Staphylococcus aureus Accessory Gene Regulator (agr) Group II: Is There a Relationship to the Development of Intermediate-Level Glycopeptide Resistance?

George Sakoulas,1,a George M. Eliopoulos,1,3 Robert C. Moellering, Jr.,1,3 Richard P. Novick,4 Lata Venkataraman,2 Christine Wennersten,1 Paola C. DeGirolami,2,3 Mitchell J. Schwaber,1,3 and Howard S. Gold1,3

1Department of Medicine, Division of Laboratory and Transfusion Medicine, and 2Department of Pathology, Beth Israel Deaconess Medical Center, and 3Harvard Medical School, Boston, Massachusetts; 4Skirball Institute of Biomolecular Medicine and New York University School of Medicine, New York

We previously determined that all 6 Staphylococcus aureus strains with confirmed intermediate-level resistance to glycopeptides (glycopeptide intermediate S. aureus [GISA]) from the United States that we tested belonged to accessory gene regulator (agr) group II. In the present study, we found that 56% of surveyed bloodstream methicillin-resistant S. aureus isolates ( ) at our hospital were agr group II, whereas only 24% of methicillin-susceptible S. aureus isolates ( ) were agr group II ( ). Population analysis of genetically engineered agr-null and parent wild-type strains of groups I, II, and IV revealed that, when agr function is lost, the agr group II knockout S. aureus was most likely to develop glycopeptide heteroresistance after growth in 1 μg/mL but not 16 μg/mL vancomycin. This strain was unique in showing decreased autolysis after growth in these conditions. This study suggests that some S. aureus strains have an intrinsic survival advantage under a glycopeptide selective pressure, which is possibly related to reduced autolysis after exposure to subinhibitory concentrations of glycopeptide.

The accessory gene regulator (agr) locus of Staphylococcus aureus is recognized as a quorum-sensing gene cluster that generally up-regulates production of secreted virulence factors and down-regulates production of cell-associated virulence factors in a growth phase–dependent manner [1–4]. A recent study suggests that agr also may be a critical global regulator of cellular metabolism [5]. Polymorphisms in agrB, agrC, and agrD define 4 S. aureus agr specificity groups (groups I–IV) [1–4]. Previous published reports have noted that the majority of clinical S. aureus isolates belong to agr group I [6, 7]. More recent data demonstrate that the vast majority of community-acquired methicillin-resistant Staphylococcus aureus (MRSA) in France and around the world belong to agr group III [8–10]. The majority of basic investigations on S. aureus have used strains belonging to agr group I (e.g., RN6390, MRSA COL, and 8325) [5, 11]. However, some investigators have noted differential regulation of agr gene expression in different S. aureus agr groups [5]. Therefore, data derived from studies of agr group I strains may not accurately represent the physiology of S. aureus strains of agr groups II, III, and IV [5].

The agr group II S. aureus subgroup was largely un-
characterized the first clinical \textit{S. aureus} isolate with intermediate-level resistance to glycopeptides (glycopeptide-intermediate \textit{S. aureus} [GISA]), Mu50, was completely sequenced [12, 13]. We previously reported that all 8 GISA and hetero-GISA strains that we studied from the United States and Japan belong to \textit{agr} group II [14]. We have since expanded our evaluation to include the 6 GISA isolates from the United States, which are available through the Network of Antimicrobial Resistance in \textit{Staphylococcus aureus} (NARSA) and have been confirmed by the Centers for Disease Control and Prevention.

We found that many clinical GISA isolates were defective in \textit{agr} function and that some isolates had point mutations within \textit{agr} that could account for this lack of function [14]. Since the publication of that article, our evaluation of more GISA isolates and selection of GISA via in vitro passage experiments suggest that the loss of \textit{agr} function is not a requirement for the development of GISA. Nevertheless, our study of 1 \textit{agr}+/\textit{agr}− group II \textit{S. aureus} isogenic pair revealed that, after growth in broth containing 1 μg/mL vancomycin, loss of \textit{agr} function was associated with a proclivity to develop glycopeptide heteroresistance and to attenuate the bactericidal activity of vancomycin in vitro at clinically relevant concentrations [14]. Another observation that supports a relationship between loss of \textit{agr} function and reduced susceptibility to glycopeptides is the >99% reduction in the levels of the effector molecule of \textit{agr}, RNA III, in a teicoplanin-resistant \textit{S. aureus} mutant, compared with the teicoplanin-susceptible parent strain [15]. Recent work has determined that most GISA strains from around the world share a common genetic ancestor [16, 17].

The present study sought to shed additional light on whether the preponderance of \textit{S. aureus} \textit{agr} group II among clinical GISA strains in the United States was a reflection of the MRSA pool of isolates from which they arose or whether there was a possible proclivity of some \textit{S. aureus} \textit{agr} group II clones to develop in intermediate-level resistance to glycopeptides the appropriate selective environment.

**MATERIALS AND METHODS**

**Clinical strains.** Unique patient bloodstream isolates of MRSA were collected at the Beth Israel Deaconess Medical Center (BIDMC; Boston) from September 1998 through November 2001. The presence of \textit{mecA} was confirmed by polymerase chain reaction (PCR), as described elsewhere [18]. Unique patient bloodstream isolates of methicillin-susceptible \textit{S. aureus} (MSSA) were collected at BIDMC from May 2000 through September 2000 and from August 2001 through January 2002. Twenty MRSA bloodstream isolates from Johns Hopkins medical institutions (July 1997 through April 2000) and 9 MRSA bloodstream isolates from a neighboring Boston hospital from the years 1998, 1999, and 2001 were included in the study for comparison. All BIDMC and Johns Hopkins MRSA bloodstream isolates were analyzed by use of pulsed-field gel electrophoresis (PFGE) using 	extit{Smal}-macrorestriction genomic DNA, as described elsewhere [19]. Vancomycin susceptibility was determined by agar dilution, according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) [20].

As part of another study, we identified 31 \textit{mecA}-positive \textit{S. aureus} with oxacillin MICs =64 μg/mL, representing 17% of our MRSA sample [18]. Because of the finding that the MICs of antistaphylococcal β-lactams against several clinical GISA strains are low, we determined the representation of \textit{agr} group II among these isolates. Expression of β-hemolysin, a marker of \textit{agr} function, was determined for all isolates, as described elsewhere [14].

**Reference strains for \textit{agr} groups.** RN6390, RN6607, and RN4850 were previously described as \textit{agr}− \textit{S. aureus} prototype strains of \textit{agr} groups I, II, and IV, respectively [1, 2]. RN6911 (RN6390 \textit{agr}::tet\textit{M}), RN9120 (RN6607 \textit{agr}::tet\textit{M}), and RN9121 (RN4850 \textit{agr}::tet\textit{M}) are the respective \textit{agr}-null derivatives that have been described elsewhere [1, 2]. Additional \textit{S. aureus} \textit{agr}− group representative strains include the following: RN9353 and RN9356 (group I); RN9354 and RN9357 (group II); and RN5881 and RN9355 (group IV). All strains were analyzed by PFGE using \textit{Smal}-macrorestriction genomic DNA and were compared with clinical strains. Vancomycin, rifampin, ciprofloxacin, and oxacillin susceptibility testing were performed on all reference strains by agar dilution, according to the recommendations of the NCCLS [20]. MICs for the reference strains were as follows: vancomycin, 0.5–1 μg/mL; oxacillin, =0.25 μg/mL; rifampin, 0.125 μg/mL; and ciprofloxacin, 0.25 μg/mL.

**Identification of \textit{agr} group II \textit{S. aureus}.** Clinical \textit{S. aureus} isolates were studied by use of PCR, using the \textit{agr} group II–specific primers reported elsewhere [6]. The 5′ primer sequence (GenBank accession no. AF001782, bp 155–163) was 5′-GTAGAGCCGTATTGATTCC-3′ and the 3′ primer sequence (GenBank accession no. AF001782, bp 600–618) was 5′-GTATTTTATCTCTTTAAG-3′. The reaction conditions used were as described elsewhere [14, 18]. The PCR program consisted of an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s; and final extension step at 72°C for 10 min [6]. Products were analyzed by use of standard methods of agarose gel electrophoresis [12, 18].

**Effect of subinhibitory concentration of vancomycin on vancomycin heteroresistance.** Single colonies of \textit{agr} groups I–IV reference strains were grown on sheep blood agar (Northeast Laboratory) and were inoculated into brain-heart infusion (BHI) broth (Becton Dickinson) containing 1 μg/mL vancomycin (lot 110469; American Pharmaceutical Partners). After 14–18 h of growth, the culture was diluted 1:1000 in fresh BHI.
broth containing 1 μg/mL vancomycin and was grown for 14–18 h. A 5-μL loop of the culture was plated on BHI agar plates containing 1 μg/mL vancomycin. Relatively stable genotypic changes affecting individual strains as a result of vancomycin exposure were confirmed by comparison of organisms isolated immediately after exposure of 1 μg/mL vancomycin to those that underwent 7 subsequent passages on antibiotic-free BHI agar plates.

**Effect of clinically desirable concentrations of vancomycin on vancomycin heteroresistance.** Overnight cultures in BHI broth were diluted 1:400 in fresh broth to obtain a starting inoculum of 10^7 cfu/mL, to which was added vancomycin, for a final concentration of 16 μg/mL. Cultures were incubated at 35°C, sampled after 72 h, and serially diluted from 10^8 to 10^2 cfu/mL. Single colonies were isolated on sheep blood agar plates and were analyzed by population analysis.

**Selection of GISA from prototype strains representing various agr groups.** Attempts to select for glycopeptide-resistant mutants of RN6390, RN6911, RN6607, RN9120, RN4850, and RN9121 involved passage in BHI broth containing incremental increases in vancomycin concentration. A single colony was inoculated into 1 μg/mL vancomycin and was grown at 35°C with agitation. All strains demonstrated growth at this concentration. Cultures demonstrating visible growth were diluted 1:5000 in fresh BHI broth containing vancomycin at increasing increments of 1 μg/mL. If no growth was visible after 72 h, the test strain was eliminated from the experiment. Cultures demonstrating visible growth at each of 1 μg/mL vancomycin increment were plated on BHI agar plates containing 1 μg/mL vancomycin, and colony morphology and pigment production were assessed. Intermediate-level glycopeptide resistance was confirmed by use of macrodilution broth methods, according to NCCLS guidelines [20].

**Population studies.** Bacteria grown overnight on sheep blood agar plates were resuspended in 2 mL of BHI broth to a density equivalent to a 1.0 McFarland standard. Twenty-five microliters of this suspension and of serial 10-fold dilutions were plated on BHI agar plates with varying concentrations of vancomycin (0–16 μg/mL). Colonies were counted after 48 h of growth at 35°C.

**Mutation frequency calculation.** The evaluation of hypermutability of chromosomally encoded genes as a basis for the rapid development of antimicrobial resistance has been evaluated elsewhere [22], and we used similar methods [21, 22]. Bacteria were grown overnight in BHI broth to a density of ~5 × 10^8. Cell pellets were resuspended in 1/10 (wt/vol) fresh BHI and serially diluted 10-fold to 10^-8 cfu/mL. Twenty-five microliters of each dilution was plated in duplicate on antibiotic-free BHI agar plates and on plates containing 1 μg/mL rifampin or 1 μg/mL ciprofloxacin. These antibiotic concentrations represented 8 times the MIC of rifampin and 4 times the MIC of ciprofloxacin for RN6390, RN6911, RN6607, RN9120, RN4850, and RN9121, as determined by agar dilution. Colonies were counted at 24 h. The rate of spontaneous mutants conferring low-level resistance was determined by dividing the colony-forming units in antibiotic by the total number of colony-forming units. The experiment was performed twice in duplicate.

**Autolysis assays.** Autolysis in Triton X-100 of S. aureus has been evaluated elsewhere in the context of intermediate-level glycopeptide resistance [23, 24] and agr function [25]. Overnight cultures in BHI broth (with or without 1 μg/mL vancomycin) were diluted 1:1000 in BHI broth (with or without 1 μg/mL vancomycin) and were grown to an OD_{600} of 0.6–0.8. Cells were aliquoted to a clean sterile microcentrifuge tube, cooled on ice, and spun for 30 s in a table-top microcentrifuge. The bacterial pellet was washed twice with cold distilled water and resuspended to an OD_{600} of ~1.0 in 0.01% Triton X-100 and 50 mM glycine (pH 8.0 buffer). The suspension (200 μL) was aliquoted in duplicate onto a sterile 96-well flat-bottomed plastic tissue culture plate (Costar) and was incubated at 37°C with gentle agitation. Optical density at 630 nm was measured at hourly intervals using an MRXII Microplate Reader (Dynex Technologies). Autolysis was determined graphically as the percentage of optical density at 630 nm at time zero versus time.

**Statistical analysis** Differences in proportions were determined by use of the 2-tailed Fisher’s exact test (http://www.matsforsk.no/ola/fisher.htm).

**RESULTS**

**Representation of agr group II among S. aureus bloodstream isolates.** We used PFGE to grossly assess the genetic similarity of our agr group I–IV representatives to clinical MRSA isolates from our hospital and GISA. We found that the predominant PFGE type of MRSA at our hospital was similar to those of clinical GISA and that the PFGE type of the predominant MRSA appeared to be most closely related to RN6607, an agr group II prototype for which an agr knockout derivative, RN9120, already existed.

Using PCR primers specific to the agr group II sequences, we determined that 56% of 148 MRSA bloodstream isolates from our hospital were agr group II. This level was statistically higher than for MSSA, of which 24% were agr group II (P = .001). Smaller samples of clinical MRSA bloodstream isolates from 2 other hospitals showed a representation of agr group II similar to our hospital's sample (figure 1).

A feature of some clinical GISA isolates has been the increase in susceptibility to antistaphylococcal β-lactams without the loss of mecA [23, 26–28]. Analysis of 31 unique mecA-positive S. aureus clinical isolates for which the oxacillin MIC was ≤64 μg/mL revealed that 83% were agr group II, which was highly
statistically significantly difference from the proportion among all bloodstream MRSA ($P = .004$; figure 1).

**Function of agr in clinical bloodstream isolates.** Among our hospital’s bloodstream isolates, 41% of MRSA but only 27% of MSSA failed to produce δ-hemolysin, which is an indicator of the lack of agr function. This difference was statistically significant ($P = .03$). A subset analysis of MSSA and MRSA based on the expression of δ-hemolysin showed no correlation with agr group II and expression of δ-hemolysin (data not shown).

**Selection of vancomycin heteroresistance in vitro in the presence of vancomycin.** We grew $agr^-/agr^-$null isogenic reference strain pairs for agr group I (RN6390/RN6911), group II (RN6607/RN9120), group IV (RN4850/RN9121), and 1 unrelated $agr^+$ representative from each group (groups I–IV) in 1 μg/mL vancomycin, as described in Materials and Methods. The respective vancomycin population analyses of the agr wild-type/knockout pairs, before and after growth in vancomycin, are given in figure 2. The population analyses of the other $agr^+$ strains are not shown. Growth in 1 μg/mL vancomycin had the greatest effect on RN9120, the agr group II knockout (figure 2B, right), compared with the knockouts for agr groups I and IV and all the $agr^+$ strains tested.

The increase in vancomycin heteroresistance of RN9120 after growth in 1 μg/mL vancomycin (figure 3; RN9120V1) probably is based on relatively stable genotypic changes, given that the shifted population analysis remained stable to 7 passages on antibiotic-free media (figure 3; RN9120V1-p7). Population analysis performed on all 10 agr group representatives isolated after 72 h of exposure to 16 μg/mL vancomycin did not demonstrate any increase in heteroresistance (e.g., the population analysis graph for RN9120, the agr group II knockout exposed to 16 μg/mL vancomycin, demonstrated in figure 3 [RN9120V16]).

Serial passage of RN9120 in incremental 1 μg/mL increases of vancomycin using BHI broth readily yielded a GISA (figure 3; RN9120-GISA). This particular method was not successful in yielding GISA for the agr groups I and IV $agr^+/agr^-$ pairs or for the group II $agr^-$ strain RN6607.

**Mutation rate.** Recent findings of mutS mutations in Mu50 and the accelerated development of vancomycin heteroresistance in a laboratory-generated mutS mutant of RN4220 suggested that RN9120 may have a higher mutation rate to explain the development of vancomycin heteroresistance in vitro under the appropriate selective environment [29, 30]. We used the frequency of spontaneous mutants with low-level resistance to rifampin and ciprofloxacin as a gauge of mutation rate. The results shown in table 1 demonstrate the rate of spontaneous mutants with low-level resistance to rifampin and ciprofloxacin in agr groups I, II, and IV S. aureus prototype strains. We found no significant differences in mutants with low-level resistance to rifampin and ciprofloxacin in agr groups I, II, and IV S. aureus prototype strains. We found no significant differences in mutants with low-level resistance to rifampin and ciprofloxacin for the group II agr strain RN6607.

**Autolysis in Triton X-100.** Vancomycin killing is dependent on autolysis, and the development of GISA has been associated with diminished autolysis [23, 24, 27]. Furthermore, loss of agr function has been determined to diminish autolysis [25]. The results of autolysis in the presence of Triton X-100 for S. aureus agr group representatives and agr-null derivatives analyzed immediately after growth in 1 μg/mL vancomycin are
Figure 2. Vancomycin population analysis of *Staphylococcus aureus* agr group representatives and agr-null derivatives before and after (V) growth in 1 μg/mL vancomycin. A, agr group I RN6390 (agr⁺; left); RN6911 (agr⁻; right). B, agr group II RN6607 (agr⁺; left); RN9120 (agr⁻; right). C, agr group IV RN4850 (agr⁺; left); RN9121 (agr⁻; right).

shown in figure 4. Most noteworthy is the marked decrease in autolysis observed with agr group II knockout RN9120 (figure 4B, right). The agr⁺/agr⁻—null representatives of agr groups I and IV showed increased autolysis after growth in vancomycin.

**DISCUSSION**

*S. aureus* is subdivided into 4 agr specificity groups on the basis of polymorphisms at the agr locus [1–4]. It is well established
that there is AgrD octapeptide autoinduction of strains within the same \textit{agr} group and bacterial interference between \textit{S. aureus} that are in different \textit{agr} groups, as well as between different staphylococcal species [3, 4]. This may result in competition for environmental resources among \textit{S. aureus} clones that are in different \textit{agr} groups and cooperation of strains within the same \textit{agr} group [3]. Therefore, it is believed that strains within a given \textit{S. aureus} \textit{agr} group are more related genetically and share similar biological properties [3]. However, these relationships need to be further defined, especially in the context of commonly used typing methods, such as PFGE and multilocus sequence typing (MLST), to serve as possible explanations for variation in experimental results obtained by independent investigators using different bacterial strains.

With this in mind, we have shown elsewhere that all 8 of the clinical GISA and hetero-GISA isolates from the United States and Japan that we studied belonged to \textit{agr} group II [14]. Our analysis to date has included all 6 confirmed US GISA (vancomycin MIC, 8–16 \textmu g/mL) contained in the NARSA repository of the total 8 confirmed US cases. All these isolates belong to \textit{agr} group II.

The \textit{agr} specificity group has not traditionally been a polymorphism used to evaluate the ancestry of current MRSA clones. Recent work by Monk et al. [16], Enright et al. [17], Crisostomo et al. [31], and Oliveira et al. [32], using the very discriminatory method of MLST shows that sequence type 5 (ST-5) MRSA is the genetic progenitor background of most GISA strains throughout the world, including Mu50, US GISA, and the New York/Japanese MRSA clone. It is noteworthy that the first clinical vancomycin-resistant \textit{S. aureus} in the United States is highly related to the New York/Japanese clone [33, 34].

Because these clones are identical, as determined by MLST, it was not surprising that we found them to be related by the less discriminatory methods of \textit{agr} typing and PFGE, which allows us to conclude that ST-5 MRSA is probably a clonal background that lies within the \textit{agr} group II specificity group. We believe, although with less certainty, that RN6607 and RN9120, the prototype \textit{agr} \textit{agr} group II strains used in this study, may be members of the ST-5 genetic background. However, more work specifically aimed at correlating \textit{agr} type, PFGE patterns, and MLST would be helpful to further substantiate this hypothesis.

In the present investigation, we showed the following. First, \textit{agr} group II represented the majority of MRSA bloodstream isolates tested from 3 US hospitals; clinical meca-positive MRSA with oxacillin MIC \textless 64 \mu g/mL are further enriched for \textit{agr} group II. Second, just short of a majority of MRSA bloodstream isolates demonstrated defective \textit{agr} function. Third, subinhibitory concentrations of vancomycin preferentially selected for heteroresistance in RN9120, an \textit{agr}-null group II \textit{S. aureus}, whereas clinically desirable vancomycin concentrations in vitro did not provide a selective environment for the development of heteroresistance for this isolate. Fourth, there was no dif-

Table 1. Frequency of spontaneous low-level resistance mutants as a measure of mutation rate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{agr} Group</th>
<th>\textit{agr} Function (mutation)</th>
<th>Ciprofloxacin (1 \mu g/mL), (2 \times) MIC</th>
<th>Rifampin (1 \mu g/mL), (8 \times) MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN6390</td>
<td>I</td>
<td>Yes</td>
<td>(7.5 \times 10^{-8})</td>
<td>(6.6 \times 10^{-8})</td>
</tr>
<tr>
<td>RN6911</td>
<td>I</td>
<td>Null (\textit{agr::tetM})</td>
<td>(6.2 \times 10^{-8})</td>
<td>(8.7 \times 10^{-8})</td>
</tr>
<tr>
<td>RN6607</td>
<td>II</td>
<td>Yes</td>
<td>(9.2 \times 10^{-8})</td>
<td>(8.8 \times 10^{-8})</td>
</tr>
<tr>
<td>RN9120</td>
<td>II</td>
<td>Null (\textit{agr::tetM})</td>
<td>(8.8 \times 10^{-8})</td>
<td>(1.6 \times 10^{-7})</td>
</tr>
<tr>
<td>RN4850</td>
<td>IV</td>
<td>Yes</td>
<td>(3.3 \times 10^{-8})</td>
<td>(8.8 \times 10^{-8})</td>
</tr>
<tr>
<td>RN9121</td>
<td>IV</td>
<td>Null (\textit{agr::tetM})</td>
<td>(4.0 \times 10^{-8})</td>
<td>(5.1 \times 10^{-8})</td>
</tr>
</tbody>
</table>
S. aureus Vancomycin Heteroresistance and agr

Figure 4. Autolysis in Triton X-100 of Staphylococcus aureus agr group representatives and agr-null derivatives before and after (V1) growth in 1 μg/mL vancomycin. A, agr group I RN6390 (agr\(^+\); left); RN6911 (agr\(^-\); right). B, agr group II RN6607 (agr\(^+\); left); RN9120 (agr\(^-\); right). C, agr group IV RN4850 (agr\(^+\); left); RN9121 (agr\(^-\); right).

...ference in mutation rate that could explain the more rapid development of heteroresistance of RN9120. Fifth, RN9120 was unique in showing a marked decrease in autolysis immediately after growth in 1 μg/mL vancomycin.

Our examination of SmaI macrorestriction PFGE patterns of MRSA bloodstream isolates from our hospital, GISA isolates from the US and Japan, and reference strains representing agr groups I–IV suggested that the PFGE pattern similar to that of prototype agr group II strain RN6607 was prevalent among clinical MRSA isolates. Therefore, on the basis of the assumption that agr grouping is a marker of ancestral relatedness, we suspected that agr group II isolates were prevalent among clini-
ical strains of MRSA. We confirmed this suspicion in this study using PCR, determining that agr group II represented 50%–56% of bloodstream MRSA from 2 Boston and 1 Baltimore hospital. This contrasts with MSSA bloodstream isolates, of which only 24% were agr group II. This also contrasts with European MRSA isolates, of which the majority are agr group I [7, 35].

Comparison of PFGE patterns of MRSA from our hospital with the predominating PFGE patterns of clinical MRSA in the United States (e.g., the New York/Japanese clone) suggested that our findings may be representative of US MRSA isolates, rather than a unique feature of MRSA in the hospitals from which our study isolates were obtained [36]. Although the survey of clinical isolates presented here is derived from only 3 hospitals and therefore may be considered to be limited, our analysis of 87 MRSA bloodstream isolates collected from 16 states in the United States as part of a study recently presented provided similar rates of agr group II S. aureus [37]. These observations are interesting in light of our finding that RN9120 has a potential intrinsic advantage in subinhibitory vancomycin concentrations in vitro and the increasing use of vancomycin in hospitals in the United States [38–40]. Of even greater significance is that the major finding of the study by Moise-Broder et al. [37] was that the agr group II specificity group was a very strong predictor in both univariate (P = .003) and multivariate (P = .005) analyses of vancomycin failure of treatment of MRSA bacteremia.

Additional evidence, albeit circumstantial at this point, on clonal selection of MRSA based on intrinsic advantages in vancomycin is the paucity of agr group II strains in community-acquired MRSA, a homogeneous population belonging to agr group III [8–10], and in MSSA. These strains undergo selection in a relatively vancomycin-free environment. The agr group II knockout RN9120, unlike its agr− parent prototype RN6607 or tested strains of agr groups I and IV, showed diminished autolysis after exposure to 1 μg/mL vancomycin. We chose this concentration of vancomycin because it was the highest concentration that allowed visible growth of all the isolates tested using BHI broth. However, because many hospital laboratory recommend vancomycin trough levels as low as 5 μg/mL to guide dosing by clinicians, because bactericidal activity of vancomycin against many MRSA isolates is low, despite optimal levels of antibiotic (authors’ unpublished data), and because vancomycin levels in the respiratory tract, a common colonization site for S. aureus in the hospital, are roughly one-third that observed in serum [41], it is very conceivable that our in vitro conditions of 1 μg/mL vancomycin would approximate concentrations that bacteria would be exposed to at colonization sites in vivo. Strains that are able to survive at these low concentrations of vancomycin through diminished autolysis would be set up for acquiring stepwise mutations toward intermediate-level glycopeptide resistance.

It is important to point out that our study was restricted to 1 agr+/agr− pair from agr groups I, II, and IV. An agr group III pair was not readily available. Determining whether the differences that we observed for RN9120 in 1 μg/mL vancomycin could be extrapolated to agr knockouts of other group II clones or whether the same results could be seen in strains within other agr groups would require study of multiple agr+ knockout pairs from all agr groups.

Prolonged administration of vancomycin is a consistent feature in all the clinical cases from which GISA were recovered. In our studies, in vitro exposure to 16 μg/mL vancomycin, a recommended target serum concentration [42], did not select for increased heteroresistance among any isolate studied, including the group II knockout strain RN9120. This finding, coupled with the population analysis changes induced by 1 μg/mL vancomycin, highlights that the selection of vancomycin heteroresistance in S. aureus probably occurs as a consequence of organisms being exposed to suboptimal concentrations of vancomycin in vivo and would explain, for example, the rapid development of GISA in a patient in the United States with a hepatic abscess in whom vancomycin trough concentrations over the course of 9 weeks of therapy were documented to range from 2.7 to 4.9 μg/mL [43].

With the pharmacokinetic properties of vancomycin in mind, we find it very interesting that patients receiving hemodialysis therapy were particularly well-represented in the GISA case series. Vancomycin serves as a cornerstone of empiric and definitive therapy of gram-positive infections in patients who have undergone hemodialysis. Although it has been common practice among some physicians to administer a once-weekly dose of vancomycin to the hemodialysis patients [44], trough concentrations at 1 week can be highly variable but typically range from 3 to 7 μg/mL [45, 46]. Concentrations as low as 1 μg/mL have been documented [47]. The pharmacokinetic profile of vancomycin in hemodialysis patients suggests that after a single dose, extremely low levels may persist for a prolonged period [45, 46, 48]. Such vancomycin dosing practices potentially provide the intravascular correlate of the in vitro studies that we performed in selecting vancomycin heteroresistance.

Although our findings and those of others suggest that some S. aureus clones with the ST-5 genetic background that are members of agr group II may have a proclivity toward glycopeptide resistance, published studies by other investigators of in vitro manipulation of MRSA COL (agr group I) suggest that glycopeptide resistance may develop in other S. aureus agr groups with sufficient selection pressure [24, 49]. We successfully passaged MRSA COL that lacked δ-hemolysin activity into GISA. In fact, agr group I MRSA with reduced susceptibility to glycopeptides have been noted in France (F. Vandenesch and
Vantage in the presence of subinhibitory levels of vancomycin, clones genetically related to this strain may have a survival advantage almost exclusively and, perhaps more specifically, strains that belong to the ST-5 genetic background. Therefore, agr group II is by no means a prerequisite for the GISA phenotype.

In summary, we undertook these studies to determine whether the preponderance of agr group II *S. aureus* among clinical GISA in the United States was a reflection of the MRSA pool of isolates from which they arose or whether there was a possible proclivity of some agr group II clones to develop intermediate-level glycopeptide resistance. It appears that both factors may be intimately intertwined. The agr group II specificity group may now represent the majority of clinical MRSA isolates in the United States. Our evaluation of an agr knockout derived from agr group II prototype *S. aureus* RN6607 suggested that some *S. aureus* clones genetically related to this strain may have a survival advantage in the presence of subinhibitory levels of vancomycin when agr function is lost, which is perhaps related to decreased autolysis in this environment. These clones also may have a proclivity to develop vancomycin heteroresistance when agr function is lost in the setting of exposure to suboptimal concentrations of vancomycin. Whether these findings can be extrapolated to other agr group II strains or to strains of agr groups I, III, or IV warrants further study.

To date, the preponderance of basic research into *S. aureus* physiology has focused on agr group I strains [5, 11]. In an era in which antimicrobial resistance in *S. aureus* is a paramount concern for both clinicians and scientists, the finding that MRSA and GISA isolates in the United States are predominantly agr group II and that community-acquired MRSA isolates are almost exclusively agr group III suggests that our choice of strains for study in the laboratory needs to keep pace with the evolution of *S. aureus* resistance. Further study of agr group II *S. aureus* and, perhaps more specifically, strains that belong to the ST-5 genetic background, will be important in our future understanding of the epidemiology and treatment of MRSA infections and may be essential for unraveling the mechanisms of glycopeptide resistance in *S. aureus*.

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