Accessory Gene Regulator (agr) Locus in Geographically Diverse Staphylococcus aureus Isolates with Reduced Susceptibility to Vancomycin

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The majority of infections with glycopeptide intermediate-level resistant Staphylococcus aureus (GISA) originate in biomedical devices, suggesting a possible increased ability of these strains to produce biofilm. Loss of function of the accessory gene regulator (agr) of S. aureus has been suggested to confer an enhanced ability to bind to polystyrene. We studied agr in GISA, hetero-GISA, and related glycopeptide-susceptible S. aureus isolates. All GISA strains from diverse geographic origins belong to agr group II. All GISA strains were defective in agr function, as demonstrated by their inability to produce delta-hemolysin. Hetero-GISA isolate A5940 demonstrated a nonsense mutation in agrA that was not present in a pulsed-field gel electrophoresis-indistinguishable vancomycin-susceptible isolate from the same patient. Various other agr point mutations were noted in several clinical GISA and hetero-GISA isolates. A laboratory-generated agr-null strain demonstrated a small but reproducible increase in vancomycin heteroresistance after growth in vitro in subinhibitory concentrations of vancomycin. This was not seen in the isogenic agr group II parent strain in which agr was intact. The in vitro bactericidal activity of vancomycin was attenuated in the agr-null strain compared to the parent strain. These findings imply that compromised agr function is advantageous to clinical isolates of S. aureus toward the development of vancomycin heterogeneous resistance, perhaps through the development of vancomycin tolerance.

Since 1997, several case reports have appeared describing Staphylococcus aureus clinical isolates with reduced susceptibility to glycopeptide antibiotics (5, 11, 13–15, 32, 35, 36). The mechanisms responsible for this low-level resistance are poorly understood but do not involve the vanA, vanB, vanC, vanD, vanE, or vanG genes that confer vancomycin resistance in enterococci (12–15). The preponderance of data suggests that the mechanism for intermediate-level vancomycin resistance in S. aureus may relate to sequestration of the antimicrobial agent to bind to polystyrene. We studied agr in GISA, hetero-GISA, and related glycopeptide-susceptible S. aureus isolates. All GISA strains from diverse geographic origins belong to agr group II. All GISA strains were defective in agr function, as demonstrated by their inability to produce delta-hemolysin. Hetero-GISA isolate A5940 demonstrated a nonsense mutation in agrA that was not present in a pulsed-field gel electrophoresis-indistinguishable vancomycin-susceptible isolate from the same patient. Various other agr point mutations were noted in several clinical GISA and hetero-GISA isolates. A laboratory-generated agr-null strain demonstrated a small but reproducible increase in vancomycin heteroresistance after growth in vitro in subinhibitory concentrations of vancomycin. This was not seen in the isogenic agr group II parent strain in which agr was intact. The in vitro bactericidal activity of vancomycin was attenuated in the agr-null strain compared to the parent strain. These findings imply that compromised agr function is advantageous to clinical isolates of S. aureus toward the development of vancomycin heterogeneous resistance, perhaps through the development of vancomycin tolerance.

Materials and Methods

Bacterial isolates. Details of strains used in this study are listed in Table 1. The first seven isolates in the table were the GISA and hetero-GISA strains evaluated in this study. The bottom set were control strains and isolates related to GISA. For the purposes of this study, GISA was defined as a strain that was determined by others and/or by us to have a vancomycin MIC of ≥8 μg/ml (11, 39). For isolates A6298 and A5940, the MIC of vancomycin was 4 μg/ml as determined by susceptibility testing methods proposed by the National Committee for Clinical Laboratory Standards (NCCLS) and therefore did not meet criteria for GISA. However, since these strains demonstrated subpopulations with the ability to grow on ≥4 μg of vancomycin/ml, they were considered hetero-GISA (39). All strains were confirmed to be S. aureus by PCR for a polymorphic region of the coagulase gene as described previously (40) with minor modifications (33). All GISA and vancomycin-susceptible isolates yielded a fragment of ca. 600 bp that was detected by 1% agarose gel electrophoresis with ethidium bromide staining examined under UV light (data not shown).

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TABLE 1. Characteristics of study isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>MIC(^a) (µg/ml) of</th>
<th>agr group</th>
<th>Delta-hemolysin activity</th>
<th>Source of infection</th>
<th>Comments (references)</th>
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<tbody>
<tr>
<td>PC-3</td>
<td>NY</td>
<td>8</td>
<td>II</td>
<td>0</td>
<td>HD gortex graft fistula (34)</td>
<td>GISA; original MIC of vancomycin, 8 µg/ml (12, 13)</td>
</tr>
<tr>
<td>Mu50(^b)</td>
<td>JPN</td>
<td>2</td>
<td>&gt;128 II</td>
<td>0</td>
<td>Mediatinitis status post surgery congenital heart disease</td>
<td>GISA; original MIC of vancomycin, 8 µg/ml (12, 13)</td>
</tr>
<tr>
<td>Mu(^b)</td>
<td>JPN</td>
<td>2</td>
<td>&gt;128 II</td>
<td>0</td>
<td>Lung</td>
<td>Hetero-GISA; original MIC of vancomycin, 3 µg/ml (12, 13)</td>
</tr>
<tr>
<td>HIP5836</td>
<td>NJ</td>
<td>8</td>
<td>128 II</td>
<td>0</td>
<td>Probable peritoneal dialysis catheter (34)</td>
<td>GISA</td>
</tr>
<tr>
<td>A5940(^c)</td>
<td>MO</td>
<td>4</td>
<td>4 II</td>
<td>0</td>
<td>Porcine aortic valve</td>
<td>Hetero-GISA</td>
</tr>
<tr>
<td>A6298(^c)</td>
<td>MA</td>
<td>4</td>
<td>&gt;128 II</td>
<td>0</td>
<td>Hemodialysis gortex graft fistula; origin knee replacement</td>
<td>Hetero-GISA; + beta-hemolysin</td>
</tr>
<tr>
<td>A6272(^c)</td>
<td>MA</td>
<td>8</td>
<td>&gt;128 II</td>
<td>0</td>
<td>Supraprenal aortic graft</td>
<td>GISA</td>
</tr>
<tr>
<td>A5937</td>
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<td>+</td>
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<tr>
<td>A6300</td>
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<td>2</td>
<td>&gt;128 II</td>
<td>0</td>
<td>Hemodialysis gortex graft fistula; origin knee replacement</td>
<td>MRSA related to A6298; + beta-hemolysin</td>
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<td></td>
<td></td>
<td>+</td>
<td></td>
<td>MRSA</td>
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<td></td>
<td>1</td>
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<td>+</td>
<td></td>
<td>Derived from growth of RN6607 in vancomycin</td>
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<tr>
<td>RN6607-V</td>
<td></td>
<td>2</td>
<td>&lt;0.25 II</td>
<td>+</td>
<td></td>
<td>Derived from growth of RN6607 in vancomycin</td>
</tr>
<tr>
<td>RN9120-V</td>
<td></td>
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<td>&lt;0.25 agr:&lt;tetM</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>RN9120-V</td>
<td></td>
<td>4</td>
<td>&lt;0.25 agr:&lt;tetM</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) VAN, vancomycin; OXA, oxacillin.

\(^{b}\) Strain authenticity was confirmed by PFGE (data not shown).

\(^{c}\) Previously unpublished strains with reduced vancomycin susceptibility: A5937 and A5940 are from the same patient isolated at different time points and are identical as determined by PFGE (Fig. 1), and A6300 and A6298 are from the same patient isolated at different time points and are identical as determined by PFGE (Fig. 1).

\(^{d}\) NY, New York; JPN, Japan; NJ, New Jersey; MO, Missouri; MA, Massachusetts.

Clonal relationships were determined by pulsed-field gel electrophoresis (PFGE) with Smal-macrorestricton genomic DNA (21). Pairs of vancomycin-susceptible and hetero-GISA isolates from the same patient were indistinguishable by PFGE despite differences in vancomycin susceptibility (Fig. 1).

Laboratory strains RN6607 and RN9120 are isogenic agr group II S. aureus except for the knockout of agr in RN9120 as described and characterized previously (19, 26). The clonal relationship between these strains was confirmed by PFGE. We obtained strains RN6607-V and RN9120-V by growing the original strains in brain heart infusion (BHI) broth with vancomycin at 1 or 2 µg/ml each for 24 h. Note that RN6607 is the tetM plasmid-minus derivative of SA502A, the source of the agr group II reference sequence for this study (16). SA32 is a clinical isolate of methicillin-resistant S. aureus (MRSA) with a vancomycin MIC of 1 µg/ml that does not demonstrate glycopeptide heteroresistance with exposure to vancomycin. It was determined to be agr group I by partial sequencing.

All subculturing was performed on sheep blood agar plates unless otherwise specified. Agar dilution susceptibility testing to oxacillin (mamolydate sodium salt) and vancomycin hydrochloride (Sigma Chemical Co., St. Louis, Mo.) were performed according to recommendations of the NCCLS (24). For oxacillin susceptibility testing, Mueller-Hinton agar (Becton Dickinson, Cockeysville, Md.) was supplemented with 2% NaCl in accordance with NCCLS recommendations (24). Quality control of each susceptibility testing experiment was performed with reference strains ATCC 35591 (MRSA), ATCC 25923 (methicillin-susceptible S. aureus [MSSA]), and ATCC 29213 (MSSA).

**Population studies.** Bacteria grown overnight on plates were resuspended in 2 ml of Mueller-Hinton broth to a density equivalent to 0.1 McFarland standard. Portions (25 µl) of this suspension and of serial 10-fold dilutions were plated on Mueller-Hinton agar and BHI agar (Becton Dickinson) plates with various concentrations of vancomycin (0 to 16 µg/ml). Colonies were counted after 48 h of growth at 35°C.

**Vancomycin bactericidal assays.** Overnight cultures were diluted 1:800 in BHI broth to obtain a starting inoculum of ca. 10^6 CFU/ml, to which was added 16 µg of vancomycin/ml. Samples obtained at 0, 4, 24, and 48 h and then daily thereafter for 5 days were serially diluted from 10 to 10^7. Next, 25 µl of each dilution was plated in duplicate onto sheep blood agar plates to determine the counts of viable bacteria.

**Polystyrene adherence assay.** Biofilm production of RN6607 and RN9120 was measured under different conditions by a polystyrene adherence assay (7, 17, 30). Overnight cultures in Trypticase soy broth (TSB) (Difco Laboratories, Detroit, Mich.) were diluted 1:200 with fresh TSB or TSB supplemented with 1% glucose or 4% ethanol. Aliquots of 200 µl were dispensed in duplicate into wells of a sterile 96-well flat-bottom plastic tissue culture plate (Costar Corp., Cambridge, Mass.) and grown aerobically for 20 h at 35°C. Comparable bacterial growth in each well was confirmed by measuring the optical density at 630 nm (OD_630) with an MRXII Microplate Reader (Dynex Technologies, Chantilly, Va.). The supernatant was removed, and each well was washed four times with 300 µl of sterile phosphate-buffered saline. The adherent cells were fixed by heating at 65°C for 30 min, air-dried, and stained with crystal violet. Relative strain adherence was determined for each strain by comparing the OD_630 of each well as a function of vancomycin concentration.

**Fig. 1.** PFGE of Smal-macrorestricton genomic DNA of isolate pair A6300 (lane 1) and A6298 (lane 2) and isolate pair A5937 (lane 3) and A5940 (lane 4). Each pair was isolated from the same patient.
A PCR product of ca. 1.2 kb containing \(3\)-CAGCGGGTACTTTAGGTT-3 was generated with the primer pair S1 (5'-ATGGTTATTAAGTTGGGATGG-3') and S2 (5'-CAGCGGTACTTTAGGTT-3'). A PCR product of ca. 1.1 kb containing \(3\)-GATTTAGGTCAGCTGTTTGGT-3' and S2 (5'-CAGCGGTACTTTAGGTT-3') was generated by the primer pair S3 (5'-GATTTAGGTCAGCTGTTTGGT-3') and S4 (5'-ACGGTGACAATTAGTTTGT-3'). Both reactions were done independently by using the same reaction conditions as follows: DNA denaturation at 95°C for 5 min, followed by 30 cycles of a 30-s denaturation step at 94°C, a 45-s annealing step at 52°C, a 60-s extension at 72°C, and finally a 10-min extension step at 72°C. Primers were derived from the published sequence of \(S. aureus\) agr group II strain N315 (GenBank accession number AP003135; bp 277870 to 277890 [primer S1], bp 279014 to 278997 [primer S2], bp 280043 to 280063 [primer S3], and bp 281219 to 281199 [primer S4]) (18).

Amplification products of ca. 1.9 kb containing \(agrB\), \(agrD\), and \(agrC\) were generated as recently described (27). DNA fragments were separated by 1% agarose gel electrophoresis and then visualized under UV light after they were stained with ethidium bromide.

PCR products were purified for RFLP and DNA sequencing by using Microcon centrifugal devices (Millipore Corp., Bedford, Mass.) as recommended by the manufacturer. \(Ahu\) and \(Rau\) (Promega) were used to perform RFLP analyses of \(agrB\)-\(agrD\)-\(agrC\) amplification products (42). Digested products were separated by 2% agarose gel electrophoresis.

DNA sequencing was performed by the standard Sanger dideoxynucleotide method (34). Sequence data was analyzed by using EDITSEQ and MEGALIGN software (DNASTAR, Inc., Madison, Wis.). Homology searches and sequence alignments were done by means of the BLAST programs provided by the National Center for Biotechnology Information (1, 2). All sequences that differed from the \(agr\) group II prototype sequence SAS02A (GenBank accession number AF801782) (16) were confirmed by sequencing two or more independent products of amplified DNA.

Nucleotide sequence accession numbers. The sequences for Mu3, PC-3, A6298, A5937, A5940, A6300, and HIP5836 were deposited in GenBank, with accession numbers AY082626, AY082627, AY082624, AY082626, AY082625, AY082629, and AY082630, respectively.

RESULTS

Hemolysis of sheep blood and delta-hemolysin expression. We noted the attenuated hemolytic properties of GISA and hetero-GISA strains when they were plated on sheep blood agar. Hetero-GISA A6298 showed production only of beta-hemolysin, the amount of which was considerably diminished compared to strain A6300, a PFGE-indistinguishable isolate from the same patient that was fully susceptible to vancomycin.
RN9120, the agr-null mutant derived from RN6607, retained its ability to produce beta-hemolysin but lost the ability to produce detectable alpha- and delta-hemolysins on sheep blood (Fig. 2, left panel). The production of delta-hemolysin by the GISA, hetero-GISA, and related vancomycin-susceptible strains are included in Table 1, and a selected subset is demonstrated in the center panel of Fig. 2. None of the GISA isolates produced delta-hemolysin, as manifested by the absence of hemolysis at the interface with RN4420. Given that the DNA sequences of hld (delta-hemolysin gene) and associated promoters were intact in PC-3, Mu3, Mu50, A5940, A6298, and A6300, the lack of delta-hemolysin expression was most likely due to the loss of agr function. The development of the hetero-GISA phenotype in strain A5940 was associated with the loss of delta-hemolysin expression compared to A5937, the vancomycin-susceptible strain indistinguishable by PFGE from the same patient (Fig. 2H and I, right panel).

**agr grouping.** Initial DNA sequencing of agrB, agrD, and agrC of A6298, A5940, and Mu3 showed these isolates to belong to agr group II. The published sequence for Mu50 (GenBank accession number AP003364) revealed that it was also of the agr group II (18). RFLP after digestion with RsaI (Fig. 3) and AluI (data not shown) of PCR fragments containing polymorphic regions of agrB, agrD, and agrC demonstrated that, despite their geographically diverse origins, all S. aureus isolates with reduced susceptibility to vancomycin and related susceptible strains belong to agr group II. This was a notable finding because other investigators have reported that this group comprises as few as 6% of clinical isolates of MSSA and MRSA (41).

**Analysis of previously published agr group II sequences.** Review of the DNA sequence of the agr locus in Mu50 compared to N315 (GenBank accession number AP003135) (18) revealed several silent mutations and a T→A that converts the last amino acid of the AgrA protein from isoleucine to lysine. No differences between Mu50, N315, and the previously published agr group II DNA sequence of strain SA502A (16) were noted in hld, agrB, and agrD and in the intragenic region between hld and agrB containing the P2 and P3 promoters (23, 25).

**agr sequences of GISA, hetero-GISA, and related vancomycin-susceptible strains.** Based on the above information, we sequenced the entire agr locus of Mu3, PC-3, A6298, A5937, and A5940. A6300 and GISA HIP5836 were partially sequenced. A summary of the mutations in translated agrA, agrB, and agrC is given in Table 2. hld, agrD, and P2 and P3 promoter DNA sequences of all study isolates were identical to those of prior agr group II prototype strain SA502A (16).

Hetero-GISA strain A5940 differed from the related vancomycin-susceptible strain A5937 by demonstrating a nonsense mutation that would be predicted to truncate 238 amino acid AgrA by 76 amino acids (Fig. 4A; Table 2). The expected loss of function conferred by this mutation is consistent with the loss of delta-hemolysin production by A5940 compared to A5937 (Fig. 2, left panel). Similarly, nonhemolytic GISA isolate HIP5836 demonstrated a nonsense mutation at amino acid 216 (Fig. 4A; Table 2).

Strains A5937 and A5940 demonstrated an identical frameshift mutation at the C terminus of agrB, resulting in a truncation of the protein by three amino acids compared to the other study strains and to N315 and Mu50 (Fig. 4B; Table 2). This does not appear to confer a complete loss of agr function since A5937 maintains detectable, although reduced, delta-hemolysin activity. PC-3 showed a unique glycine-to-aspartic acid change at position 225 of agrC (Fig. 4C; Table 2).

The exact site of initiation of agrC is unknown and can only be deduced from the DNA sequence. We modified the most likely initiation site of agrC of SA502A (16) after discussions...
with the authors of the initial sequence (Fig. 4C). The translated extreme C terminus of AgrC of all of the strains that we sequenced was identical to that of N315 and Mu50 and differed in the last 10 amino acids from SA502A as shown in Fig. 4C and in Table 2, footnote b.

**agr function and vancomycin heteroresistance.** In order to determine whether loss-of-function agr mutations would contribute directly to the vancomycin heteroresistance, we performed population analyses of agr group II prototype strain RN6607 and the isogenic agr-null strain RN9120. Additionally, we grew RN6607 and RN9120 in BHI broth containing vancomycin and recovered subclones designated RN6607-V and RN9120-V, respectively. Results of population analyses in BHI agar of all four strains in Fig. 5 demonstrated an increase in heteroresistance to vancomycin in RN9120-V that is not seen in RN6607-V. The differences in the population analyses be-
between the strains were consistent but less pronounced when Mueller-Hinton agar was used (data not shown). Agar dilution susceptibility testing according to NCCLS guidelines (24) demonstrated the vancomycin MICs for strains RN6607, RN6607-V, RN9120, and RN9120-V to be 1, 2, 2, and 4 μg/ml, respectively (Table 1).

**Consequences of loss of agr function.** To delineate the direct consequences of the loss of agr function on the ability of *S. aureus* to produce biofilm, we subjected RN6607 and RN9120 to a polystyrene binding assay. Figure 6A demonstrates that the loss of agr confers a weak ability to bind polystyrene in TSB and TSB-glucose, as determined according to criteria proposed by others (7). There was a greater disparity between RN6607 and RN9120 under conditions of ethanol stress, where the agr-null RN9120 showed a greater ability to bind polystyrene.

Figure 6B demonstrates that RN9120 was less susceptible to killing upon prolonged incubation at 16 μg of vancomycin/ml compared to the wild-type parent strain RN6607. Differences between the two strains did not become apparent until 48 h. After 5 days, the RN6607 culture was sterile, whereas the RN9120 counts remained unchanged.

**DISCUSSION**

We demonstrated here that geographically diverse hetero-GISA and GISA isolates displayed similarities at the agr locus.
FIG. 4—Continued.
All belong to agr group II, a subgroup of S. aureus currently believed to represent a small minority of clinical isolates (22, 41). All of the GISA and hetero-GISA isolates lacked delta-hemolysin expression. Delta-hemolysin is unique among the secreted virulence factors regulated by agr because is encoded by hld within the agr locus and is derived from translation of RNAIII, the effector molecule of agr (25). Therefore, in an S. aureus strain in which hld is structurally intact, as we have shown in several strains by DNA sequencing, delta-hemolysin expression can be used as a marker of agr function. The findings of absent delta-hemolysin expression in GISA and hetero-GISA isolates suggests that agr function is suppressed in these isolates. Compromised function of agr suggests either significance of the point mutations we detected, the loss of function of other genes required for agr function, agr suppression by mutations in upstream regulatory genes, disruption of agr-mediated quorum sensing, or disruption of delta-hemolysin secretion secondary to abnormalities in the cell walls of these isolates.

Based on susceptibility testing of agr-null RN9120, the loss of agr function appeared to confer a small decrease in susceptibility to vancomycin. Population analysis of the agr-null strain RN9210-V demonstrated a small but reproducible shift toward vancomycin heteroresistance after exposure to vancomycin, a phenomenon not seen in isogenic strain RN6607-V in which agr was intact (Fig. 5). Inactivation of agr seems to have conferred a degree of vancomycin tolerance, with attenuated bactericidal activity in vitro at clinically relevant concentrations of vancomycin (Fig. 6). There was a modest but reproducible increase in polystyrene adherence in RN9120 compared to the agr<sup>+</sup> parent strain RN6607, confirming the findings of others (Fig. 6) (42).

Analysis of S. aureus strains A5937 and A5940 (isolated from the same patient and indistinguishable by PFGE) showed that development of vancomycin heteroresistance was accompanied by loss of delta-hemolysin expression. The DNA sequence of agr revealed a nonsense mutation in agrA of hetero-GISA A5940 not present in A5937. This mutation would result in the deletion of the 76-amino-acid C terminus of AgrA and presumably the loss of agr function. We also noted a nonsense mutation in agrA in GISA HIP5836 and a nonconserved glycine-to-aspartic acid change at position 225 of agrC of strain PC-3, a clinical GISA isolate from New York. We did not have

<table>
<thead>
<tr>
<th>Strain</th>
<th>agrB (aa)</th>
<th>agrC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>agrA (aa)</th>
</tr>
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<tbody>
<tr>
<td>Mu50&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>WT</td>
<td>I-238-K</td>
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<td>WT</td>
<td>WT</td>
<td>I-238-K</td>
</tr>
<tr>
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<td>HIP5836</td>
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<sup>a</sup> There were no mutations in P2, P3, hld, and agrD, aa, amino acids; WT, wild-type.

<sup>b</sup> C terminus: S. aureus SA502A, -FYSKS; all GISA and hetero-GISA, -FIQKVEINN; S. epidermidis A086, -FIQKVEILNNP, and S. lugdunensis, -FVQKVEINN.

<sup>c</sup> Mu50 sequences were published by Kuroda et al. (18).

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**FIG. 5.** (A) Population analyses of agr group II strain RN6607 before (RN6607) and after (RN6607-V) growth in subinhibitory concentrations of vancomycin. (B) Same experiment as in panel A but performed with the agr::tetM isogenic agr-null strain RN9120 before (RN9120) and after (RN9120-V) growth in subinhibitory concentrations of vancomycin.
clinical isolates could translate into prolonged survival of the vancomycin-resistant organism, recent observations and our previously noted clinical and microbiologic observations associated with these isolates. The majority of S. aureus isolates with elevated vancomycin MICs were isolated from sites of infection involving biomedical devices, including the three strains (A5940, A6298, and A6222) first described here (5, 11, 15, 32, 35, 36). While the presence of a biomedical device in a patient may be associated with a higher likelihood of receiving vancomycin-resistant organism, recent observations and our polystyrene adherence assay of RN6607 and RN9120 suggest that the loss of agr function may independently confer an advantage to these isolates to persist on biomedical devices (42). Therefore, a biomedical device and vancomycin could potentially serve as dual-positive selection pressures for agr-null isolates. Additionally, the diminished virulence of agr-null clinical isolates could translate into prolonged survival of the host and provide the opportunity for prolonged and/or repeated exposure to vancomycin, increasing the risk of glycopeptide resistance.

Attenuation of the bactericidal activity of vancomycin that we observed with the loss of agr function may explain the bacteriostatic activity of vancomycin observed in GISA (R. L. Akins and M. J. Rybak, Abstr. Meet. Infect. Dis. Soc. Am., abstr. 1771, 2001) and the difficulty encountered in the treatment of endovascular infections with these organisms. For example, vancomycin failed to sterilize the bloodstream of the patient from whom A5940 was isolated despite a minimal increase in the vancomycin MIC to 4 \mu g/ml (unpublished results). We propose that the loss of agr function may be an early step in the development of vancomycin tolerance that may set the stage for vancomycin treatment failure and, in patients with persistent infection, for the subsequent development of mutations that confer the GISA phenotype.

Hemolysin production is upregulated by agr and sar (6, 25). Therefore, the loss of agr is expected to confer attenuated hemolytic properties (25, 31). Consistent with this, RN6607 was strongly hemolytic and strain RN9120 showed production only of beta-hemolysin. Hetero-GISA A6298 produced only beta-hemolysin. GISA isolates showed a complete absence of hemolysis. This suggests either the loss of function of multiple steps in the pathways that mediate hemolysin production or the inability of these isolates to secrete hemolysins through a thickened cell wall (8, 13), as proposed by others (Flayhart et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001). Additionally, the thickened cell wall may result in the inability to release the quorum sensing autoinducing cyclic octapeptide derived from agrD, thereby rendering this function of agr inactive in these isolates.

Although the mechanism is unknown, mutations in agr could partially explain the decrease in oxacillin MICs observed in S. aureus with increased vancomycin MIC (Flayhart et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001). Genetically engineered agr mutants demonstrate a reproducible decrease in beta-lactam resistance compared to isogenic parent strains (10). Transcription profiling technology has identified femB, a gene required for the full expression of mec-mediated methicillin resistance, to be under the regulation of agr (9). Agr-null strains may display lower levels of femB and therefore may display heteroresistance to antistaphylococcal beta-lactams, as we noted in PC-3 and A5940 and as reported by others (Flayhart et al., Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001).

The recent findings of increased \( \sigma^B \) activity in teicoplanin-resistant S. aureus mutants selected in vitro (3) and the negative regulatory effect of \( \sigma^B \) on agr expression (4) may provide one possible upstream mechanism of agr suppression that would account for our finding of a structurally preserved agr locus in strains which do not produce delta-hemolysin. It will be of interest to determine whether the teicoplanin resistance phenotype observed by these authors depends on \( \sigma^B \)-mediated agr suppression and to evaluate \( \sigma^B \) activity in A6298, Mu3, and Mu50.

Molecular analyses by PFGE of geographically diverse GISA and hetero-GISA isolates by us (data not shown) and others show that strains from North America are highly related (F. C. Tenover, unpublished data). The Japanese strains Mu3 and Mu50 display a lesser degree of similarity to the North American strains. These data may suggest the presence of a
small subset of S. aureus strains with the genetic predisposition to become vancomycin resistant under the appropriate selection pressures. One finding of our study was that all of the GISA and hetero-GISA isolates belong to agr group II. A recent study of 192 MRSA and MSSA clinical isolates sampling eight worldwide collections showed that agr group II isolates constitute only 6% of surveyed S. aureus isolates (42), and another study from England showed that agr group II represents 26% of nosocomial MSSA strains (22). Whether our finding represents a predisposition of agr group II strains to become vancomycin resistant, a higher tropism of agr group II strains to the patient population in whom GISA strains have been isolated (e.g., hemodialysis or the presence of a foreign body infection), or an underappreciation of the general frequency of agr group II among highly related clinical MRSA isolates remains to be determined.

Since GISA and hetero-GISA isolates appear to be very closely related, the more traditional typing methods, such as PFGE and RFLP, may not be sensitive enough for epidemiological evaluation. Based on our findings, small DNA sequence differences at the agr locus can be used to determine the epidemiological relationship of different GISA and hetero-GISA isolates, including the determination of true clonality.

In summary, the globally diverse GISA isolates we have studied display similarities at the agr locus. All belong to agr group II even though this group has been demonstrated by others to represent a minority of clinical S. aureus strains. All of the GISA and hetero-GISA strains failed to produce delta-hemolysin, implying a lack of agr function in these isolates. One hetero-GISA strain and one GISA strain displayed nonsense mutations in agrA. Other GISA and hetero-GISA isolates demonstrated point mutations in agr, the functional significance of which remains to be determined. Compromised agr function, either directly through agr mutations or indirectly through mutations in other genes, may represent a step in the heterogeneous pathway leading to vancomycin resistance in S. aureus. The loss of agr alone appears to confer a degree of vancomycin tolerance. Further studies are needed to study the role of agr in the regulation of cell wall synthesis and autolysis and to characterize the relationship between agr function and the development of glycopeptide resistance.

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ADDITION IN PROOF

A recent publication (M. B. Avison, P. M. Bennett, R. A. Howe, and T. R. Walsh, J. Antimicrob. Chemother. 49:255–260, 2002) emphasizes that modifications have been made to the genomic sequence of Mu50 from the time of its original publication by Kuroda et al. in April 2001 (18). Our analysis of the agr sequence of Mu50 reflects changes that were made in the sequence as of 5 March 2002. These findings support the possible role of agr in the evolution of vancomycin resistance by providing a list of genes in which there are loss-of-function mutations in Mu50. Several of these genes have been determined to be under the regulation of agr (9).

REFERENCES


