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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*

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Running title: Vancomycin and *in vitro* MRSA biofilm model

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Abstract

Staphylococcus aureus (SA) 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 SA (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 failures. The present studies examined potential relationships between MRSA susceptibility to VAN in biofilms *in vitro* and non-susceptibility to VAN in endovascular infection *in vivo*. Using ten VAN-susceptible MRSA bloodstream isolates previously investigated for VAN responsiveness in experimental IE, we studied mechanism(s) of such *in vivo* VAN resistance, including: **i**) VAN binding to MRSA; **ii**) the impact of VAN on biofilm formation and biofilm composition; **iii**) VAN efficacy in an *in vitro* catheter-related biofilm model; and **iv**) cell-wall thickness. As a group, the five strains previously categorized as VAN Non-Responders (Non-Rsp) in the experimental IE 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 presence or absence of catheters, and greater biofilm reduction with proteinase K treatment. Interestingly, sub-MICs of VAN significantly promoted biofilm formation only in Non-Rsp isolates. Cell wall thickness was similar among all MRSA strains. These results suggest that sub-lethal VAN levels induce biofilm formation and reduce efficacy of VAN in the *in vitro* catheter-associated biofilms may contribute to suboptimal treatment outcomes in endovascular infections caused by "VAN-susceptible" MRSA strains.

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Introduction

Staphylococcus aureus is the leading cause of biofilm-associated infections such as intravascular catheter-related sepsis and endocarditis (IE), which are associated with unacceptably high morbidity, mortality and cost (1, 2). Two key factors have been linked with suboptimal outcomes in treating such invasive *S. aureus* infections: the organism's abilities to develop resistance to multiple antibiotics (e.g., MRSA; VISA; VRSA) (3), and 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).

Vancomycin (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 $\leq$ 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 ten MRSA clinical bloodstream isolates in a catheter-induced IE model in rabbits (15). All ten MRSA strains were VAN-susceptible (MICs 0.5-1.0 µg/ml) and non-tolerant to this agent *in vitro*. However, VAN treatment outcomes versus 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 (Non-Responders [**Non-Rsp**]) (15). Thus, the current

study was designed to evaluate mechanism(s) that may contribute to these disparate VAN treatment outcomes previously observed in experimental IE, focusing on biofilm-associated phenotypes and events.

Materials and Methods

Bacterial strains. The ten MRSA bloodstream study isolates are shown in **Table 1**; these data have been described in detail previously (15-17). All ten strains had VAN MICs within the susceptible range (0.5-1.0 μg/ml). The five Rsp strains were defined as isolates in which VAN treatment caused ≥ 5 $\log_{10}$ CFU mean reduction per gram of vegetations, and ≥ 3 $\log_{10}$ CFU mean reductions per gram of kidneys and spleen in a rabbit IE model (15, 17). The five Non-Rsp strains were defined as isolates in which < 1.5 $\log_{10}$ CFU mean reduction per gram of vegetations, kidneys, and spleen due to VAN treatment in the IE model were observed (15, 17). To assure that the phenotypic properties of these 10 strains were maintained upon long-term storage at -80°C, we re-tested a prototypic phenotype (delta-hemolysin production); this phenotype remained identical to those we have previously published (15) (data not shown).

Population analyses. VAN population analyses of two *S. aureus* control strains and the ten MRSA study stains were performed by standard protocols (18, 19). *S. aureus* strains ATCC 25923 (non-VISA) and MU50 (known VISA) (20) were used as a negative and positive control isolates, respectively, for this assay. Briefly, an overnight culture of *S. aureus* cells were washed, adjusted to OD_{600nm} at 1.000 (~10^9 CFU/ml) and diluted in saline to 10^{-6}. Fifteen microliters of the bacterial suspension (from no dilution to 10^{-6}) was placed on Mueller-Hinton broth agar (MHBA) plates containing VAN, ranging from 0.125 to 16 μg/ml to encompass sublethal-to-lethal drug levels. Colonies counts were quantified after 48 h incubation at 37°C, and the viable count plotted against VAN concentrations to create population analysis profiles (PAP).

***In vitro* time-kill curve of VAN.** To mimic human treatment scenarios in which high trough serum concentrations are targeted for severe MRSA infections, VAN at 15 µg/ml was used in timed-kill analyses in cation-adjusted MHB. In these assays, a starting inoculum of 10^8 CFU/ml early exponential phase cells was used to mirror target tissue counts in IE (15); surviving bacterial colony counts were quantified at 2, 4, 6 and 24 h incubation at 37°C. Results are expressed as the $\Delta\log_{10}$ CFU/ml (± SD) as compared to the initial inoculum.

VAN binding to MRSA. VAN binding to MRSA strains was measured using a modified boron dipyrromethene difluoride-labelled VAN strategy (Bodipy®FL VAN; Invitrogen Corp., Carlsbad, CA, USA) (21). The maximum excitation and emission spectra of the Bodipy®FL VAN are 504nm and 511nm, respectively. Briefly, overnight cultured MRSA cells (10^8 CFU/ml) were exposed to Bodipy®FL 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) (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 x190,000 magnification (model 100CX; Jeol, Tokyo, Japan), and data was analyzed to determine mean cell wall thickness (± SD). All cell wall measurements were performed by one of the authors (C.C.N) 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 ten MRSA strains to attach to a polystyrene surface by using the method previously described (27, 28). Briefly, overnight *S. aureus* cultures were adjusted to an OD_{600nm} of 0.5 (~ 10^8 CFU/ml) and diluted to 10^3 CFU/ml. 100 µl of the suspension was spread on Primaria™ tissue culture polystyrene petri dishes (BD Falcon™ REF 353803). After 30 min incubation at 37°C, the petri dishes were gently rinsed three times with PBS and then covered with 15 ml of 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 host defense system. In addition, some investigations have shown that sub-MIC levels of selected antibiotics can actually prompt biofilm formation (29). In this regards, Hsu *et al* (14) have 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 ten MRSA strains in the presence or absence of 0.5x MICs 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 BHI supplemented with 0.5% glucose (BHIG; containing ~ 10^7 CFU/ml). Then, 200 µl of this suspension (with or without 0.5x MICs of VAN) were 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%

149 acetic acid and absorption was measured at OD_{490nm} to quantify biofilm formation.

150

151 **Biofilm formation under flow conditions.** To test biofilm formation under flow conditions,
152 two randomly selected Rsp and Non-Rsp strain pairs were assessed in an *in vitro* once-through
153 flow cell model (30). Briefly, polycarbonate flow cells (channel dimensions 5 x 35 x 1 mm) with
154 acid-washed glass cover slips serving as a substratum were used. Sterile culture media (2% BHI
155 with 0.4% glucose) was pumped at laminar flow (0.17 ml/min) through a bubble trap, a flow cell,
156 and into a final collection device. The system was inoculated with 1 ml overnight culture of each
157 Rsp or Non-Rsp strain diluted 1:20 in sterile water, and bacteria were allowed to attach for 1 h at
158 22° C before starting flow. After 48 h, biofilms were post-stained using the Syto 9 "live" and
159 Topro-3 "dead" fluorescent stains (Molecular Probes), then visualized by confocal microscopy
160 using a Nikon Eclipse E600 microscope equipped with a Radiance 2100 imaging system®
161 (Biorad). Z series of images were collected, and three-dimensional renderings of the confocal
162 images were generated with Volocity® software (Improvision). Average biomass and thickness
163 of biofilms was determined by the COMSTAT program (31).

164

165 **Biofilm composition.** Biofilms formed by different bacteria have been shown to have distinctly
166 different chemical compositions (i.e., carbohydrate-; protein- or extracellular DNA-dominants)
167 (2, 32). These compositional differences have been described to contribute functionally and
168 structurally to the organization of biofilm (2, 32). Therefore, relative differences in the stability
169 of the biofilms formed by our MRSA study strains were determined as a function of specific
170 biochemical components, using carbohydrate, protein, or DNA dispersal agents (16, 33). In
171 addition, the impact of sub-MIC VAN on such differences was assessed. In brief, the
172 supernatants of 18 h old biofilms generated by MRSA in the presence or absence of 0.5x MICs

VAN exposure were replaced by fresh medium supplemented with either: **i**) 10 mM sodium metaperiodate (Alfa Aesar, Ward Hill, MA) to assess relative carbohydrate content; **ii**) 100 µg/ml protease K (Acros Organics) to assess relative protein content; or **iii**) 140 U/ml RNase-free DNase I (Takara Bio Inc., Shiga, Japan) to assess relative DNA content. All incubations were for 2 h at 37°C as detailed (16, 33). Media without the above supplements served as respective negative controls. After biochemical dispersal treatments, the biofilms were quantified as described before.

***In vitro* catheter-associated biofilm model.** To simulate the *in vivo* scenario of the catheter-induced experimental IE model, an *in vitro* catheter-associated biofilm formation model was used to test the relative capacity of the MRSA strains to form biofilm and to respond to VAN exposure (34). Briefly, sterile 1-cm segments of polyethylene catheters (14-gauge, BD Insyte Autoguard, BD), the same material as was used in the experimental IE model (15), were placed in a 12-well microtiter plate containing 4 ml of BHIG with $5x10^6$ CFU/ml of MRSA cells and incubated overnight with shaking (100 rpm) at 37°C. After 24 h incubation, infected catheters (n = 3 for each group) were rinsed with PBS to remove nonadherent *S. aureus*, then placed into a sterile tube containing 5 ml of PBS, and sonicated for three 30 s cycles with a Branson Sonifier 450 (power output setting 3; Branson Ultrasonic Corporation, Danbury, Connecticut, USA). After sonication, samples were quantitatively cultured, and the mean (± SD) of adherent bacteria then calculated from three independent experiments.

The same *in vitro* catheter-associated biofilm model was used to assess susceptibility to VAN killing. Catheters covered with established biofilms after the initial overnight incubation as described above were rinsed in PBS, and then transferred to fresh BHIG with or without VAN

(15 µg/ml), and incubated with shaking (100 rpm) at 37°C for three 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. A 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 (PAPs). VAN PAPs of the two control *S. aureus* and ten MRSA strains are shown in **Fig. 1.** As expected, *S. aureus* ATCC 25923 had no sub-populations with decreased VAN susceptibility. In contract, the VISA MU50 had a notable rightward shift of the survival curve, with large subpopulations that were 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 Non-Rsp and Rsp groups over the 24 h incubation. As a group, the Non-Rsp isolates had significantly less MRSA density reductions at all time-points during the 24 h VAN exposures as compared to Rsp strains (**Fig. 2**; $P < 0.05$).

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

Cell wall thickness. All ten MRSA strains exhibited similar cell wall thickness profiles. The means (± SD) of cell wall thickness for the Non-Rsp and Rsp groups are 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 capacity of MRSA strains to form biofilms in the presence vs absence of sub-MIC of VAN is summarized in **Fig. 4**. The mean absorbance without VAN exposure for Non-Rsp and Rsp 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 x MIC of VAN, four of the five Non-Rsp MRSA strains exhibited significantly enhanced biofilm formation vs. their respective controls ($P < 0.05$). In contrast, none of the Rsp strains significantly increased biofilm formation in the presence of sub-MICs VAN exposure as compared to their respective controls (**Fig. 4**).

Biofilm formation under flow conditions. To verify the static biofilm assay observations, 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 $\mu m^3/\mu m^2$ and an average thickness of 44 µm among the strain 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 capacity in the flow cell system. Strain 010-016 measured a biomass of just 0.0001 $\mu m^3/\mu m^2$, and the second Rsp strain, 301-188, accumulated slightly more biomass than strain 010-016 (0.02 $\mu m^3/\mu m^2$) (**Fig. 5**).

Biofilm composition. The percent reductions in biofilm for the 10 MRSA strains in the presence of sodium metaperiodate, proteinase K and DNase I are shown in **Fig. 6**. Without sub-MIC of VAN exposure, 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 vs. Rsp strains (mean reductions were 73.1 ± 17.3% vs. 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 vs. the Rsp group in the absence of VAN. Sub-MIC of VAN exposure 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 vs. without VAN exposure (**Fig. 6B**).

Activity of VAN within catheter-associated biofilms *in vitro*. We further assessed the capacity of the ten MRSA strains to form biofilm and the efficacy of VAN on preformed biofilms in the presence of catheters (**Fig. 7**). Without VAN exposure, a 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 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 $\log_{10}$ CFU/catheter, respectively. By comparing the number of viable bacteria recovered from catheters infected with the strains over additional three days

experimental period without VAN exposure, a similar capacity to grow within biofilm was also observed (**Fig. 7**). Taken together, these analyses indicated that the Non-Rsp and Rsp strains had a similar ability to form catheter-associated biofilms (mirroring similar intrinsic virulence in comparing the Non-Rsp and Rsp groups in the experimental IE model) (15).

In the presence of VAN, the number of viable MRSA cells recovered from catheters infected by all five Rsp isolates was markedly reduced during three days incubation (**Fig. 7**). In addition, this biofilm clearance effect of VAN in the Rsp group reached statistical significance in four of the five Rsp isolates versus 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 as 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 ≤ 2 µg/ml (35), reports of clinical failure with VAN treatment are relatively frequent (36, 37). For instance, VAN treatment success rates of only 10-20% and 56-77% have been demonstrated for MRSA bacteremia caused by isolates with MICs of 1-2 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 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 sub-MIC VAN exposure; ***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), using ten 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 using *in vitro* conditions approximating key *in vivo* parameters: **i**) utilizing VAN at 15 µg/ml to mirror targeted trough concentrations for severe MRSA infections (40); and **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 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 vs. the Rsp strains; **ii**) the Non-Rsp strains displayed more extensive *in vitro* biofilm formation in the absence of catheters; **iii**) biochemical analyses revealed a significantly greater biofilm formation reduction with proteinase K treatment by Non-Rsp vs. Rsp strains; **iv**) sub-MIC VAN exposures significantly enhanced biofilm formation, and substantially and globally increased biofilm composition only in the Non-Rsp group; and **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 above between Non-Rsp vs. 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 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 sub-MIC of VAN exposure in Non-Rsp strains are not well known. In contrast to *S. epidermidis*, few studies have examined the effect 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, extracellular DNA release, and promote biofilm formation (14). We have tested the effect of sub-MIC of VAN on autolysis, but no differences were found between the Non-Rsp and Rsp groups (data not shown). In addition, Rachid *et al* have reported that sub-inhibitory

339 concentrations of tetracycline and quinupristin/dalfopristin increase *ica* expression and
340 subsequent biofilm formation in *S. epidermidis* (45). Taken together, biofilm formation appears
341 to be triggerable by certain antibiotic exposures in both coagulase-positive and coagulase-
342 negative staphylococci, albeit by different mechanisms. Studies to further examine the
343 phenotypic and genotypic impacts of sub-MIC of VAN upon biofilm formation are in progress in
344 our laboratory.
345
346 The data also demonstrated that biofilms formed by Non-Rsp and Rsp isolates have
347 different vulnerabilities to proteinase as compared to carbohydrate and DNA perturbation. It has
348 been reported that PNAG, the major polysaccharide component of the *S. aureus* biofilm matrix,
349 does not limit VAN penetration (46). However, to our knowledge, the role of biofilm protein
350 content in the context of VAN penetration or intra-biofilm bacterial clearances has not been
351 defined. The major fibronectin binding proteins (FnBPA and FnBPB) are induced with sub-MIC
352 VAN (14); these surface proteins are known to be essential for MRSA biofilm formation (47),
353 and could be a factor in the increased susceptibility of Non-Rsp strains to proteinase K (Fig. 6).
354 An alternate explanation could be that exposure to sub-MIC VAN may affect the expression of
355 genes involved in biofilm production in a differential manner across distinct MRSA strains.
356
357 The *in vitro* catheter-associated biofilm model provided a mean to assess VAN treatment
358 in a setting akin to *in vivo* endovascular infections. Importantly, all ten MRSA strains formed
359 similar quantities of biofilm in the *in vitro* biofilm model based on bacterial counts. This
360 comparable biofilm formation between the two groups parallels the similar intrinsic virulence of
361 the Non-Rsp and Rsp strains in the IE model (based on similar target tissue bacterial counts)
362 (15). However, in some strains, MRSA densities ($\log_{10}$ CFU) within catheter biofilms in the

absence of VAN tended not to parallel the static biofilm assay (OD_{490nm}). 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**) chemical composition of materials used in the assays (polyethylene vs. polystyrene); ii) biofilm growth conditions (shaking vs. static) and/or iii) presence vs. 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 are not well understood, but will likely include their distinct biofilm compositions, limitations of VAN biofilm penetration, the evolution of "persister" cell populations within the depths of the biofilm, and/or VAN-induced enhancement of biofilm formation (13, 48-50).

In conclusion, our results suggest that several factors, including *in vitro* VAN activity, VAN binding, and 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 translated important observations from *in vitro* to *in vivo* conditions. Given the importance of biofilm phenotypes in persistent bacteremia and endocarditis, these insights may also be useful as 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 strains exhibiting a VAN "susceptible" phenotype *in vitro*.

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576 **Table 1. Clinical methicillin-resistant *Staphylococcus aureus* strains used in this study**

Strain	Genotype	VAN MIC (μg/ml)	VAN response in IE model[a]
300-087	*agr*-I, SCC*mec* IV, *spa*-NEW4, CC45	0.5	Non-Responder
300-169	*agr*-I, SCC*mec* IV, *spa*-NEW1, CC45	0.5	Non-Responder
324-136	*agr*-I, SCC*mec* IV, *spa*-NEW5, CC45	0.5	Non-Responder
300-103	*agr*-I, SCC*mec* IV, *spa*-NEW4, CC45	0.5	Non-Responder
300-246	*agr*-II, SCC*mec* I, *spa*-385, CC5	0.5	Non-Responder
301-188	*agr*-I, SCC*mec* IV, *spa*-NEW1, CC45	0.5	Responder
010-016	*agr*-II, SCC*mec* II, *spa*-2, CC5	1.0	Responder
077-107	*agr*-II, SCC*mec* II, *spa-2*, CC5	1.0	Responder
088-180	*agr*-II, SCC*mec* II, *spa*-2, CC5	0.5	Responder
088-237	*agr*-II, SCC*mec* II, *spa*-2, CC5	0.5	Responder

577

578 [a]. Vancomycin response in an experimental endocarditis (IE) model as previously described

579 (*15*). See Methods and Materials section for further descriptions

580

581

582

583 **Figures**

584 **Figure 1.** Population analyses of two control *S. aureus* strains ATCC 25923 (◇) and MU50 (■)
585 upon exposure to a range of VAN concentrations (0-16 µg/ml) are shown in Panel A. VAN
586 population analyses of ten MRSA study strains are shown in Panel B (Non-Responders) and C
587 (Responders). Non-responders: ◆ 300-087; ■ 300-169; ▲ 324-136; × 300-103; ○ 300-246;
588 Responders: ◆ 301-188; ■ 010-016; ▲ 077-107; × 088-180; ○ 088-237. These data represent the
589 means (± SD) for two separate assays.
590
591 **Figure 2.** *In vitro* time-kill curve of VAN (15 µg/ml) vs 10 MRSA study strains at high initial
592 inoculum (~10^8 CFU/ml) using exponential phase cells. Data are expressed as $\Delta\log_{10}$ CFU/ml
593 (± SD) at 2, 4, 6 and 24 h incubation vs. initial inoculum. Non-Rsp control: white; Non-Rsp with
594 VAN exposure: black; Rsp control: light gray; Rsp with VAN exposure: dark gray. * $P < 0.05$
595 between Non-Rsp vs. Rsp group comparisons with VAN exposure.
596
597 **Figure 3.** *In vitro* VAN binding of the ten MRSA study strains, using Bodipy-labeled VAN. The
598 mean of VAN binding for each group is indicated by a horizontal dashed line.
599
600 **Figure 4.** Biofilm formation of the ten MRSA strains under static condition with (■) and without
601 (□) 0.5x MIC of VAN exposure by measuring the absorbance values (A_{490nm} ± SD). * $P < 0.05$
602 vs. their respective controls without VAN exposure.
603

604 **Figure 5.** Biofilm formation of the two selected Non-Rsp and Rsp strain pair under flow
605 conditions. Confocal microscopy was used to obtain images of MRSA isolates grown in a flow
606 cell biofilm model.
607
608 **Figure 6.** Biofilm stability of pre-formed biofilm by the ten MRSA strains following sodium
609 metaperiodate, proteinase K or DNase I treatment (as quantified by the percent of A_{490nm}
610 reduction vs. controls). **Panel A**: without VAN exposure; **Panel B**: with 0.5x MIC VAN
611 exposure during the overnight biofilm formation. □: Non-Rsp group; ■ Rsp group. * $P < 0.05$ vs.
612 Non-Rsp group.
613
614 **Figure 7.** Effect of VAN (15 μg/ml) in the *in vitro* catheter-associated biofilm formation model.
615 Catheters infected with ten MRSA strains without VAN exposure (□) and with VAN exposure
616 (■) were harvested at daily intervals. The MRSA counts are measured in comparisons of the
617 changes of $\log_{10}$ CFU/catheter vs. initial density for each strain. D1, D2 and D3 represent one,
618 two and three days with/without VAN exposure on established biofilm, respectively. * $P < 0.05$
619 vs. respective controls without VAN exposure.
620

Figure 1

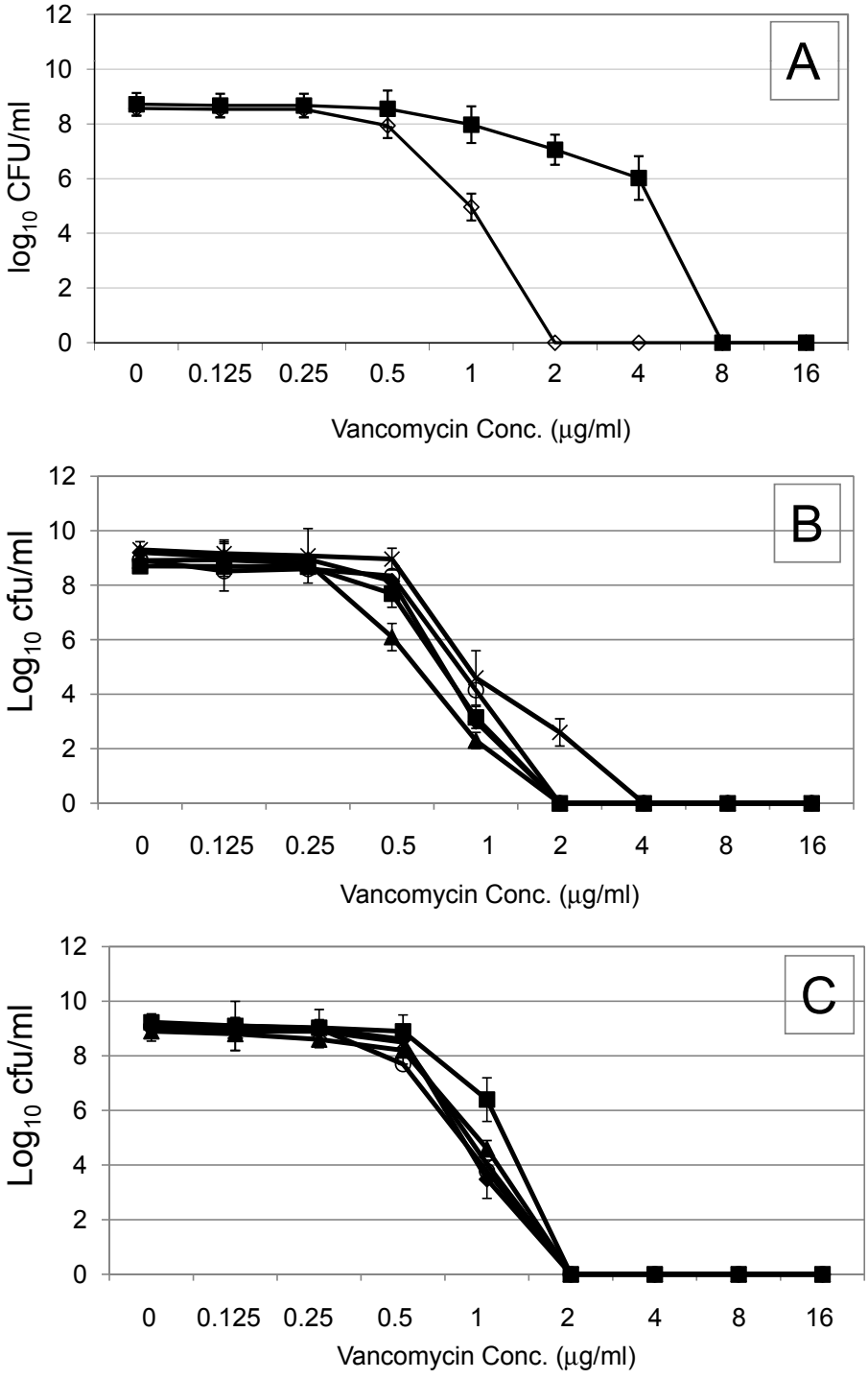

Figure 2

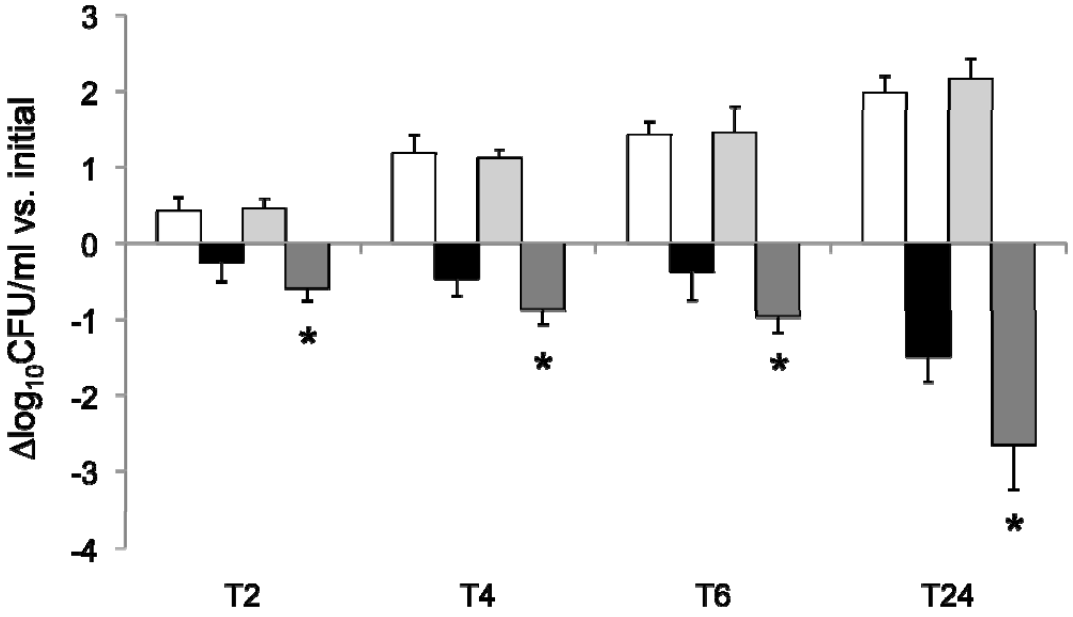

Figure 3

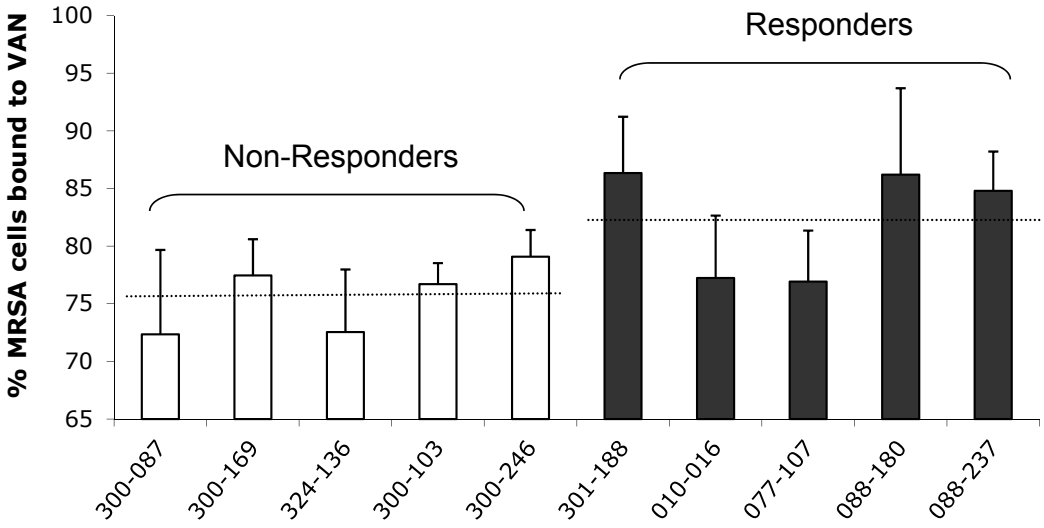

Figure 4

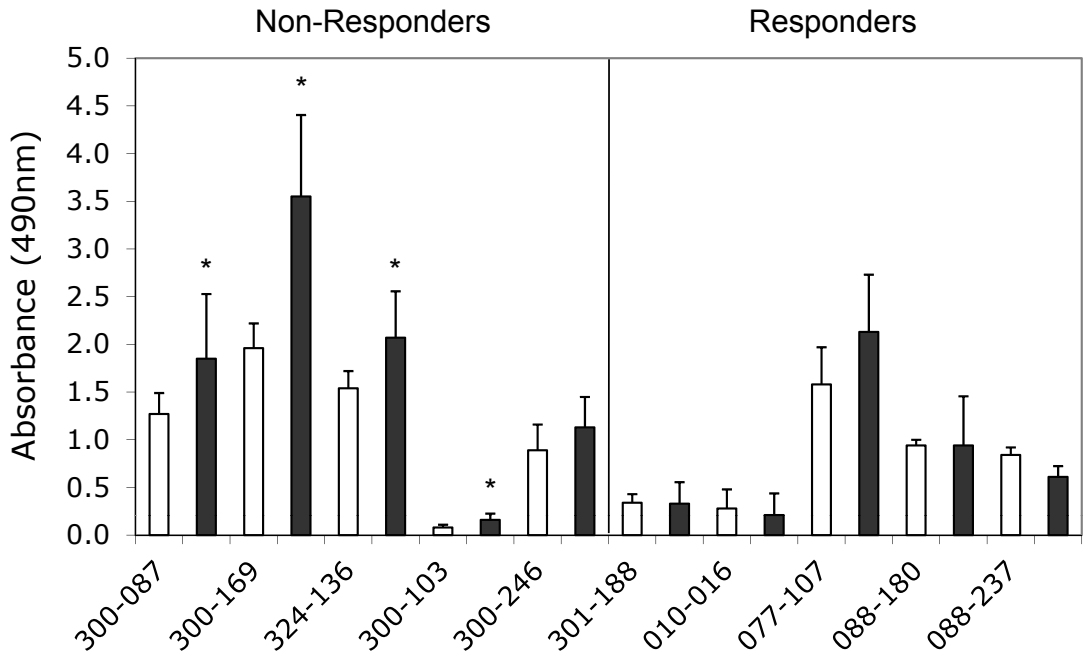

Figure 5

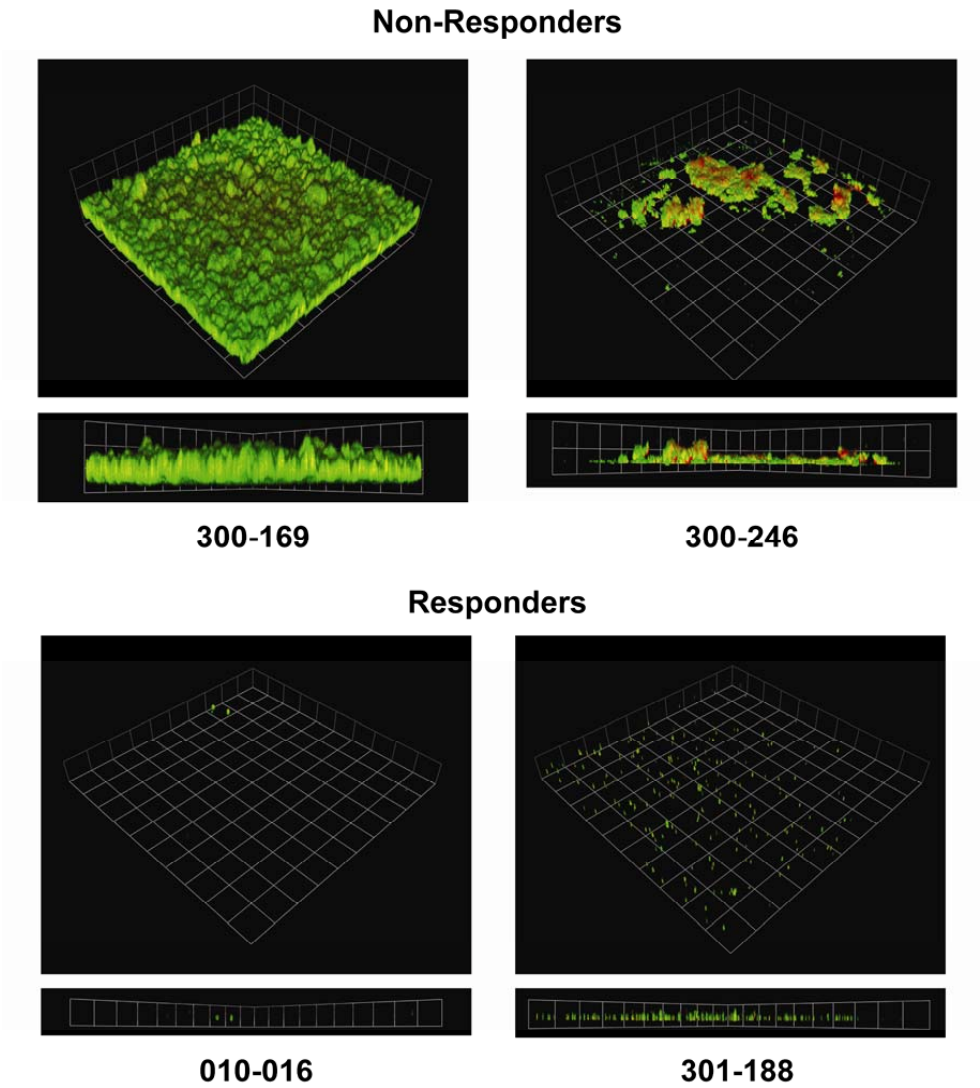

Figure 6

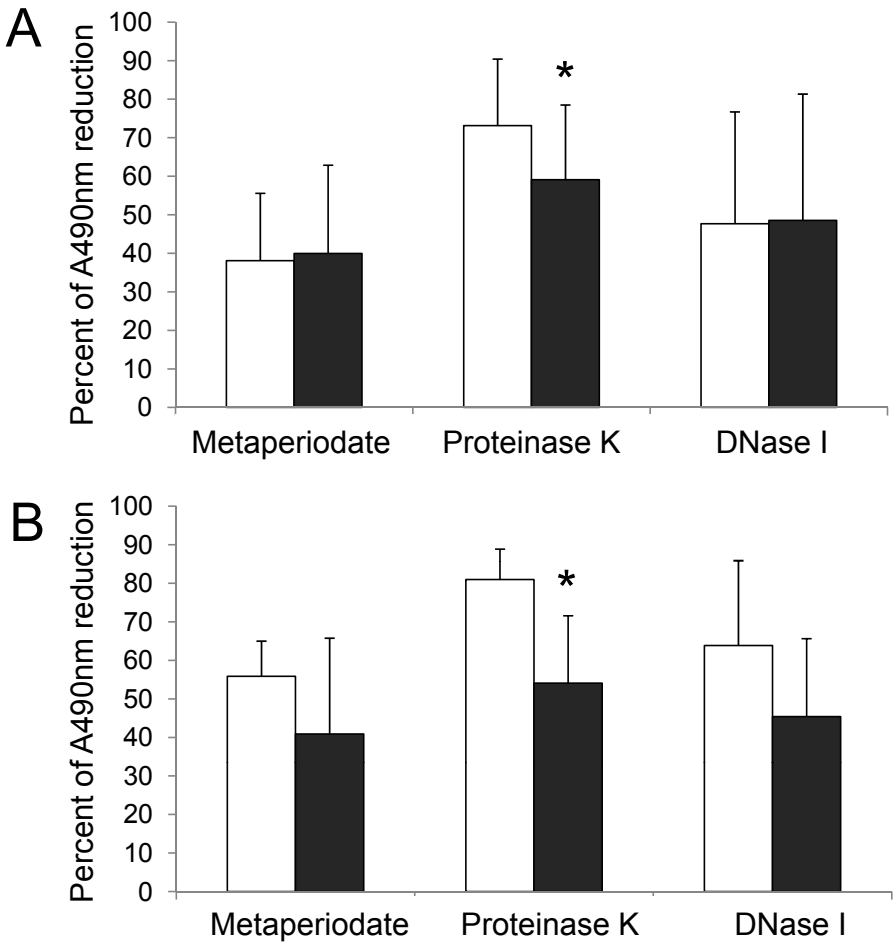

Figure 7