Combination Antibiotic Exposure Selectively Alters the Development of Vancomycin Intermediate Resistance in *Staphylococcus aureus*

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ABSTRACT Invasive methicillin-resistant *Staphylococcus aureus* (MRSA) treated with vancomycin (VAN) is associated with reduced VAN susceptibility and treatment failure. VAN combination therapy is one strategy to improve response, but comprehensive assessments of combinations to prevent resistance are limited. This study identifies optimal combinations to prevent the emergence of VAN-intermediate *Staphylococcus aureus* (VISA). Two standard MRSA and two heterogeneous VISA (hVISA) strains were exposed for 28 days *in vitro* to VAN alone, VAN with cefazolin (CFZ), fosfomycin, gentamicin, meropenem, rifampin, piperacillin-tazobactam (TZP), or trimethoprim-sulfamethoxazole. In addition to VAN susceptibility testing, cell wall thickness (CWT), carotenoid content, and membrane fluidity were determined for Mu3. VAN plus any β -lactam limited the VAN MIC increase to 1 to 4 mg/liter throughout the 28-day exposure, with CFZ and TZP being the most effective agents (VAN MIC = 1 to 2 mg/liter). Similar MIC trends occurred with the lipo-/glycopeptide agents daptomycin and telavancin, where β -lactam combinations with VAN prevented MIC increases to these agents as well. Combinations with non- β -lactams were ineffective in preventing VAN MIC increases with VAN MICs of 4 to 16 mg/liter emerging during weeks 2 to 4 of treatment. VAN plus β -lactam decreased CWT significantly, whereas VAN plus other antibiotics significantly increased the CWT. No correlation was observed between carotenoid content or membrane fluidity and antibiotic exposure. Only the combination exposures of VAN plus β -lactam suppress the development of VISA. Rational selection of VAN plus β -lactam should be further explored as a long-term combination treatment of MRSA infections due to their ability to suppress VAN resistance.

KEYWORDS glycopeptides, vancomycin resistance, antimicrobial combinations, *Staphylococcus aureus*

Methicillin-resistant *Staphylococcus aureus* (MRSA) has spread rapidly around the world and has become an important and common pathogen in both hospital and community settings. It is now a major causative agent of several invasive bacterial infections, including pneumonia, skin and soft tissue infection, osteomyelitis, septic joints, bloodstream infections, and infective endocarditis, among others (1).

Vancomycin (VAN) has been considered the frontline therapy for empirical and definitive treatment of severe MRSA infections for many years. Although VAN resistance remains rare, isolates with reduced susceptibility to VAN have appeared, including VAN-intermediate *Staphylococcus aureus* (VISA), heterogeneous VAN-intermediate *Staphylococcus aureus* (hVISA), and vancomycin-tolerant strains. VAN treatment can select for

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FIG 1 VAN susceptibility at day 28 for all four isolates following VAN or combination exposures. Data represent the medians and ranges (when applicable) of the triplicate replicates for each exposure. The dashed lines indicate the initial VAN MICs for each isolate at the beginning of the experiment (day 0). (A) hVISA Mu3; (B) ATCC 33591; (C) hVISA W1082; (D) W217.

expansion of this small subpopulation, resulting in VAN treatment failure and a transition to a homogeneous VISA population. These isolates have been associated with poor response to VAN therapy in patients and are frequently more resistant to secondary treatment options (2–6). VAN treatment failure is a significant clinical concern, and strategies to prevent the emergence and spread of these organisms are needed.

Despite these reduced susceptibility phenotypes, VAN still remains the principal antimicrobial agent of choice in the treatment of MRSA (7). In addition, it is often used initially in patients with hVISA since the identification of these isolates appear as susceptible by standard testing (1). To improve VAN clinical outcomes in the treatment of MRSA, many studies have focused on the *in vitro* or *in vivo* activities of VAN combination strategies which demonstrate synergy, antagonism, or indifference. These include VAN plus rifampin, gentamicin, fosfomycin, linezolid, or a variety β -lactams (8–19). However, relatively few studies have comprehensively studied these combinations for their ability to delay or prevent reduced susceptibility to VAN over prolonged exposures. This study identifies optimal combinations with VAN to prevent the emergence of VISA from either VAN-susceptible or hVISA isolates.

RESULTS

***In vitro* antibiotic susceptibility.** The VAN susceptibility for all four isolates after 4 weeks of serial passages with VAN or with combination exposures are presented in Fig. 1. Among all four isolates tested, VAN alone resulted in VISA at day 28. VAN combinations with fosfomycin (FOS), gentamicin (GEN), rifampin (RIF), or trimethoprim-sulfamethoxazole (SXT) were ineffective in preventing VISA development and in some cases hastened the development of reduced VAN susceptibility compared to VAN alone. The β -lactam combinations of cefazolin (CFZ), meropenem (MEM), or piperacillin-tazobactam (TZP) with VAN largely prevented VISA emergence in all isolates with the exception of VAN+MEM in Mu3. Overall, the β -lactam combinations were effective in preventing any change in VAN susceptibility, including strains with an initial VAN MIC of 1 mg/liter (Mu3 and ATCC 33591) or 2 mg/liter (W1082 and W217).

FIG 2 VAN MIC development in *Mu3* during 28-day antibiotic exposures *in vitro*. Solid line, VAN alone; dashed line, VAN plus a secondary antibiotic. (A) VAN+CFZ; (B) VAN+FOF; (C) VAN+GEN; (D) VAN+MEM; (E) VAN+RIF; (F) VAN+SXT; (G) VAN+TZP. Data points are the median MIC values from three independent replicates of each exposure.

The susceptibility changes over the entire 4-week experiment with VAN alone or with secondary antibiotic combinations are represented in the prototypical hVISA *Mu3* (Fig. 2). VAN exposure alone quickly resulted in the development of VISA (VAN MICs of 4 mg/liter) at the first 7-day time point tested during passage. All of the combination exposures prevented VAN resistance at day 7, except VAN+FOF. Although resistance development was delayed, the combinations VAN+GEN, VAN+RIF, and VAN+SXT were unable to prevent VAN resistance development from hVISA with VAN MICs of 4 mg/liter emerging by day 14. Among all of the combinations of VAN and β -lactams, VAN+CFZ and VAN+TZP prevented the development of VAN resistance throughout the 28-day exposure (MIC = 2 mg/liter), but VAN+MEM produced strains with VAN MICs of 4 to 8 mg/liter between days 21 to 28. At the end of the 28-day exposure, exposure to VAN alone or VAN+FOF, GEN, RIF, or SXT increased the *Mu3* VAN MIC to 8 to 16 mg/liter.

The MICs of daptomycin (DAP), linezolid (LZD), and telavancin (TLV), as well as all of the secondary antibiotics used in passage, were determined against all four strains at days 0 and 28 with the results represented by *Mu3* in Table 1. The combination of VAN and any β -lactam exposure tested resulted in minimal DAP and TLV MIC increases (<1-fold) and in some cases paradoxically reduced MICs to DAP and TLV. In contrast, the non- β -lactam combinations increased the MICs of DAP and TLV. These increases resulted in strains that had become nonsusceptible to DAP and/or TLV with MICs greater than at least one doubling of the MIC breakpoints of 1 and 0.12 mg/liter,

TABLE 1 Mu3 MIC values before and after 28-day antibiotic exposures

Treatment	Median MIC (mg/liter) ^a									
	DAP	LZD	TLV	CFZ	TZP	MEM	GEN	SXT	FOF	RIF
Mu3	1 (1)	2 (2)	0.125 (0.125)	>256 (>256)	>256/4 (256/4)	128 (128)	>256 (>256)	0.064 (0.064)	>256 (>256)	0.03 (0.03)
VAN	4 (4)	2 (2–4)	0.25 (0.25)	ND	ND	ND	ND	ND	ND	ND
VAN+CFZ	1 (1)	2 (2)	0.19 (0.19)	>256 (>256)	ND	ND	ND	ND	ND	ND
VAN+TZP	1 (1)	4 (4)	0.125 (0.125)	ND	>256/4 (>256/4)	ND	ND	ND	ND	ND
VAN+MEM	0.5 (0.5–1)	4 (4)	0.047 (0.032–0.047)	ND	ND	256 (256)	ND	ND	ND	ND
VAN+GEN	4 (4–8)	2 (2–4)	0.25 (0.25)	ND	ND	ND	256 (256–>256)	ND	ND	ND
VAN+SXT	4 (4)	2 (2)	0.5 (0.5–0.75)	ND	ND	ND	ND	0.125 (0.125)	ND	ND
VAN+FOF	8 (2–16)	2 (2–4)	0.38 (0.38)	ND	ND	ND	ND	ND	>256 (>256)	ND
VAN+RIF	4 (4–8)	2 (2)	0.38 (0.38–0.5)	N	ND	ND	ND	ND	ND	4 (0.5–16)

^aRanges are indicated in parentheses. ND, not determined.

TABLE 2 CPT susceptibility determined by broth dilution and Etest before and after 28-day antibiotic exposures of VAN alone or in combination with β -lactams in Mu3, W1082, W217, and ATCC 33591

Treatment	Median CPT MIC (mg/liter) ^a							
	Mu3		W1082		W217		ATCC 33591	
	Broth	Etest	Broth	Etest	Broth	Etest	Broth	Etest
Initial MIC (day 0)	2	3	1	1.5	1	1	1	1
VAN	0.5	0.75 (0.5–0.75)	1	1.5 (1–1.5)	0.5	0.75 (0.75–1)	1 (0.5–1)	0.75 (0.5–1)
VAN+CFZ	2	2 (2–3)	1	1.5	1	1.5	1	1.5
VAN+MEM	4 (2–4)	3 (2–4)	1	1.5 (1.5–2)	2 (1–2)	2 (1.5–2)	1 (1–2)	1.5 (1.5–2)
VAN+TZP	4	3	1	1	1 (1–2)	1.5	2 (1–2)	2

^aThe data represent the medians of three replicate passages. Ranges are indicated in parentheses.

respectively. Development of LZD MIC changes was distinct from development of VAN, DAP, or TLV MIC changes. The combinations VAN+TZP and VAN+MEM resulted in a doubling of the median LZD MICs at the end of exposure, whereas VAN alone and other VAN combination exposures did not alter the median LZD MIC. No changes to the MIC of any secondary antibiotic used in each passage were observed in any combination except VAN+RIF, which increased the RIF MIC 512-fold (Table 1). Similar results were found for the other three strains in this study (data not shown).

Since the β -lactam antibiotics were effective in combination with VAN in preventing the emergence of VISA over 28 days, we were interested in the effect of these exposures on the susceptibility to ceftaroline (CPT), a recently developed cephalosporin antibiotic with activity against MRSA through inhibition of penicillin-binding protein 2a (PBP2a). The CPT MICs at initiation (day 0) and at 28 days for all four isolates passaged with antibiotics are presented in Table 2. VAN alone resulted in high VAN MICs and often lower CPT MICs at 4 weeks, which is consistent with the noted see-saw effect with glycopeptides and β -lactams (20). Interestingly, the CFZ combination had little effect on CPT MICs, whereas MEM and TZP combinations often increased CPT MICs. The resulting CPT MICs from MEM or TZP exposure were noted to be intermediate (2 mg/liter) or resistant (≥ 4 mg/liter).

CWT. In order to assess the relationship between reduced VAN susceptibility and cell wall thickening, the cell wall thickness (CWT) of Mu3 with each antibiotic exposure was examined after the 28-day study (Table 3 and Fig. S1 in the supplemental material). The CWT increased significantly after the 28-day VAN only exposure. Of the combinations tested, only VAN+CFZ and VAN+TZP exposures prevented cell wall thickening. When we compared CWT values for the 28-day passage with VAN versus VAN plus combination antibiotics, all β -lactam combinations resulted in significantly reduced CWT, while the other combinations had cultures containing cells with CWT similar to

TABLE 3 Mu3 strain characteristics after 28-day antibiotic exposures

Treatment	Median VAN MIC (mg/liter) ^a	Mean $\pm$ SD ^b		
		CWT (nm)	Carotenoid content (ng/mg)	Membrane fluidity (p)
No antibiotic	1 (1)	52.1 $\pm$ 6.5	13.3 $\pm$ 1.2	0.213 $\pm$ 0.009
VAN	8 (8)	60.1 $\pm$ 6.7 [‡]	13.6 $\pm$ 1.6	0.215 $\pm$ 0.011
VAN+CFZ	2 (2)	52.0 $\pm$ 4.9**	13.8 $\pm$ 1.2	0.209 $\pm$ 0.015
VAN+TZP	2 (2–4)	53.9 $\pm$ 6.0**	16.1 $\pm$ 1.0* [‡]	0.208 $\pm$ 0.008*
VAN+MEM	4 (4–8)	58.3 $\pm$ 8.0* [‡]	14.3 $\pm$ 0.9	0.201 $\pm$ 0.012** [‡]
VAN+GEN	8 (8)	63.5 $\pm$ 8.7** [‡]	14.3 $\pm$ 0.7	0.214 $\pm$ 0.010
VAN+SXT	8 (8–16)	64.3 $\pm$ 9.6** [‡]	11.8 $\pm$ 2.1	0.224 $\pm$ 0.014* [‡]
VAN+FOF	16 (8–16)	66.4 $\pm$ 6.7** [‡]	16.2 $\pm$ 1.6* [‡]	0.214 $\pm$ 0.012
VAN+RIF	16 (8–16)	68.5 $\pm$ 8.8** [‡]	21.2 $\pm$ 3.9** [‡]	0.217 $\pm$ 0.017

^aRanges are indicated in parentheses.

^bAsterisks indicate statistically significant differences between the VAN single exposure and combination exposures (*, $P < 0.05$; **, $P < 0.001$). Dagger indicate statistically significant differences between the any exposure and Mu3 progenitor strain ([†], $P < 0.05$; [‡], $P < 0.001$). Lower polarization index (p) values indicate a greater degree of membrane fluidity.

VAN passage alone. As expected, a significantly positive correlation between the CWT and the VAN MIC ($r = 0.939$) was observed.

Carotenoid content and membrane fluidity. The carotenoid content and membrane fluidity of Mu3 after antibiotic exposure was tested to determine a potential correlation with VAN resistance (Table 3). The hVISA Mu3 exposed to the combinations VAN+FOF, VAN+RIF, and VAN+TZP led to a significantly higher carotenoid content compared to both Mu3 and VAN alone. As for membrane fluidity, VAN+SXT obtained a lower degree of fluidity compared to both Mu3 and VAN alone, whereas VAN+MEM resulted in enhanced membrane fluidity. However, no significant correlation was observed between carotenoid content ($r = 0.267$) or membrane fluidity ($r = 0.680$) and VAN MIC.

DISCUSSION

The causes of reduced susceptibility to VAN in patients with complex MRSA infections are often multifactorial, but this reduced susceptibility can be attributed in part to suboptimal and/or prolonged VAN exposure. Although combination therapy is one proposed strategy to prevent the loss of VAN activity, this approach has not been evaluated in a comprehensive fashion with commonly used combinations. In this study, antibiotics in combination with VAN significantly altered the trend toward the emergence of VISA (Fig. 1 and 2). Traditionally used combination therapies with VAN, such as GEN, RIF, and SXT, were not effective in preventing VAN resistance. However, the combinations of VAN plus β -lactams prevented the emergence of VAN resistance significantly during the 28 days of study, particularly the combinations VAN+CFZ and VAN+TZP. These results were consistent in four total strains regardless of hVISA background or initial VAN MIC.

The combination of VAN with β -lactams has displayed synergy against *S. aureus* both *in vivo* and *in vitro* (12–19). In addition, a phenomenon termed the “see-saw effect” was observed in some studies where a VAN MIC increase is accompanied by a β -lactam MIC decrease in select clinical MRSA, VISA, and VRSA strains, as well as *in vitro*-derived VISA (20–26). The mechanism for this is not completely known, and not all *S. aureus* demonstrate this phenomenon. However, there is evidence pointing toward a contribution of the VraSR two-component regulatory system on the seesaw effect, leading to enhanced sensitization of β -lactam antibiotics (27, 28). In our study, we evaluated CPT susceptibility following VAN alone and combined with the β -lactams in the study. Our results support this seesaw effect with CPT, since the MIC increased up to 4-fold when VISA (VAN MIC 8 mg/liter) developed with VAN exposure alone. We are currently evaluating what regulatory adaptations coincide with this effect.

The four isolates tested displayed reduced susceptibility to VAN, which can be related to several phenotype changes. One of the most common changes is the thickened cell wall, which correlates with VISA development (29, 30). Similarly, significant cell wall thickening of the *in vitro*-derived VISA strains under the selective pressure of VAN alone or any combination was observed in our study; a thicker cell wall correlated with a higher VAN MIC ($r = 0.939$). It is evident that the combination of VAN and β -lactams limited cell wall thickening; however, in all of the VAN+ β -lactam combinations, only VAN+CFZ maintained the same CWT as Mu3 and prevented the VAN MIC in all replicates from increasing beyond 2 mg/liter. To assess other phenotypic changes previously observed in VISA, we tested the carotenoid content and membrane fluidity of the *in vitro* derived stains compared to the progenitor hVISA strain Mu3. Several studies (31, 32) have shown that *in vitro*-derived DAP-resistant MRSA strains frequently display excess carotenoids and decreased membrane fluidity as the DAP MIC increases in parallel with increasing VAN MICs. Of note, we did observe that exposure to VAN alone and VAN plus the secondary antibiotics resulted in DAP nonsusceptible MRSA (DAP MIC > 1 mg/liter) in parallel with increasing VAN MICs in Mu3 unless a β -lactam was present during the exposure. Similar observations were observed in the additional strains tested (data not shown). There was no correlation between VAN MICs and carotenoid content or membrane fluidity. Overall, our data confirm that the

thickened cell wall may play a pivotal role in the VAN-intermediate resistance and the combination of VAN and β -lactams can limit the cell wall thickening. This evidence supports the combination of VAN with β -lactams not only for antimicrobial synergy purposes but also to prevent the mechanism associated with the transition to VISA.

Although the mechanism of DAP activity is different than VAN in that it acts as a functional cationic peptide on the cell membrane, several studies have identified a similar synergistic effect with DAP plus β -lactams (24, 33). This includes the ability of oxacillin to prevent the development of DAP resistance during prolonged exposure simulations (25). Recent evidence suggests that inhibition of PBP1 is important for maximal synergistic activity (34, 35). In our study with VAN, penicillin-binding protein inhibition specificity did not appear to reflect a more potent suppression of VAN resistance. CFZ and TZP, nonspecific inhibitors of *S. aureus* PBPs, prevented any increase in VAN MIC beyond 2 mg/liter, the breakpoint for susceptibility. MEM, which has affinity for PBP1/PBP2, was less effective against Mu3 (VAN MICs increases to 4 to 8 mg/liter) but showed results similar to those for other β -lactams in the other isolates. In addition, FOF, which has been shown to sharply reduce the expression of PBP1 (36), was not effective at suppressing VISA at any point during the exposure. These results support the existing literature that the mechanisms for β -lactam synergy with VAN versus DAP are different (34, 37).

Combining VAN with a second antibiotic may be an alternative strategy to improve clinical outcomes. The majority of VAN combination therapy research has focused on the combinations of VAN plus β -lactams that typically have demonstrated synergy (9–16). In contrast, few recent studies have compared this approach to other combinations using such agents as RIF, GEN, and SXT, and these findings have been inconsistent (38). In our study, the combinations of RIF, SXT, and FOS in combination with VAN hastened the development of VISA compared to VAN alone in two of the four strains tested and therefore appears to be strain dependent. Although the mechanisms for this are currently being explored, we note that the susceptibility to SXT and RIF increased 2- and 128-fold in Mu3, respectively.

Since complicated infections caused by MRSA are associated with long-term treatment and relapse, combination therapy may play an additional role beyond synergy to delay or prevent this transition. In this regard, it is significant that our findings show that the VAN-induced transition to VISA during the 28-day exposure can be delayed or prevented by the addition of β -lactams but not by the addition of other antibiotics. Clinical studies of this combination have been promising, primarily with piperacillin-tazobactam with vancomycin, for empirical coverage leading to improved outcomes (13). Although minimal clinical data are available with vancomycin combined with cefazolin, the ongoing CAMERA-2 study includes cefazolin as one of three β -lactam combinations with vancomycin and may provide clarity on the optimal approach (39). However, the phenomenon of β -lactam-induced vancomycin resistance (BIVR) has been described in the literature; this may conflict with applying our findings to all MRSA. In surveillance studies, BIVR, which is strain and concentration dependent, occurs in 6 to 20% of MRSA (40–42). This phenotype has been explored mostly in isolates from Japan, and studies of recent isolates are lacking. The mechanism for BIVR is attributed to depletion of free vancomycin when combined with the β -lactams ceftizoxime or imipenem (43). Further, testing for BIVR appears to lack consistency and standardization (44), so the clinical significance of this effect remains unknown and difficult to study.

We noted that the VAN combinations also altered susceptibility to other MRSA therapies such as DAP, LZD, TLV, and CPT (Tables 1 and 2). For example, only the β -lactam agents combined with VAN largely prevented collateral DAP and TLV resistance (MICs of >1.0 and >0.12 mg/liter, respectively). In particular, VAN+MEM reduced DAP MIC by 1-fold and TLV MIC by >2 -fold compared to the initial MICs. This is consistent with MEMs ability to affect DAP activity (40), but this is the first time to our knowledge that this has been shown to possibly be related to TLV. These results also suggest that MEM or TZP exposures could alter the activity of CPT, by a mechanism that

is under investigation (A. Rosato et al., unpublished results), since we noted that VAN combinations with these agents after 28 days resulted in CPT MICs up to 4 mg/liter. We are currently exploring how these secondary β -lactam agents may induce mutations in PBPs that could result in this observed reduced CPT effectiveness phenotype.

In conclusion, this study clearly demonstrated that combination treatment with VAN could significantly impact the transition to VISA *in vitro*. The combination of VAN and β -lactams most effectively prevented the emergence of VAN resistance, although some exceptions were noted with MER. In contrast, combination treatment with other agents, including VAN+FOF, VAN+GEN, VAN+RIF, and VAN+SXT, at best delayed VISA emergence only in the first week of treatment. Our study is limited to the *in vitro* environment and the concentrations of antibiotic tested, but it provides a comprehensive assessment of commonly used combinations with VAN in multiple isolates. The combination of VAN plus β -lactams should be further explored as a long-term treatment of serious MRSA infections due to their ability to suppress VAN resistance. This may include evaluation of mechanisms on the cell wall to determine peptidoglycan and teichoic acid composition with this combination (45), *in vitro* models with different media types known to affect VISA development and antibiotic activity (46, 47), and animal models of prolonged infection types. However, collateral effects of this approach on other MRSA treatment options should be investigated.

MATERIALS AND METHODS

Bacterial strains, antimicrobials, and media. This study used two standard MRSA and two hVISA isolates to determine whether reduced susceptibility derived from passage is due to initial susceptibility differences or the hVISA phenotype. The two hVISA isolates were Mu3, the first clinically identified hVISA from Japan reported by Hiramatsu et al. with an initial VAN MIC of 1 mg/liter (2) and a clinical hVISA (confirmed by population analysis profile testing) blood isolate collected at the University of Wisconsin Hospital (W1082) with a VAN MIC of 2 mg/liter. The standard MRSA isolates consisted of a clinical MRSA blood isolate collected at the University of Wisconsin Hospital with a VAN MIC of 2 mg/liter (W217) and the ATCC strain 33591 with a VAN MIC of 1 mg/liter. VAN, SXT, and RIF were purchased from Research Products International Corp (Mount Prospect, IL). GEN, FOF, and D-glucose 6-phosphate potassium salt were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). DAP was provided by Merck (Merck & Co., Inc., Kenilworth, NJ). LZD was obtained from Pfizer (Pfizer Inc., New York, NY). Allergan (Dublin, Ireland) provided CPT active powder. CFZ, MEM, and TZP were purchased from clinical stock powders. Mueller-Hinton Broth II (BD, Sparks, MD) supplemented with 25 mg/liter calcium (as CaCl_2) and 12.5 mg/liter magnesium (as MgCl_2) (cation-adjusted Mueller-Hinton broth [CAMHB]) was used to grow *S. aureus* in liquid culture.

Susceptibility testing. MIC values of study bacteria to SXT were determined by Etest as suggested by the manufacturer (bioMérieux, Marcy l'Étoile, France). TLV (Theravance Biopharma, South San Francisco, CA) and CPT (Allergan) susceptibilities were determined by Etest. CPT susceptibilities were also confirmed by broth dilution testing. MICs of other antibiotics were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (48). CAMHB was adjusted to 50 mg/liter calcium for DAP testing and supplemented with D-glucose-6-phosphate for FOF testing. All samples were incubated at 37°C for 24 h. *S. aureus* ATCC 29213 was included simultaneously as a quality control strain. All MIC values determined were within the acceptable quality control range for this organism.

VAN exposure conditions. All isolates were exposed in triplicate to VAN concentrations increasing from $0.5 \times \text{MIC}$ (0.5 or 1 mg/liter) to 6 mg/liter according to visual growth *in vitro* in CAMHB for 28 days. This passage protocol, adapted from a previously described method (25), consisted of preparing a standard inoculum of 10^6 in fresh CAMHB containing $0.5 \times \text{VAN MIC}$. Cultures were grown overnight in triplicate at 37°C with shaking at 160 rpm. Cultures that showed visual turbidity were collected and diluted 1:100 into fresh CAMHB containing VAN. Cultures that did not show visual turbidity were discarded and the previous day's turbid culture was thawed from frozen stocks and used to inoculate the next day's culture. This process was repeated as a series of distinct daily passages for 28 days. The VAN concentration in the CAMHB was escalated every 7 days to 2, 4, and 6 mg/liter. When no visual growth occurred following escalated VAN concentration, cultures were recovered from the previous passage and continued in the lower VAN concentration with visual growth and continued for an additional 7 days. VAN susceptibility was determined on days 0, 7, 14, 21, and 28, whereas DAP, LZD, TLV, and CPT susceptibilities, along with the secondary antibiotics used in each treatment, were determined on days 0 and 28.

Combination exposure conditions. For combination exposures, all four isolates were exposed to VAN and passaged for 28 days, as outlined above, except that the media contained a constant concentration of a secondary antibiotic. As the VAN concentration increased every 7 days from 0.5 or 1 mg/liter up to 6 mg/liter, the concentration of the secondary antibiotic was held constant throughout. The secondary antibiotics were added at concentrations corresponding to the value of the serum free average concentration (fc_{avg}) obtained using standard dosing. In the case where the fc_{avg} was above the

MIC, secondary antibiotic concentrations of $1/2 \times$ MIC were used during serial passage. Antibiotics added at the fC_{avg} included CFZ (9.5 mg/liter), FOF (145 mg/liter), GEN (2.6 mg/liter), MEM (24 mg/liter), and TZP (105 mg/liter of piperacillin and 12 mg/liter of tazobactam). Antibiotics added at $1/2 \times$ MIC included RIF (0.015 mg/liter) and SXT (0.032 mg/liter of trimethoprim and 0.776 mg/liter of sulfamethoxazole).

CWT. Mu3 was used as the model organism to correlate cell wall thickness (CWT) to antibiotic exposure and resulting VAN MIC changes. CWT was determined by transmission electron microscopy (TEM) as described previously (49). Samples were collected from day 28 exposure cultures and compared to Mu3 prior to passage in antibiotics. Images were analyzed and measured using ImageJ 1.50b software. CWT measurements were made on a minimum of 25 cells per exposure using four separate quadrants of each cell.

Carotenoid extraction. Mu3 was also used as the standard organism to compare carotenoid changes in the cell membrane to antibiotic exposure and resulting VAN MIC. The modified protocol of Castro et al. (50) was followed for the extraction and quantification of carotenoids. Bacteria were cultivated on brain heart infusion agar, incubated at 37°C for 24 h, and transferred to room temperature for an additional 48 h of carotenoid development. At 72 h, colonies amounting to 5 to 10 mg of wet cell weight were transferred from plates to microcentrifuge tubes, resuspended in 1 ml of methanol, and incubated at 55°C for 20 min. Clarified supernatant was assessed for carotenoid content spectrophotometrically at 450 nm using an extinction coefficient for staphyloxanthin of $250,000 \text{ M}^{-1} \text{ cm}^{-1}$. Values were normalized to nanograms of staphyloxanthin detected per milligrams of wet cell weight. The assay was repeated with a minimum of three analytical replicates, and three biological replicates were evaluated on separate days.

Cell membrane fluidity determination. Mu3 was used to correlate membrane fluidity alterations to antibiotic exposure and resulting VAN MIC. Membrane fluidity was determined by fluorescence polarization spectrofluorimetry using 1,6-diphenyl-1,3,5-hexatriene (DPH) as previously described (51, 52) using a Synergy H1 spectrofluorometer (BioTek, Winooski, VT). The results were corrected by subtracting data from an unlabeled control reaction. The polarization index (p) was determined using the formula $p = (I_v - I_h)/(I_v + I_h)$, where I is the fluorescence intensity, and subscripts V and H indicate vertical and horizontal orientation instrument values, respectively (52). Data represent averages and standard deviations of three experiments performed on separate days with eight readings from every sample each day. There is an inverse relationship between polarization index and the degree of membrane fluidity (the lower fluorescence polarization value, the higher degree of membrane fluidity).

Statistical analysis. Continuous variables were summarized using the means and standard deviations and compared using a two-tailed unpaired Student t test. Comparisons of MIC values were conducted with Wilcoxon rank sum tests. P values of ≤ 0.05 were considered significant. The relationships among the CWT, carotenoid extraction, membrane fluidity, and the VAN MIC were compared using Spearman's rank correlation.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02100-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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