Adaptation of Methicillin-Resistant *Staphylococcus aureus* in the Face of Vancomycin Therapy

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For the past 2 decades, vancomycin has served as the cornerstone of therapy against serious methicillin-resistant *Staphylococcus aureus* infections. This role is increasingly challenged by questions of efficacy, including reduced efficacy against infections caused by glycopeptide-intermediate *S. aureus* strains. In an evaluation of clinical glycopeptide-intermediate *S. aureus* isolates and serial, clinical methicillin-resistant *S. aureus* isolates obtained from patients receiving vancomycin for the treatment of bacteremia, we found that loss of function of the accessory gene regulator operon may confer a survival advantage to *S. aureus* under vancomycin selection pressure, particularly in strains with the accessory gene regulator group II genotype. Other advantages in a nosocomial setting may include enhancement of biofilm formation and promotion of physiologic changes supporting colonization. We conclude that loss of accessory gene regulator function in methicillin-resistant *S. aureus* might, in part, explain the decreased efficacy of vancomycin in the therapy of methicillin-resistant *S. aureus* bacteremia, thus highlighting the need to reevaluate the criteria of susceptibility to vancomycin.

In recent years, antimicrobial resistance in *Staphylococcus aureus* has posed great challenges to clinicians treating serious infections, particularly endovascular infections (e.g., infective endocarditis), for which bactericidal therapy is pivotal for a successful clinical outcome [1–3]. This issue is complicated by the continued increase in the prevalence of methicillin-resistant *S. aureus* (MRSA) in both community-acquired and nosocomially acquired infections [4]. The efficacy of vancomycin, the agent long considered to be the first choice for such infections, is limited by slow bactericidal effects, reduced efficacy against infections caused by glycopeptide-intermediate *S. aureus* (GISA) strains [1, 5–7], and the recent emergence of *vanA*-mediated, fully glycopeptide-resistant strains [8].

The GISA phenotype is somewhat unique, compared with phenotypes associated with resistance to other antibacterial agents, because it appears to be the result of global metabolic changes affecting cell wall synthesis, cross-linking, and overall structure [5, 6, 9, 10]. We refer readers to several excellent reviews that offer detailed descriptions of cell wall metabolism in GISA [5, 9, 10]. The instability of the GISA phenotype in the absence of glycopeptide selective pressure has made the characterization of the genetic mutations leading to the GISA phenotype difficult [5].

Our studies examining how MRSA strains adapt under vancomycin selection pressure began with the observation that all 6 GISA strains that were available to us lacked the ability to hemolyze sheep blood. Furthermore, in some instances in which progenitor strains were available, the loss of hemolysis appeared to correlate with the emergence of increased vancomycin heteroresistance. In other cases, both the progenitor and the vancomycin heteroresistant strains failed to demonstrate hemolysis of sheep blood. This led us to investigate the accessory gene regulator (*agr*) operon as a potential site of mutation or upstream repression under vancomycin selection pressure [11].

**THE AGR OPERON**

The *agr* operon in *S. aureus* is a key global regulon that coordinates many critical virulence pathways [12–15].
The structure of the operon and the interrelationship of the various gene products of the operon are shown in figure 1. For a complete description of this complex regulatory circuit, we refer the reader to several articles by Richard P. Novick’s group, which was instrumental in the discovery and characterization of this operon [12–15]. In brief, transcription at the agr locus is driven by the divergent promoters P2 and P3, the first of which promotes transcription of AgrA, AgrB, AgrC, and AgrD, and whose function is to produce the gene products required to activate the circuit via the assistance of SarA. However, it is transcription at P3 that produces RNAIII, the effector molecule in transcriptional regulation of numerous downstream genes.

As a general rule, activation of the agr operon induces the production of secreted virulence factors (e.g., hemolysins, exoproteins, and exotoxins) and decreases the production of cell-associated virulence factors (e.g., adhesions). S. aureus strains in which the agr operon is inactivated through insertional inactivation, mutation, or upstream down-regulation are expected to show decreased hemolysin expression [14, 15]. The effector molecule of agr is RNAIII, which, in addition to its role as a transcription factor, may be translated into the delta-hemolysin protein. The activity of agr in a given S. aureus strain can be assayed quantitatively by measuring RNAIII levels in RNA extracts or delta-hemolysin protein levels by use of chromatography or, more qualitatively, by observing delta-hemolysin activity on sheep blood agar. Delta-hemolysin activity can be measured as hemolysis that is augmented in the presence of beta-hemolysin. Therefore, streaking an S. aureus strain in close proximity to (but not touching) a beta-hemolysin–hyperproducing strain (such as the well-characterized strain S. aureus RN4220) can serve as a crude measure of agr activity [11].

In addition, it is important to note that, for the purposes of this discussion, the agr gene cluster demonstrates polymorphism in the agr-D-C region, which defines 4 major agr groups in S. aureus, termed agr groups I–IV. AgrD is processed into a cyclic autoinducing peptide that serves to up-regulate the operon when it binds to the receptor histidine kinase AgrC from the same agr group. However, in general, AgrD is inhibitory to the operon when bound to AgrC of other agr groups [12].

S. aureus strains of specific agr groups have been strongly associated with certain clinical characteristics. For example, agr group IV S. aureus strains have been associated with exfoliative toxin production, resulting in scaled skin syndrome. S. aureus in agr group III were traditionally associated with menstrual toxic shock syndrome and now include the USA400 strains of community-acquired MRSA that have emerged in the United States [4]. Endocarditis isolates are usually of the group I or II subtypes [16, 17].

Until recently, agr was considered almost exclusively as a quorum-sensing mechanism regulating staphylococcal virulence factors. However, it is clear from the study by Dunman et al. [18] that the agr is a global regulon of various metabolic pathways and autolysin expression, in addition to its effects on virulence regulation.

**FUNCTION OF AGR IN GISA AND SERIAL ISOLATES FROM VANCOMYCIN-TREATED PATIENTS**

We determined the delta-hemolysin activity on sheep blood agar plates (as a measure of agr function) of serial MRSA bloodstream isolates obtained from patients who were treated with vancomycin. In addition, we assayed delta-hemolysin in several clinical GISA isolates. Some representative experiments are shown in figure 2: strain RN6607 (streaked horizontally in figure 2A and 2E) is an agr-positive, group II, methicillin-susceptible S. aureus isolate that serves as a positive control, and strain RN9120 (streaked horizontally in figure 2B) is an agr II knockout strain derived from RN6607 that serves as a negative

![Figure 1. Structure and function of the accessory gene regulator (agr) locus of *Staphylococcus aureus*. Transcription at the P2 promoter results in the production of RNAII, which encodes 4 gene products: AgrB, AgrD, AgrC, and AgrA. AgrC is the receptor histidine kinase, and AgrA is the response regulator of a 2-component signal transduction pathway. This pathway is induced by a posttranslationally modified cyclic octapeptide (autoinducing peptide [AIP]) that is processed from the AgrD gene product and secreted into the extracellular space via the 26-kDa transmembrane protein, AgrB. Activation of the circuit by the binding of the AIP to AgrC leads to phosphorylation of AgrA, which, with the assistance of SarA, results in the production of RNAIII. The effector molecule that modulates transcription of numerous genes, including virulence factors. RNAIII is also translated into delta-hemolysin; therefore, delta-hemolysin activity can serve as a marker for production of RNAIII. Reprinted from Lyon et al. [13] with permission from the National Academy of Sciences.](https://academic.oup.com/cid/article-abstract/42/Supplement_1/S40/275715)
control. Note that inactivation of the agr operon resulted in complete lack of hemolysin expression in RN9120. However, because the expression of hemolysins-alpha and -beta is under the control of multiple factors, in addition to agr, we use delta-hemolysin activity as the surrogate marker of agr function. GISA and heteroresistant GISA (different strains streaked horizontally in figure 2 C, D, F, and G) do not express delta-hemolysin activity, as measured by hemolysis in proximity to a vertical streak of RN4220, a beta-hemolysin hyperproducer.

On the right side of figure 2 are shown 2 sequential bloodstream isolates: A5937 (figure 2H) and A5940 (figure 2I). A5937 is an MRSA strain isolated from the bloodstream of a patient with aortic valve endocarditis. A5940 is an MRSA strain grown from the valvular tissue of the same patient after he had received vancomycin therapy for 50 days. The isolates are indistinguishable genotypically by PFGE. One can see that there is no hemolysin activity on sheep blood agar plates of the later isolate, A5940, which emerged after prolonged vancomycin therapy. There is a clear shift toward vancomycin heteroreistance in A5940 shown in vancomycin population analysis (figure 3). The vancomycin MIC increased from 2 μg/mL (in A5937) to 4 μg/mL (in A5940). The presence of a subpopulation in A5940 that grows at vancomycin concentrations >4 μg/mL characterizes this strain as heteroreistant GISA, unlike the progenitor A5937. We have sequenced the agr operon of these 2 strains and have determined that a loss-of-function mutation in A5940 in agrA accounts for the loss of agr function [11].

We found that a loss of delta-hemolysin activity coincided with the development of glycopeptide heteroresistance in other MRSA isolate series. We are unaware of any clinical GISA isolate that expresses delta-hemolysin. However, our sequencing of the agr operon of several GISA failed to demonstrate any major mutations that would potentially account for loss of function. We hypothesize that suppression of agr function may occur through upstream regulators.

Since publication of our initial report, decreased agr activity under glycopeptide selection pressure has been reported by other investigators. Renzoni et al. [19] reported a >98% reduction in RNAIII expression in GISA selected from a rat chronic tissue cage MRSA infection model after treatment with teicoplanin. Adhikari et al. [20] noted a complete loss of RNAIII transcription after selection of GISA in vitro. In both of these cases, there were no mutations identified in the agr operon, but sigB expression was increased. The phenotype selected from the animal model appeared to be unstable, because a glycopeptide-susceptible revertant was identified with partially restored RNAIII expression [19]. It has been speculated that agr function may have been suppressed by upstream regulators, such as sigB, in these instances in the absence of detectable loss-of-function mutations [19–22].

Figure 2. Production of delta-hemolysin, as a measure of accessory gene regulator (agr) function, of glycopeptide-intermediate Staphylococcus aureus (GISA) isolates. The vertical streak denotes strain RN4220, which, by virtue of its beta-hemolysin hyperproduction, allows augmentation of delta-hemolysin activity and inhibition of alpha-hemolysin activity of the test isolate. A and E, RN6607 is the agr II-positive control. B, RN9120 is the agr II knockout, negative control. C, A6222 is a GISA strain from Boston. D, HIP8846 is a GISA strain from New Jersey. F, Mu3 is a heteroresistant GISA strain from Japan. G, Mu50 is a GISA strain from Japan. H and I, Delta-hemolysin activity of sequential methicillin-resistant S. aureus endocarditis clinical isolates: H, A5937 was obtained from blood before vancomycin therapy. I, A5940 was obtained from valvular tissue after 50 days of vancomycin therapy and is a heteroresistant GISA strain. Reprinted from Sakoulas et al. [11] with permission from the American Society for Microbiology.

Figure 3. Population analysis of serial, clinical methicillin-resistant Staphylococcus aureus isolates recovered from a patient with aortic valve endocarditis. A5937 is the strain initially isolated from the patient’s bloodstream isolate. A5940 is the strain isolated from the patient’s valve tissue after the patient received vancomycin therapy for 50 days.
GISA AND AGR GROUP II GENOTYPE

Our sequencing of the agr locus of various GISA and heteroresistant GISA strains was completed almost simultaneously with the publication of the complete genome of Mu50, the first clinical GISA strain, which revealed that all the strains belonged to agr group II [11, 23]. At the time, this was considered to be a remarkable finding, because agr group II S. aureus strains were considered to represent the minority of clinical isolates, although a systematic evaluation of agr typing among clinical MRSA and methicillin-susceptible S. aureus strains from around the world was lacking [24–26]. Our subsequent evaluation of MRSA isolates from 3 US hospitals showed that the majority belonged to agr group II, a finding confirmed by other researchers [4]. Although this apparent clonal spread of agr group II strains was unexpected, there appears to remain considerable variability in their prevalence within different hospitals; we found that, at one medical center, the majority belonged to agr group III (G.S., V. F. Fowler, and V. Meka, unpublished observations). Furthermore, MRSA isolates from Europe and USA300 community MRSA in the United States reflect agr group I [25].

It would appear that the finding of an agr group II genotype predominating in GISA isolates from the United States is a reflection of the MRSA population from which these strains emerged. We know that the agr group II genotype is not a prerequisite for the GISA phenotype. For example, MRSA COL, a well-characterized agr group I S. aureus strain, can be passaged in vitro to select for GISA. We point out that, in our laboratory, MRSA COL does not express delta-hemolysin. Furthermore, agr group I clinical GISA strains have emerged in Europe [27]. However, it is reasonable to ask whether it is a coincidence that GISA first emerged among agr group II S. aureus, a genotype previously thought to represent a minority of clinical isolates.

EVALUATION OF PROTOTYPE STRAINS AND AGR KNOCKOUTS REPRESENTING DIFFERENT AGR GROUPS

As discussed above, several studies have now described decreased agr activity in S. aureus strains recovered after exposure to glycopeptides both in vitro and in vivo. To determine whether decreased agr function provided any selective advantage to S. aureus by enhancing survival during glycopeptide exposure, we investigated agr knockouts and their agr functional parental strains that were available from other studies [12, 13, 15].

We found no difference in susceptibilities to vancomycin, either by evaluation of MICs or by population analysis, between the respective agr-positive and agr-knockout pairs RN6390b/ RN6911 (group I), RN6607/RN9120 (group II), and RN4850/RN9121 (group IV) [28]. However, after organisms were grown in broth media containing subinhibitory vancomycin concentrations of 0.5–1 μg/mL, we observed a shift toward vancomycin heteroresistance that was very pronounced for the agr group II knockout strain RN9120, whereas little difference was observed with group I or group IV strains. The population analyses of these strains before and after growth in subinhibitory concentrations of vancomycin are shown in figure 4 [28].

Attempts to obtain GISA derivatives by serially passaging these 6 strains through incrementally higher concentrations of vancomycin were successful only for the agr II knockout strain RN9120. RN9120 was able to grow in broth containing up to 10 μg/mL vancomycin. None of the other 5 strains was able to grow in broth containing ≤5 μg/mL vancomycin. Population analysis of RN9120-GISA, relative to its isogenic progenitors, is shown in figure 5 [28].

We and others have recently shown that GISA strains exhibit notable defects in their propensity to undergo autolysis in vitro [28–30]. Because the agr global regulon positively regulates the expression of many murein hydrolases that are involved in autolysis [31], we hypothesized that the differences in vancomycin heteroresistance of the agr prototype strains and their respective knockouts, particularly of the increased resistance of RN9120 after growth in vancomycin, might possibly be related to differences in autolysis. In figure 6A and C, we noted that, for the agr group I and IV pairs, growth in subinhibitory concentrations of vancomycin resulted in minimal changes in the autolysis phenotype. However, with the agr group II knockout strain RN9120 (figure 6B), growth in 1 μg/mL vancomycin selected for a population of bacteria that showed a phenotype of significantly decreased autolysis [28].

These results suggest that agr group II S. aureus may have an intrinsic survival advantage during exposure to glycopeptides and may be more likely to develop incremental glycopeptide resistance. However, it is difficult to make a sound conclusion on the basis of experiments performed on only a single agr knockout representing each agr group. We already know that non–group II strains can develop into GISA. Conclusions from experiments using only 1 representative strain for each agr group can be confounded by unknown baseline mutations in the test strains. One potential confounding variable that could hamper the selection of the RN6390/RN6911 agr group 1 pair into GISA is that these strains are sigB mutants [32]. Other investigators have noted overexpression of sigB in S. aureus selected under glycopeptide expression in vitro and in vivo, suggesting that this gene may play a role in the development of GISA [19, 20, 22]. The mechanism by which sigB could assist the development of glycopeptide heteroresistance is unknown but may involve upstream repression of agr. More agr knockout representatives from each agr group need to be generated and studied before the agr group
Figure 4. Vancomycin population analysis of accessory gene regulator (agr) prototype strains and agr knockout strains before and after (V) growth in 1 μg/mL vancomycin. A, RN6390 is the agr group I strain, and RN6911 is the knockout strain. B, RN6607 is the agr group II strain, and RN9120 is the knockout strain. C, RN4850 is the agr group IV strain, and RN9121 is the knockout strain. Reprinted from Sakoulas et al. [28].

GENOTYPIC AND PHENOTYPIC ANALYSIS OF VANCOMYCIN EFFECTIVENESS AGAINST MRSA

Despite the limitations of our data, our finding that agr group II represented the majority of MRSA in some centers and all GISA from the United States, taken together with results of in vitro studies with the agr group II prototype strain and its knockout, prompted our interest in evaluating further the clinical efficacy of vancomycin against MRSA infection, with particular attention given to agr group, and in examining other possible changes in microbiological phenotype.

One retrospective study [33] revealed that vancomycin therapy was significantly more likely to fail for infections caused by MRSA of the agr group II genotype, compared with infections due to MRSA not of the agr group II. According to multivariate analysis, other predictors of vancomycin treatment failure were renal failure ($P = .047$) and coronary artery disease ($P = .150$). The agr group II genotype was the most powerful predictor of vancomycin treatment failure in that study ($P = .005$) [33].

A subgroup analysis of the same population showed that both decreased in vitro killing by 15 μg/mL vancomycin and a higher vancomycin MIC were predictors of vancomycin treat-
Figure 6. Triton-X (0.01%)–induced autolysis of accessory gene regulator (agr) prototype strains and agr knockout strains before and after (V1) growth in 1 \(\mu\)g/mL vancomycin. Graphs denote a percentage of the initial optical density (OD) present over time. A, RN6390 is the agr group I, and RN6911 is the knockout strain. B, RN6607 is the agr group II strain, and RN9120 is the knockout strain. C, RN4850 is the agr group IV strain, and RN9121 is the knockout strain. Reprinted from Sakoulas et al. [28].

Figure 7. Killing of RN6607, an accessory gene regulator (agr) group II wild-type strain, and RN9120, an agr group II knockout strain, in 16 \(\mu\)g/mL vancomycin. Reprinted from Sakoulas et al. [11] with permission from the American Society for Microbiology.

The correlation of agr group II with vancomycin treatment failure is not universal, however. In a separate study limited to MRSA bloodstream infections occurring at a single institution [35], where the predominant clone was not agr group II, the agr group II genotype was not predictive of prolonged bacteremia during vancomycin therapy. In that study, however, both loss of agr function, as assayed by delta-hemolysin expression, and a phenotype of diminished autolysis were predictive of prolonged MRSA bacteremia [35].

A collective analysis of these data allows us to hypothesize that the loss of agr function and a phenotype of diminished autolysis may prove to be a more universal predictor of vancomycin treatment failure that may be applicable to strains across all agr groups. The finding that agr group II is a strong predictor of treatment failure [3]. None of the organisms in these investigations had vancomycin MICs of >2 \(\mu\)g/mL, so all were fully susceptible [34]. However, these studies showed that MRSA strains with vancomycin MICs of <0.5 \(\mu\)g/mL responded much more readily (56%) than did MRSA strains with MICs of 1–2 \(\mu\)g/mL (10%). Thus, subtle changes in the MIC that were within the “susceptible” range and differences in susceptibility to killing (which is rarely determined in routine clinical practice) may contribute to clinical failure. It is important, however, not to use these results prematurely to predict the likelihood of clinical failure with glycopeptide therapy in patients with MRSA infections. Our studies were based on a population of patients who, for the most part, experienced glycopeptide therapy failure or who were intolerant to such therapy and who, therefore, were referred for investigational drug protocols. These results should be confirmed by prospectively conducted studies.
vancomycin treatment failure may be confounded by the fact that this genotype may simply be more vancomycin “experienced,” because it is the predominant agr type in US hospitals. An MRSA agr group II strain is more likely to have been present in a nosocomial setting and is, therefore, more likely to have been exposed previously to vancomycin and more likely to develop physiological changes that may confer advantages in the face of subsequent vancomycin exposure. Nevertheless, it may still be possible that agr group II strains have some advantage under vancomycin selection pressure and, perhaps, may have emerged as a result of the burgeoning use of vancomycin.

To answer this question, more agr knockouts of each agr group need to be analyzed in terms of in vitro susceptibility to vancomycin and bactericidal activity. In addition, it would be useful to have information on how effective vancomycin treatment is in animal models of infection using agr wild-type and knockouts of different S. aureus agr genotypes.

**IS THE AGR OPERON UNNECESSARY “EXCESS BAGGAGE” FOR A NOSOCOMIAL PATHOGEN?**

If function of the agr operon were to exert a metabolic demand on S. aureus, one could envision that it would be suppressed in situations where this demand exceeds survival benefits conferred to the organism. It is known that agr up-regulates production of secreted virulence factors that mediate host invasion and down-regulates cell-associated virulence factors, such as microbial surface components recognizing adhesive matrix molecules [14]. It is a reasonable conjecture that, in the hospital environment, where iatrogenic invasion with surgical procedures, mechanical ventilation, and indwelling catheters and devices might seem to render bacterial virulence factors almost redundant, greater survival advantage to the organism would be conferred by properties resulting in greater resistance to killing by antibiotics and greater adherence to foreign materials.

Our evaluation of delta-hemolysin production in different groups of S. aureus strains supports this hypothesis. We found that 41% of 148 MRSA bloodstream isolates, but only 27% of 33 methicillin-susceptible S. aureus bloodstream isolates, failed to produce delta-hemolysin, a difference that was statistically significant [28]. We noted that 10 (100%) of 10 of bloodstream, community-acquired MRSA isolates produce delta-hemolysin. Evaluation of 200 community-acquired MRSA found that 96.5% produce delta-hemolysin (M. Rybak, personal communication). In other words, the probability that an S. aureus strain will produce delta-hemolysin is related to the probability that the strain is a community-acquired pathogen, rather than a nosocomial pathogen.

We believe that abrogating agr function not only relieves an organism of unnecessary metabolic demands but actually confers some beneficial properties in a nosocomial setting. We have already discussed that loss of agr function in the agr group II prototype, coupled with exposure to subinhibitory concentrations of vancomycin, is associated with the development of vancomycin heteroresistance. We took this a step further by showing that in vitro killing of S. aureus by clinically desirable concentrations of vancomycin (15 μg/mL) [36] is attenuated by the loss of agr function. Figure 7 shows that vancomycin kills the agr group II prototype RN6607 and the respective agr knockout RN9120 [11].

The notion that loss of agr function attenuates vancomycin killing in S. aureus is strengthened by our findings from the study of 3 different groups of clinical isolates, which showed, at 72 h in vitro, a decreased mean rate of killing of S. aureus lacking delta-hemolysin expression (table 1). Statistical significance was achieved in 2 of the 3 experiments. The third study, which did not achieve significance, was an analysis of the previously published isolates that was heavily weighted toward vancomycin treatment failure. Not surprisingly, the group of strains was weighted heavily in favor of strains that did not produce delta-hemolysin. This observation was supported by results of a clinical study showing that loss of delta-hemolysin activity was associated with persistent MRSA bacteremia during vancomycin therapy [35].

In addition, loss of agr function has been found by other investigators to be associated with increased biofilm production [37]. We confirmed this observation with the agr-positive/agr-knockout pairs. Figure 8 depicts polystyrene adherence, a marker of biofilm production, of the isogenic pairs discussed above: the agr group II prototype RN6607 and the agr knockout RN9120, and the MRSA endocarditis isolates A5937 and heteroresistant GISA A5940 [11]. In both of these pairs, inactivation of agr either through genetic manipulation or through in vivo selection resulted in a greater ability to adhere to polystyrene.

**Table 1. Delta-hemolysin expression and vancomycin killing in vitro among clinical methicillin-resistant Staphylococcus aureus isolates.**

<table>
<thead>
<tr>
<th>Experiment, delta-hemolysin expression status</th>
<th>No. of isolates</th>
<th>Vancomycin killing at 72 h, mean ± SD, log₁₀ cfu/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt; Positive</td>
<td>13</td>
<td>5.71 ± 1.40</td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
<td>4.54 ± 1.24</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt; Positive</td>
<td>12</td>
<td>5.58 ± 1.38</td>
</tr>
<tr>
<td>Negative</td>
<td>13</td>
<td>4.43 ± 1.22</td>
</tr>
<tr>
<td>3&lt;sup&gt;c&lt;/sup&gt; Positive</td>
<td>9</td>
<td>5.64 ± 1.03</td>
</tr>
<tr>
<td>Negative</td>
<td>21</td>
<td>5.00 ± 2.02</td>
</tr>
</tbody>
</table>

**NOTE** cfu, colony-forming unit.

<sup>a</sup> P = .0296.

<sup>b</sup> P = .038.

<sup>c</sup> P = .382.
Expression of *agr* has been shown by several investigators to be inversely related to fibronectin-binding protein (FnBP) expression [38, 39]. FnBPA and FnBPB are known to play important roles in the ability of *S. aureus* to colonize host tissues and implanted biomedical devices and, even, to allow the organism to survive as an intracellular pathogen by promoting endocytic uptake of *S. aureus* by epithelial, fibroblast, and endothelial cell lines [40–42]. FnBP expression, therefore, offers selective advantage to nosocomial pathogens by facilitating both adherence to biomedical devices (e.g., catheters) and nasal colonization of patients and staff members. Furthermore, antimicrobial therapy would be compromised against biofilm-associated and intracellular organisms. FnBP expression may also be affected by administration of fluoroquinolones, which some epidemiologic studies have shown to be associated with MRSA infection [43].

Finally, recent work shows that loss of *agr* function may be associated with resistance to endogenous thrombin-induced platelet microbicidal proteins, perhaps by decreasing murein hydrolase–mediated autolysis [44]. Thrombin-induced platelet microbicidal proteins are small, cationic, staphylocidal peptides secreted by mammalian platelets in response to agonists generated at sites of endovascular damage and infection (e.g., thrombin) [45, 46]. Staphylococcal strains that exhibit stable low-level in vitro resistance to killing induced by thrombin-induced platelet microbicidal proteins appear to have a survival advantage in experimental endocarditis [47, 48]. Moreover, human MRSA strains from patients with endocarditis and infections associated with the use of vascular devices tend to exhibit similar, stable, low-level, thrombin-induced platelet microbicidal protein resistance in vitro, compared with strains isolated from patients with nonvascular infections [45, 46]. Resistance to these endogenous antimicrobial properties has been associated with both increased virulence in animal models of endovascular infection and, more recently, persistence of MRSA bacteremia during vancomycin therapy [35, 47, 48].

It would appear from these emerging data that loss of *agr* function might well confer immense survival benefit to a nosocomial organism, such as MRSA. Some investigators have even suggested that *agr* is physiologically suppressed in vivo [49].

**DID PAST UNDERDOSING OF VANCOMYCIN CONTRIBUTE TO ITS POOR EFFICACY TODAY?**

Pharmacokinetic properties of vancomycin, as well as the prescribing practices of physicians over the years, may have contributed to selective pressure for the evolution of MRSA strains that are now relatively refractory to the antimicrobial effects of vancomycin. First, vancomycin is a large, complex molecule. Its large size and its protein binding rate of 30%–55% are thought to explain its relatively poor penetration into lung tissue [50]. Cruciani et al. [51] showed that, after an infusion of 1 g of vancomycin, 16% of patients had no detectable levels of drug in the lung; after 12 h, 43% of patients had undetectable concentrations. In general, vancomycin concentrations in lung tissue were 20%–30% of the concentrations in serum [51]. Given that MRSA may frequently colonize the lower respiratory tract of patients undergoing ventilation and the upper respiratory tract of other hospitalized patients, this pharmacokinetic property of vancomycin may allow MRSA in the respiratory tract to be exposed to subinhibitory concentrations of vancomycin.

Vancomycin is usually administered to adult patients every 12 h when renal function is normal. In younger patients, increased clearance may result in very low trough serum concentrations; therefore, younger patients require dosing every 6 h. For patients undergoing hemodialysis, vancomycin traditionally has been administered once every 7–10 days [52, 53]. This dosing interval has been shown to result in serum trough concentrations of vancomycin that are as low as 1 μg/mL [54]. Patients undergoing hemodialysis using high-flux membranes require vancomycin supplementation after each hemodialysis treatment [55]. Thus, both rapid clearance in some patients with normal renal function and poor penetration into secretions would result in exposure of MRSA to subinhibitory concentrations of vancomycin.

Finally, vancomycin has long been feared to be a potent ototoxic and nephrotoxic agent. However, with improved purification methods in manufacturing and by use of serum concentrations <50 μg/mL, these adverse effects are quite rare in the absence of coadministration of aminoglycosides [50].
ertheless, many physicians still severely underdose the drug, driven largely by reference laboratory values for peak and trough concentrations that are far lower than what many experts believe are clinically optimal. Although there have never been any definitive data correlating vancomycin efficacy and concentration, most experts will aim to achieve trough levels of \( \sim 15 \mu g/mL \). It is not uncommon, however, to find many hospital and reference laboratories accepting trough serum concentrations of \( 5 \mu g/mL \). These properties make it very conceivable that MRSA would be exposed to vancomycin concentrations of 1–2 \( \mu g/mL \) in the respiratory tract epithelia of patients receiving vancomycin therapy. Subinhibitory concentrations of vancomycin may be the critical element in selecting for GISA. \textit{S. aureus} recovered from killing assays in which vancomycin concentrations are 16 \( \mu g/mL \) show no changes in vancomycin heteroresistance, compared with the parent strain. Other investigators have come to similar conclusions when studying the development of vancomycin heteroresistance by use of an in vitro pharmacodynamic model (B. Tsuji and M. Rybak, unpublished observations). Additional evidence is derived from the unique experience of a patient with chronic, endovascular MRSA infection in whom a GISA phenotype did not develop, despite the receipt of vancomycin therapy for 9 months, which is a duration much longer than that for patients who developed GISA. In this patient, vancomycin trough serum concentrations of \( \geq 10 \mu g/mL \) were meticulously maintained.

We conclude that a driving force in the development of GISA is very likely to be the duration of exposure to subinhibitory concentrations of vancomycin, rather than the duration of exposure to clinically desirable vancomycin concentrations. The pharmacokinetic properties of vancomycin, conservative dosing regimens used by clinicians over the years because of toxicity concerns, and marked increase in use over the past 2 decades [56–58] may have allowed exposure to subinhibitory vancomycin concentrations, which facilitate the selection of heteroresistant colonies and GISA in vitro.

CONCLUSION

Increasing frequencies of both community-acquired and nosocomially acquired MRSA infections have resulted in enormous increases in vancomycin use for therapeutic, prophylactic, and empirical strategies [56–58]. Not unexpectedly, the isolation of MRSA strains exhibiting decreased susceptibility to vancomycin in association with therapeutic failures in patients with invasive infections has now been well chronicled [59–62]. MRSA strains appear to have developed several adaptive measures to enhance their survival in the nosocomial setting. This adaptation appears to include, but is likely not limited to, the abrogation of \textit{agr} function. This response appears to confer to the organism an enhanced ability to survive under vancomycin selection pressure. Recent work suggests that \textit{agr} serves to regulate more than virulence factor expression. Does \textit{agr} itself have regulatory effects on cell wall synthesis, or does loss of \textit{agr} function simply provide the platform for the development of glycopeptide resistance by diminishing autolysis through decreased murein hydrolase expression, thereby increasing the probability of survival in a glycopeptide environment where subsequent mutations may be selected?

Whether certain MRSA genotypes are more likely than others to evolve into GISA is a question of great interest that deserves further study. What appears obvious, however, is that MRSA has undergone a physiological evolution, perhaps fueled in part by increased vancomycin use and suboptimal dosing, that has resulted in only very subtle microbiological changes, in terms of susceptibility to vancomycin, but has conferred an enhanced ability to survive our antimicrobial therapies.

Acknowledgments

Potential conflicts of interest. G.S. serves on speakers’ bureaus for Sanofi-Aventis Pharmaceuticals and Cubist Pharmaceuticals; R.C.M., Jr. and G.M.E. have received support through Pfizer Pharmaceuticals.

References


33. Tarwood JM, McCormick JK, Paustian ML, Kapur V, Schlievert PM.


