantities of biofilm in the in vitro biofilm model, based on bacterial counts. This comparable biofilm formation activity between the two groups parallels the similar intrinsic virulence of the non-Rsp and Rsp strains in the IE model (based on similar target tissue bacterial counts) (15). However, in some strains, MRSA densities (in log10 CFU) within catheter biofilms in the absence of VAN tended not to parallel the static biofilm assay (the results for which are reported as the OD490). For example, while isolate 077-107 produced more biofilm mass than other non-Rsp isolates (Fig. 3), its bacterial density within the catheter biofilms was not higher than other non-Rsp strains (Fig. 7). Such differences between the results of the two assays may be due to difference in (i) the chemical composition of materials used in the assays (polyethylene versus polystyrene); (ii) biofilm growth conditions (shaking versus static), and/or (iii) the presence versus absence of catheters, which may alter cell attachment or biofilm accumulation profiles. Consistent with the differential VAN treatment outcomes in the IE model, VAN exposure substantially reduced bacterial counts within established catheter biofilms infected by all five Rsp isolates (P < 0.05 for four of five Rsp isolates). In contrast, there were no significant differences in MRSA clearance by VAN within catheter biofilms formed by non-Rsp isolates. The precise mechanism(s) of this suboptimal MRSA clearance by VAN within non-Rsp-induced catheter biofilms is not well understood, but it will likely include their distinct biofilm compositions, limitations of VAN biofilm penetration, the evolution of “persistor” cell populations.
within the depths of the biofilm, and/or VAN-induced enhancement of biofilm formation (13, 48, 49).

In conclusion, our results suggest that several factors, including in vitro VAN activity, VAN binding, the impact of VAN on biofilm formation, and the efficacy of VAN within catheter-associated biofilms, may contribute to differential VAN treatment outcomes in experimental IE. The results presented herein provide important observations for comparing in vitro to in vivo conditions. Given the importance of biofilm phenotypes in persistent bacteremia and endocarditis, these insights may also provide useful surrogate predictors of clinical outcomes in VAN treatment of human MRSA infections. However, additional mechanistic studies are needed to better understand the precise factors responsible for VAN treatment failures in invasive MRSA infections caused by strains exhibiting a “VAN-susceptible” phenotype in vitro.

ACKNOWLEDGMENTS
This study was funded in part by American Heart Association (Grant-In-Aid 09GRNT2180065 to Y.Q.X.) and research grants from the National Institutes of Health (R21AI097657 to Y.Q.X; RO1AI-39108 to A.S.B; Project 3 of P01 AI083211 to A.R.H; RO1AI-39001 to M.R.Y).

REFERENCES
computer program COMSTAT. Microbiology (Reading, England) 146:2395–2407.


