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

George Sakoulas,[1,2]* George M. Eliopoulos,[1,2] Robert C. Moellering, Jr.,[1,2] Christine Wennersten,[1] Lata Venkataraman,[3] Richard P. Novick,[4] and Howard S. Gold[1,2]

Department of Medicine[1] and Division of Laboratory and Transfusion Medicine, Department of Pathology,[3] Beth Israel Deaconess Medical Center, and Harvard Medical School,[2] Boston, Massachusetts 02115, and Skirball Institute of Biomolecular Medicine and New York University School of Medicine, New York, New York 10016[4]

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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 heteroresistance, 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 by nonamidated muropeptides within a thickened cell wall (8, 12, 13).

Our review of clinical case histories of several patients with glycopeptide intermediate-level resistant *S. aureus* (GISA) strains revealed that, in addition to prolonged exposure to vancomycin, most of these patients had infections originating in or involving biomedical devices such as artificial heart valves, central venous catheters, biliary stents, and dialysis catheters (5, 11, 13–15, 32, 35, 36).

The accessory gene regulator (*agr*) locus of *S. aureus* is a quorum-sensing gene cluster of five genes (*hld*, *agrB*, *agrD*, *agrC*, and *agrA*) that upregulates production of secreted virulence factors, including the alpha-, beta-, and delta-hemolysins, and downregulates production of cell-associated virulence factors (16, 23, 25, 26, 28, 31). Polymorphisms in *agrD* and *agrC* define four *S. aureus agr* groups (25). Published reports have noted that *agr* group I strains comprised a significant majority of clinical isolates (22, 41). In recent work, *agr* mutants of *S. aureus* had a greater ability to adhere to polystyrene, a frequently utilized marker of biofilm production (42).

We hypothesized that the ability to persist on biomedical devices and the attenuated hemolytic properties that we and others (D. Flayhart, A. Hanlon, T. Wakefield, T. Ross, L. Borio, and J. Dick, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. A39, 2001) had observed in GISA strains could be linked to loss of *agr* function. This led us to investigate the structure and function of *agr* in several clinical GISA and hetero-GISA isolates. Our results with these organisms of diverse geographic origins revealed considerable similarities.

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).

* Corresponding author. Mailing address: Division of Infectious Diseases, Beth Israel Deaconess Medical Center, Kennedy Bldg., 6th Floor, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 632-0760. Fax: (617) 632-0766. E-mail: gsakoula@caregroup.harvard.edu.

TABLE 1. Characteristics of study isolates

Strain	Origin[d]	MIC[a] (μg/ml) of:		*agr* group	Delta-hemolysin activity	Source of infection (reference)	Comments (references)
		VAN	OXA				
PC-3	NY	8	4	II	0	HD gortex graft fistula (34)	GISA
Mu50[b]	JPN	2	>128	II	0	Mediastinitis status post surgery congenital heart disease	GISA; original MIC of vancomycin, 8 μg/ml (12, 13)
Mu3[b]	JPN	2	>128	II	0	Lung	Hetero-GISA; original MIC of vancomycin, 3 μg/ml (12, 13)
HIP5836	NJ	8	128	II	0	Probable peritoneal dialysis catheter (34)	GISA
A5940[c]	MO	4	4	II	0	Porcine aortic valve	Hetero-GISA
A6298[c]	MA	4	>128	II	0	Hemodialysis gortex graft fistula; origin knee replacement	Hetero-GISA; + beta-hemolysin
A6222[c]	MA	8	>128	II	0	Suprarenal aortic graft	GISA
A5937	MO	1	128	II	+	Porcine aortic valve	MRSA related to A5940
A6300	MA	2	>128	II	0	Hemodialysis gortex graft fistula; origin knee replacement	MRSA related to A6298; + beta-hemolysin
SA32		1	>128	I	++		MRSA
RN6607		1	<0.25	II	++		
RN6607-V		2	<0.25	II	++		Derived from growth of RN6607 in vancomycin
RN9120		2	<0.25	*agr*::*tetM*	0		*agr* knockout derived from RN6607; + beta-hemolysin
RN9120-V		4	<0.25	*agr*::*tetM*	0		Derived from growth of RN9120 in vancomycin

[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 *Sma*I-macrorestricted 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 (monohydrate 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 33591 (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 a 1.0 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

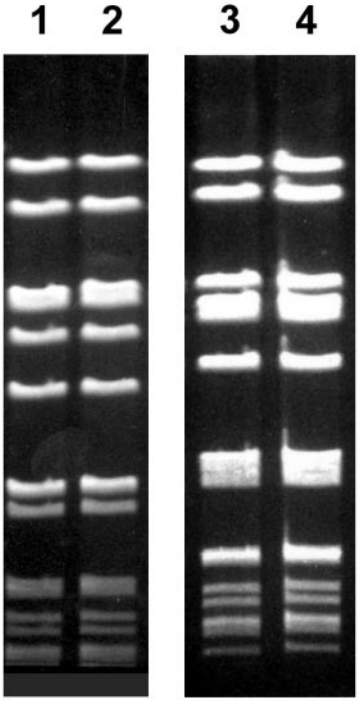

FIG. 1. PFGE of *Sma*I-macrorestricted genomic DNA of isolate pair A6300 (lane 1) and A6298 (lane 2) and isolate pair A5937 (lane 3) and A5940 (lane 4). Each pair was isolated from the same patient.

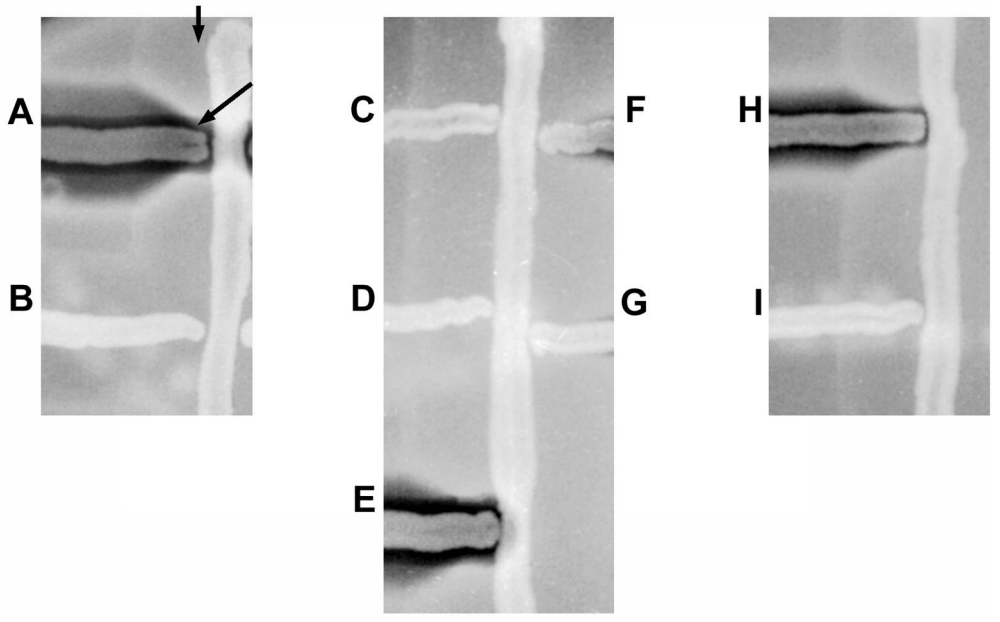

FIG. 2. Delta-hemolysin assays. Each panel is marked by the test strain streaked horizontally and beta-hemolysin-producing RN4420 streaked vertically. The left panel shows positive control RN6607 (A) and negative control *agr*-null RN9120 (B). The short arrow denotes the beta-hemolysin of RN4420, and the long arrow demonstrates the enhanced zone of hemolysis created by the interaction of the beta-hemolysin of RN4420 and the delta-hemolysin of the test strain. Note that RN9120 retains the ability to produce beta-hemolysin. The center panel shows A6222 (C), HIP5836 (D), RN6607 positive control (E), Mu3 (F), and Mu50 (G). The right panel shows hetero-GISA A5940 (I) and the related vancomycin-susceptible strain A5937 (H).

1 h. Cells were stained with 200 μl of Gram crystal violet (Becton Dickinson), and residual stain was removed with tap water. Plates were air dried, and the OD_{540} of the stained adherent bacterial films was measured.

Adherence was also measured after extraction of the stain by the addition of 160 μl of 33% (vol/vol) glacial acetic acid in water and gentle pipetting (38). The OD_{540} determined by this method was found to correlate linearly with the OD_{540} of the solid-phase bacterial film. We reported the OD_{540} of the dried plates after staining because these values were more applicable for comparison to prior reports.

Hemolysis assays. Gross hemolytic properties of individual bacterial strains were assessed by spotting 2 μl of a 10^8 CFU/ml suspension of organisms in Mueller-Hinton broth onto sheep blood agar plates. Hemolysis was assayed after 20 to 24 h at 35°C and again after an additional 20 to 24 h at 4°C. The latter step was performed to evoke the "hot-cold lysis" phenomenon observed with beta-hemolysin (4, 20, 37).

Delta-hemolysin expression. The function of the *agr* operon was measured by delta-hemolysin production. Delta-hemolysin expression was determined by using *S. aureus* RN4420, a strain that produces a large zone of beta-hemolysin without the interference of alpha- or delta-hemolysins. Beta- and delta-hemolysins of *S. aureus* act synergistically in the lysis of sheep red blood cells (20). Therefore, delta-hemolysin produced by a test strain results in a zone of enhanced hemolysis in areas where this lysin overlaps with the beta-hemolysin zone of RN4420.

PCR, restriction fragment length polymorphism (RFLP) analysis, and DNA sequencing of the *agr* locus. Genomic DNA, isolated by using the guanidium thiocyanate method (29), was used as the PCR template. Each reaction consisted of a 100-μl mixture of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, a 0.2 mM concentration of each nucleotide (Promega, Madison, Wis.), 30 pmol of each primer (Life Technologies, Rockville, Md.), and 0.6 U of *Taq* polymerase (Promega).

A PCR product of ca. 1.1 kb containing *hld*, the P2 and P3 promoters, and *agrB* was generated with the primer pair S1 (5′-ATGGTTATTAAGTTGGGATGG-3′) and S2 (5′-CAGCGGGTACTTTAGGTT-3′). A PCR product of ca. 1.2 kb containing *agrC* and *agrA* was generated with the primer pair S3 (5′-GATTTA AGTC-GCAGTATTGGT-3′) and S4 (5′-ACGCGTCATATTTAATTTTGT-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. *Alu*I and *Rsa*I (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 SA502A (GenBank accession number AF001782) (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 AY082628; 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

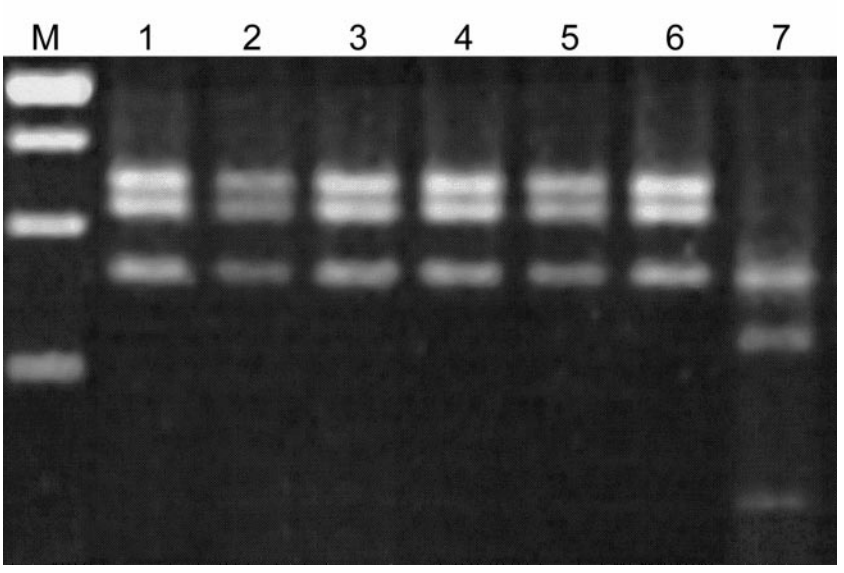

FIG. 3. RFLP analysis with *Rsa*I of an amplified portion of the *agr* locus of GISA and hetero-GISA strains with diverse geographic origins (indicated in parentheses). Lanes: 1, PC-3 (New York, N.Y.); 2, HIP5836 (New Jersey); 3, A5840 (St. Louis, Mo.); 4: A6298 (Boston, Mass.); 5, A6222 (Boston, Mass.); 6, Mu3 (Japan); 7, SA32 (*agr* group I). Molecular weight markers displayed are 200 to 800 bp increasing at 200-bp intervals.

(data not shown). 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 analysis after digestion with *Rsa*I (Fig. 3) and *Alu*I (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

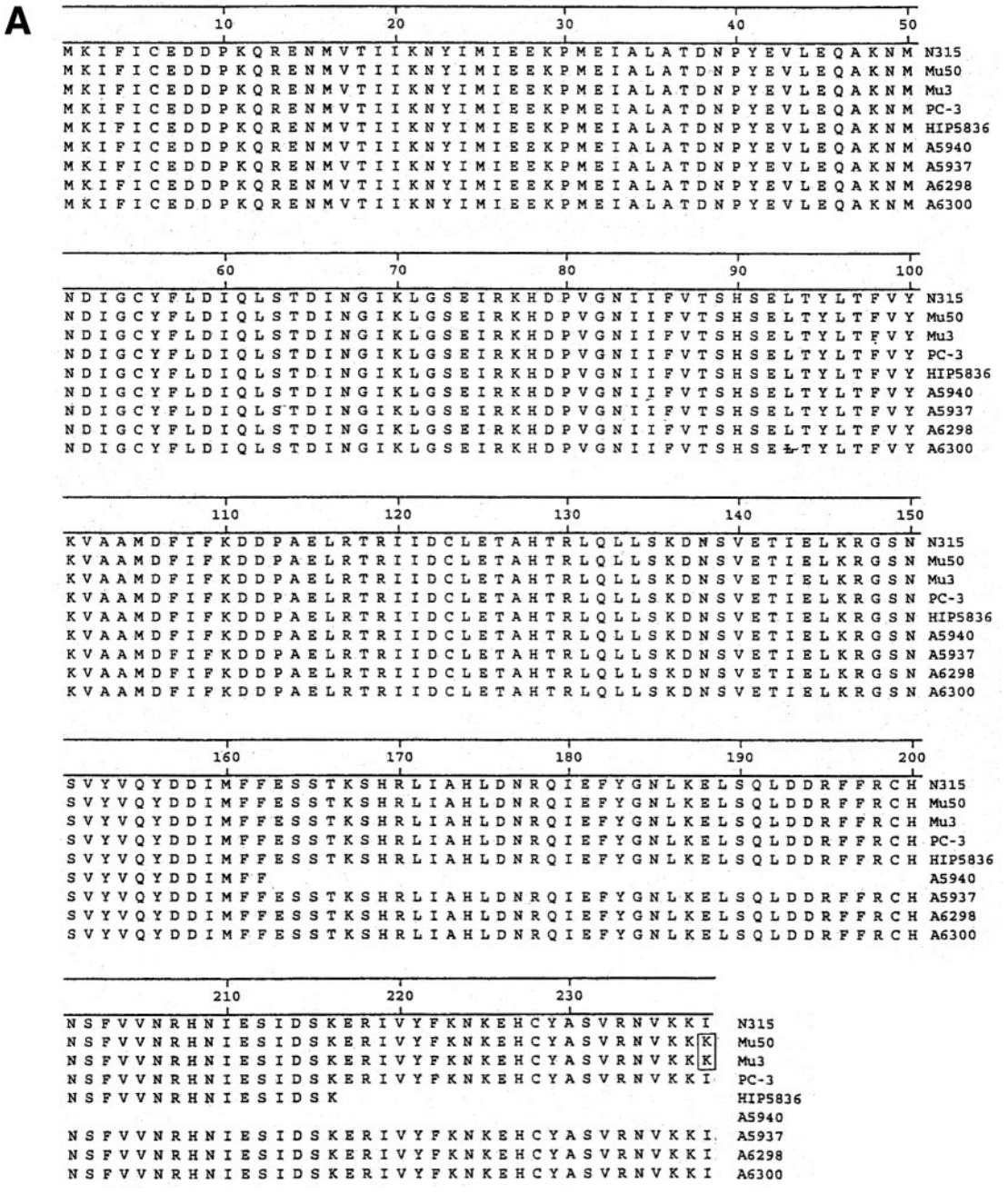

FIG. 4. (A) Translated protein sequences of *agrA* from GISA and related non-GISA *agr* group II strains. Differences in amino acids from the consensus are boxed. (B) Translated protein sequences of *agrB*. Differences from prototype *agr* group II strain SA502A are shown in boxes. (C) Translated protein sequences of *agrC*. Differences from prototype *agr* group II strain SA502A are shown in boxes. Sequences for SA502A, N315, and Mu50 were derived from GenBank as referenced in the text. Note that the sequence of *agrC* of SA502A was modified after communication with the authors of the original sequence as stated in the text (16).

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-

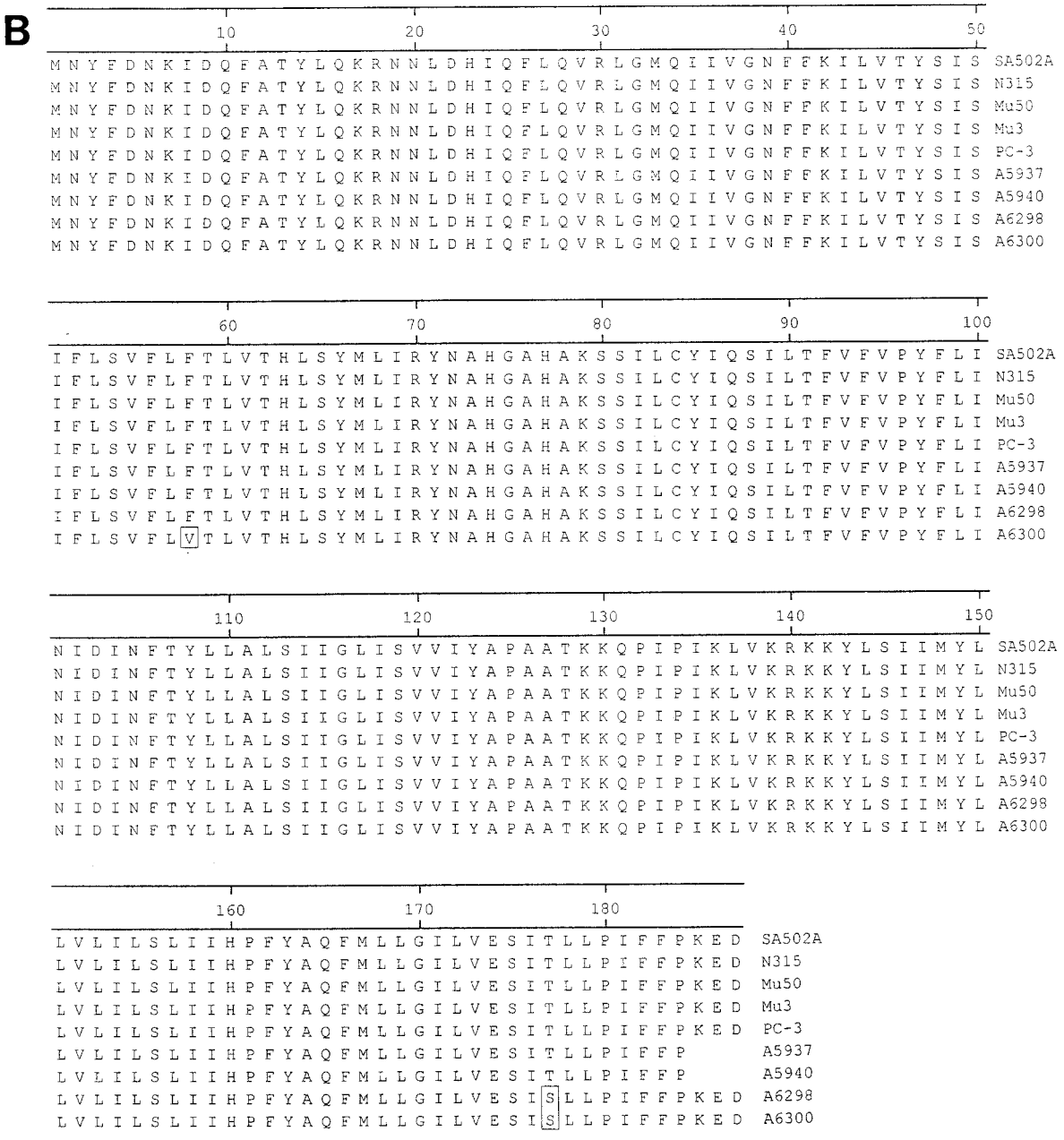

FIG. 4—*Continued.*

tween 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.

C

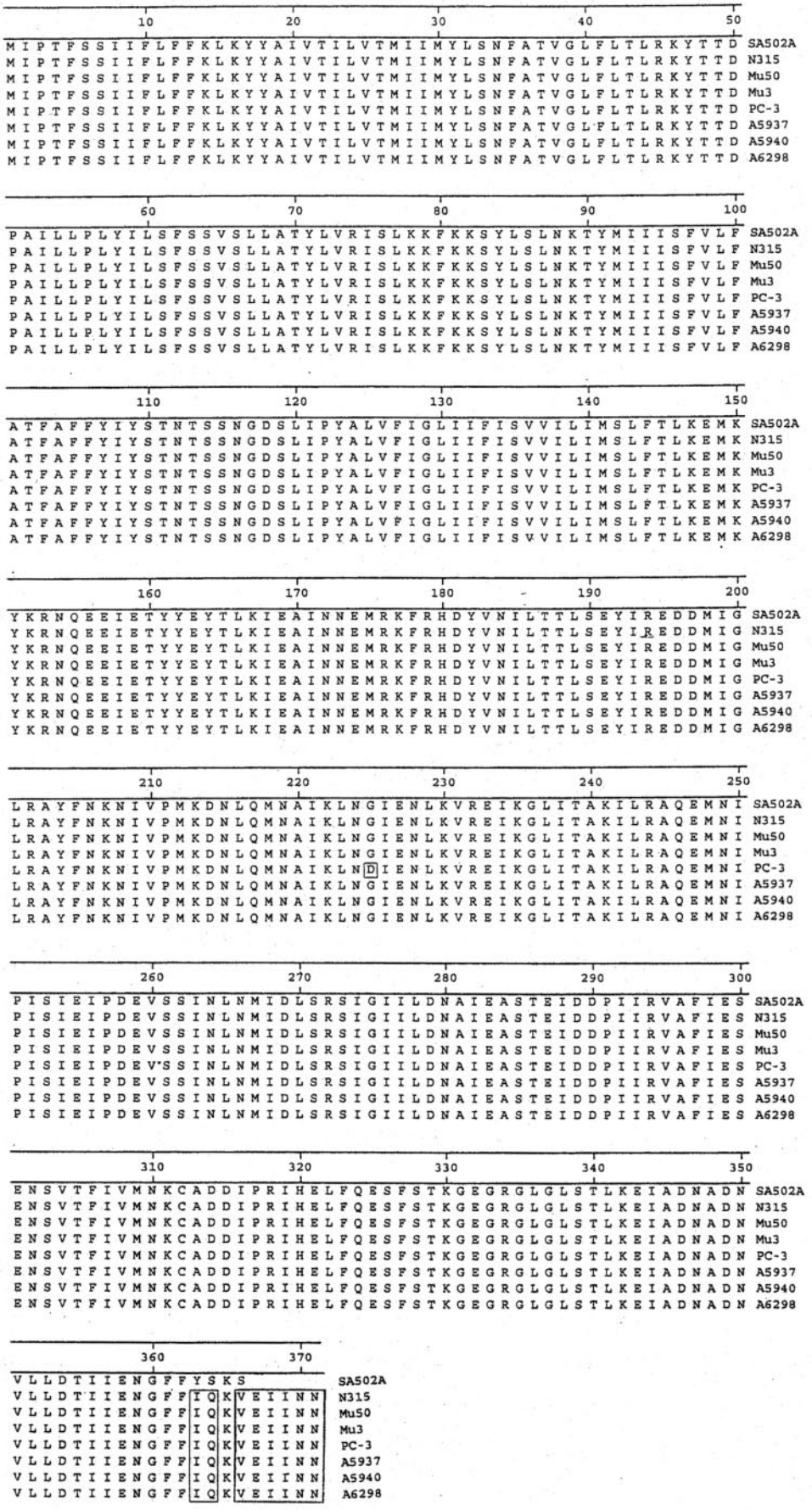

FIG. 4—*Continued.*

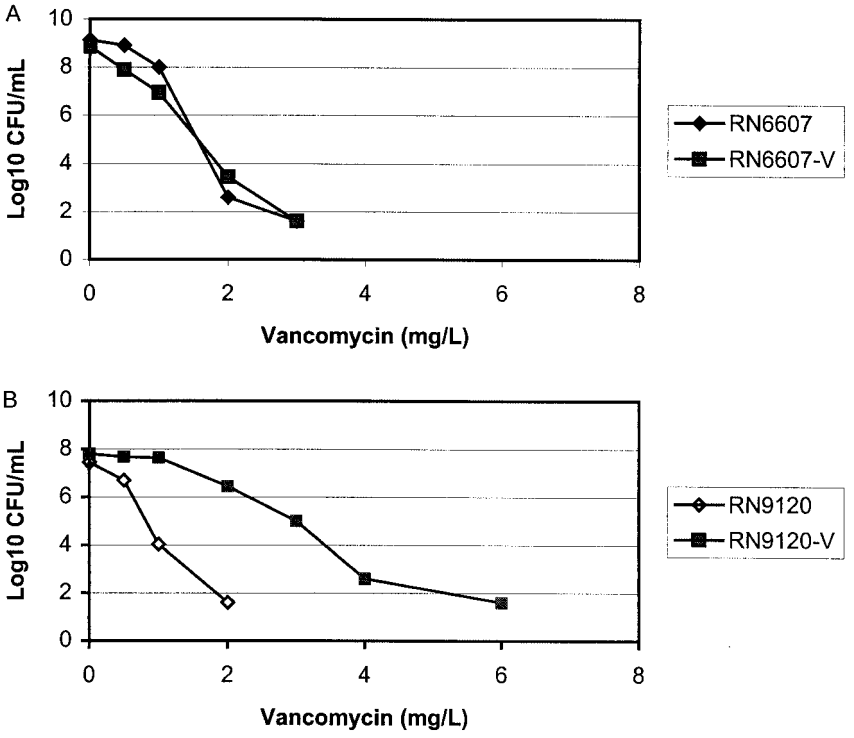

FIG. 5. (A) Population analyses of *agr* group II strain RN6607 before (RN6607) and after (RN6607-V) growth in subinhibitory concentrations of vancomycin. (B) Same experiment as in panel A but performed with the *agr*::*tetM* isogenic *agr*-null strain RN9120 before (RN9120) and after (RN9120-V) growth in subinhibitory concentrations of vancomycin.

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*$^+$ 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 2. Summary of *agr* mutations in GISA strains[a]

Strain	Mutations in:		
	agrB (aa)	*agrC*[b]	*agrA* (aa)
Mu50[c]	WT	WT	I-238-K
Mu3	WT	WT	I-238-K
PC-3	WT	G-225-D	WT
A6298	T-177-S	WT	WT
A5940	Nonsense (184)	WT	Nonsense (162)
HIP5836			Nonsense (216)

[a] There were no mutations in P2, P3, *hld*, and *agrD*. aa, amino acids; WT, wild-type.

[b] C terminus: *S. aureus* SA502A, -FYSKS; all GISA and hetero-GISA, -FIQKVEIINN; *S. epidermidis* A086, -FIQKVEILNNIP; and *S. lugdunensis*, -FVQKVEINNKES.

[c] Mu50 sequences were published by Kuroda et al. (18).

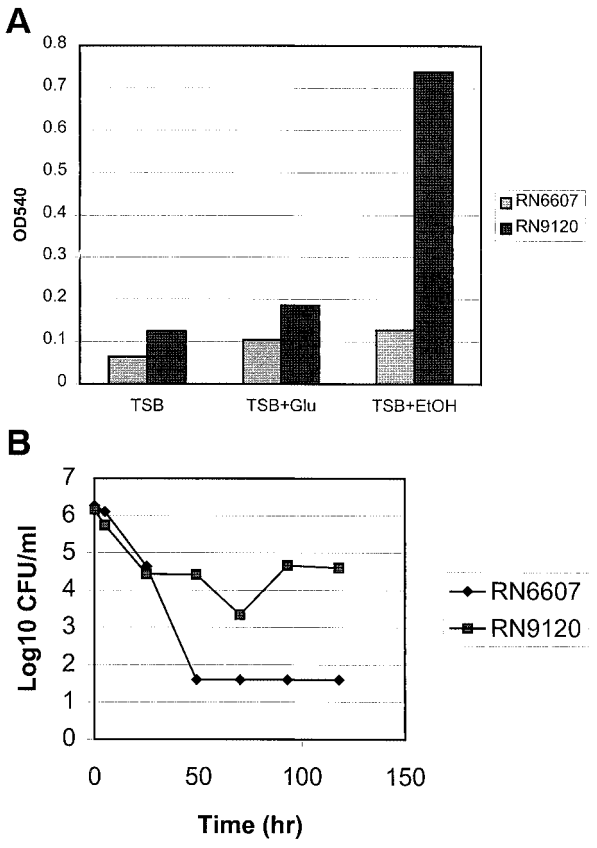

FIG. 6. (A) Biofilm assay as determined by the ability to bind polystyrene of RN6607 and RN9120 in TSB, TSB supplemented with 1% glucose (Glu), and TSB supplemented with 4% ethanol (EtOH). The values reported are the mean OD_{540}. Christensen et al. classified strains with various ODs obtained by this method as follows: OD < 0.120, nonadherent; OD 0.120 to 0.240, weakly adherent; and OD > 0.240, strongly adherent (7). (B) Bactericidal assay of RN6607 and RN9120 in BHI broth with vancomycin at 16 μg/ml. The lower limit of detection in this assay was at 1.6 $\log_{10}$ CFU/ml.

a vancomycin-susceptible progenitor strain of PC-3 to determine whether this mutation was functionally significant.

The possibility that loss of *agr* function may be involved in glycopeptide heteroresistance in *S. aureus* may explain several 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 and therefore a higher chance of selecting for a 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 μg/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 the 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 σ^B activity in teicoplanin-resistant *S. aureus* mutants selected in vitro (3) and the negative regulatory effect of σ^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 σ^B-mediated *agr* suppression and to evaluate σ^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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ADDENDUM 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).

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