ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2000, p. 272–277 Vol. 44, No. 2
0066-4804/00/$04.00+0

Reversion of the Glycopeptide Resistance Phenotype in *Staphylococcus aureus* Clinical Isolates

SUSAN BOYLE-VAVRA,[1]* SARAH K. BERKE,[1] JEAN C. LEE,[2] AND ROBERT S. DAUM[1]

The University of Chicago Children's Hospital, Department of Pediatrics, Chicago, Illinois 60637,[1] and Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 1 June 1999/Returned for modification 14 July 1999/Accepted 29 October 1999

The recent identification of glycopeptide intermediate-resistant *Staphylococcus aureus* (GISA) clinical isolates has provided an opportunity to assess the stability of the glycopeptide resistance phenotype by nonselective serial passage and to evaluate reversion-associated cell surface changes. Three GISA isolates from the United States (MIC of vancomycin = 8 μg/ml) and two from Japan (MICs of vancomycin = 8 and 2 μg/ml) were passaged daily on nutrient agar with or without vancomycin supplementation. After 15 days of passage on nonselective medium, vancomycin- and teicoplanin-susceptible revertants were obtained from each GISA isolate as determined by broth dilution MIC. Revertant isolates were compared with parent isolates for changes in vancomycin heteroresistance, capsule production, hemolysis phenotype, coagulase activity, and lysostaphin susceptibility. Several revertants lost the subpopulations with intermediate vancomycin resistance, whereas two revertants maintained them. Furthermore, although all of the parent GISA isolates produced capsule type 5 (CP5), all but one revertant tested no longer produced CP5. In contrast, passage on medium containing vancomycin yielded isolates that were still intermediately resistant to vancomycin, had no decrease in the MIC of teicoplanin, and produced detectable CP5. No consistent changes in the revertants in hemolysis phenotype, lysostaphin susceptibility, or coagulase activities were discerned. These data indicate that the vancomycin resistance phenotype is unstable in clinical GISA isolates. Reversion of the vancomycin resistance phenotype might explain the difficulty in isolating vancomycin-resistant clinical isolates from the blood of patients who fail vancomycin therapy and, possibly, may account for some of the difficulties in identifying GISA isolates in the clinical laboratory.

Recently, intermediate resistance to glycopeptides was identified in methicillin-resistant *Staphylococcus aureus* (MRSA) isolates obtained from patients in Japan and the United States who failed treatment with vancomycin (3, 6, 7, 18, 21, 25). The isolation of these glycopeptide-intermediate-resistant *S. aureus* (GISA) isolates has raised concern since, after vancomycin and teicoplanin, few therapeutic options exist for treatment of MRSA infections (13).

The term GISA was used to describe these glycopeptide-intermediate isolates in a recent report by Tenover et al. (29) to reflect the various patterns of resistance to vancomycin and teicoplanin as determined by broth dilution MIC analysis performed and interpreted according to the recommendations from the National Committee on Clinical Laboratory Standards (NCCLS) (16). Using the broth MIC data published by Tenover et al. (29) and others (6, 8, 12, 18, 21, 25), we have grouped the clinical GISA isolates into three distinct classes of glycopeptide resistance and refer to them as classes A, B, and C. Class A isolates are intermediate for both vancomycin (MIC = 8 to 16 μg/ml) and teicoplanin (MIC = 16 μg/ml), class B isolates are intermediate for vancomycin but are teicoplanin susceptible (MIC ≤ 8 μg/ml). Class C isolates are susceptible to vancomycin (MIC ≤ 4 μg/ml) and are intermediate for teicoplanin. Clinical *S. aureus* strains from all three classes are heteroresistant for vancomycin since they contain minority subpopulations that can grow on agar medium containing >4 μg vancomycin/ml (6, 7, 21), a concentration above the NCCLS susceptibility breakpoint (17). The basis for heteroresistance, a phenomenon previously described for the methicillin resistance phenotype (30), is poorly understood for either methicillin or glycopeptides.

Prior to the identification of GISA clinical isolates, it was observed that staphylococci could persist in the blood of patients despite vancomycin therapy and that such isolates appeared to be susceptible to vancomycin (11, 19, 27). This paradox raised the possibility that GISA clinical isolates have been present for some time but have escaped detection. We hypothesized that vancomycin-resistant *S. aureus* might undergo a transient adaptation in the presence of vancomycin, which might be reversed upon withdrawal of the drug.

We evaluated the stability of the resistance phenotype as well as exoprotein phenotypes in GISA isolates by serially passaging them on nonselective medium and monitoring the glycopeptide resistance phenotype of the passaged isolates by broth dilution MIC determination and population analysis.

MATERIALS AND METHODS

Culture conditions, GISA parent isolates, and passaging procedure. *S. aureus* isolates were routinely cultured at 37°C and stored as frozen stocks in skim milk (Difco Laboratories, Detroit, Mich.) at −70°C as described previously (4). To evaluate hemolysis phenotype, strains were streaked onto sheep blood agar, incubated overnight at 37°C, and transferred to a refrigerator for 24 h. As shown in Table 1, parent isolates used in passaging belonged to the three classes of GISA phenotypes isolated from patients who did not respond to vancomycin treatment. Serial passaging was initiated by reviving frozen stocks of each GISA clinical isolate onto brain heart infusion (BHI) agar (Difco Laboratories) and incubating them overnight. Several colonies chosen randomly from each plate were passaged on BHI or BHI containing subinhibitory levels of vancomycin (Sigma, St. Louis, Mo.) (2 μg/ml for isolates Mu3 and PC and 4 μg/ml for isolates MI, NJ, and Mu50). After overnight incubation several colonies were again randomly selected from each plate and passaged onto the identical medium. This procedure was repeated daily, and isolates were frozen for storage at 5-day

* Corresponding author. Mailing address: University of Chicago Children's Hospital, MC 6054, 5841 S. Maryland Ave., Chicago, IL 60637. Phone: (773) 702-6401. Fax: (773) 702-1196. E-mail address: sboyleva@midway.uchicago.edu.

TABLE 1. Bacterial strains[a]

Strain	Description	MIC (μg/ml)[b]		Source[c]	Reference
		Vancomycin	Teicoplanin		
MI	Class A GISA clinical isolate	8 (I)	16 (I)	CDC isolate HIP5827, Fred Tenover (CDC)	2, 3, 25
NJ	Class B GISA clinical isolate	8 (I)	8 (S)	CDC isolate HIP5836, Fred Tenover (CDC)	3, 25
PC	Class B GISA clinical isolate	8 (I)	2–4 (S)	CDC isolate HIP 6297, Fred Tenover (CDC)	18, 21
Mu50	Class B GISA isolate	8 (I)	8 (S)	K. Hiramatsu (Juntendo University)	5–7
Mu3	Class C GISA clinical isolate	2 (S)	16 (I)	K. Hiramatsu (Juntendo University)	5–7

[a] Classes A, B, and C refer to glycopeptide resistance patterns described in the introduction which are based on previously published broth dilution MIC values of vancomycin and teicoplanin based on twofold dilutions of drug. The values shown for MI, NJ, Mu50, and Mu3 are reproduced from Tenover et al (29).

[b] The MICs of vancomycin for strain PC were reproduced from Rotun et al. (18) and Sieradzki et al. (21). The teicoplanin MICs for PC were reproduced from Rotun et al. (18). S, susceptible; I, Intermediate as determined by NCCLS criteria (17). CDC, Centers for Disease Control and Prevention, Atlanta, Ga. Juntendo University is in Tokyo, Japan.

intervals. At days 5, 15, and 25 of passage, vancomycin and teicoplanin broth dilution MICs were determined. Serial passaging was discontinued when it was determined the MIC of vancomycin or teicoplanin had decreased to the lowest possible limit or until 60 days. Isolates which had broth dilution MICs of vancomycin at or below the susceptibility breakpoint after repeated passage of the GISA parent strains were referred to as revertants. Identification to species was confirmed with the use of the Staphaurex latex agglutination test (Abbott Laboratories, Chicago, Ill.), and pulsed-field gel electrophoresis (PFGE) was performed to confirm that clonal identity was maintained between each parent and the respective passaged isolate. GISA parent strains and revertants were coagulase and Staphaurex positive and susceptible to lysostaphin (Sigma).

Antimicrobial susceptibility testing. The MICs of glycopeptides and oxacillin were determined with the use of medium formulations recommended by the NCCLS for broth macrodilution protocols: Mueller Hinton broth (Difco Laboratories) was supplemented with Ca^{2+} and Mg^{2+} and, additionally, 2% NaCl for oxacillin (16). An inoculum of approximately 10^5 CFU/ml was seeded into the wells of a sterile 24-well cell culture plate (Corning Incorporated, Corning, N.Y.) containing medium with various dilutions of antibiotic. To discern small changes in resistance phenotype, our procedure differed from the NCCLS protocol in our testing of glycopeptides in arithmetic dilutions instead of twofold dilutions. For oxacillin, the concentrations tested were twofold dilutions from 1 to 1,024 μg/ml. A *mecA*-negative clinical isolate with an oxacillin MIC of ≤1 (strain 523) (4) was used as a methicillin-susceptible control isolate in each oxacillin MIC test. MIC testing was performed at least three times for each isolate, and the results were confirmed in blinded experiments. Isolates were classified as susceptible, intermediate, or resistant to each agent by using NCCLS criteria (17). Isolates were classified as susceptible if the MICs of vancomycin were ≤4 μg/ml or if the MICs of teicoplanin were ≤8 μg/ml. Since arithmetic concentrations of glycopeptides were used, all MICs of vancomycin of >4 and <32 μg/ml or all MICs of teicoplanin of >8 and <32 μg/ml were considered intermediate. No *S. aureus* clinical isolate has yet been reported to be resistant to vancomycin or teicoplanin by NCCLS criteria (MIC ≥32 μg/ml). All GISA isolates had an MIC of oxacillin of ≥16 μg/ml and were therefore classified as MRSA (≥4 μg/ml) by NCCLS standards (17).

PFGE. Clonal identity between parent and passaged isolates was confirmed by PFGE performed as described previously (14). Briefly, electrophoresis was performed in a Bio-Rad Chef DRII apparatus at 200 V for 22 h with an initial pulse of 1 s and a final pulse of 40 s. Banding patterns were visualized by ethidium bromide staining and UV transillumination. The clonality of isolates was judged by using previously described criteria (28) from visual comparisons of banding patterns of samples run together in the same gel.

Population analysis. The vancomycin heteroresistance phenotype for each strain was determined by population analysis (6). Briefly, overnight broth cultures were diluted and plated at final cell counts of 10^7, 10^6, 10^5, 10^3, and 10^1 CFU onto Trypticase soy agar (TSA) containing vancomycin at concentrations ranging in arithmetic increments from 0 to 12 μg/ml and additionally at 24 and 48 μg/ml. Colonies were enumerated after 48 h of incubation.

Capsule serotyping and coagulase tests. GISA clinical isolates before and after serial passage were submitted in blinded code for capsular serotyping. All GISA parent isolates were maintained on TSA containing 2 to 4 μg of vancomycin/ml prior to serotyping. Serotyping was performed by a colony immunoblot method (9) with polyclonal antisera specific for the type 5 (CP5) and the type 8 (CP8) capsules. The reactivity of each isolate was evaluated by comparison with that of control *S. aureus* strains (serotypes 1, 2, 5, and 8 and nontypeable isolates) included on each filter membrane. The reactivity was confirmed by immunodiffusion between capsule type-specific antisera and capsular extracts made from each strain (10). Isolates with no reaction against CP5 or CP8 antisera were reported as nontypeable.

Tube coagulase tests were performed from overnight cultures as previously described (4).

RESULTS

PFGE of GISA parent strains and revertants. PFGE was performed to confirm that the *Sma*I restriction pattern of each GISA strain was consistent with the published pattern and to confirm the clonality of each revertant to the respective parent. Although the *Sma*I restriction patterns of all GISA isolates were published previously (6, 18, 21, 25), the PC isolate was not compared directly to the other GISA isolates in the same gel. Therefore, we confirmed that the *Sma*I restriction patterns in our laboratory were similar to those previously published (6, 18, 21, 25). We also determined that the restriction patterns of the PC and MI GISA isolates were identical when compared on the same pulsed-field gel; a difference of just two bands could be discerned when these two isolates were compared with NJ (data not shown). The *Sma*I restriction patterns of the GISA parent strains also confirmed the conclusions of others that the U.S. isolates MI, PC, and NJ, are closely related to each other and that they are more distantly related to the Japanese isolates. The *Sma*I restriction patterns of each revertant was identical to its respective parent strain.

Serial passaging associated with decreased glycopeptide MICs. Table 2 compares the broth dilution MICs of vancomycin and teicoplanin for the GISA clinical strains, the susceptible revertant isolates, and the isolates passaged on medium containing vancomycin. GISA clinical strains MI, NJ, PC, and Mu50 each produced a revertant isolate with an MIC of vancomycin in the susceptible range after 15 days of passaging (as indicated by "[P15]"). However, isolate MI[P15] was less susceptible than the other P15 revertants. Whereas the MIC of vancomycin for isolate MI[P15] and MI[P25] was at the susceptibility breakpoint (4 μg/ml), the MICs of vancomycin for revertants NJ[P15], PC[P15], and Mu50[P15] were 2 to 3 μg/ml. Passaging of the MI[P15] isolate for an additional 45 days produced an isolate, MI[P60], which had an MIC of vancomycin of 2 μg/ml. Further passaging of NJ[P15] until day 60 did not lead to a further decrease in the MIC of vancomycin; the MIC of vancomycin for isolates NJ[P15], NJ[P60], and MI[P60] were each measured at least three times, and the MIC was never greater than 3 μg/ml.

Although the MICs of teicoplanin for the parent GISA strains Mu50, MI, NJ, and PC were lower than those previously published (6, 18, 21, 25), we nevertheless detected a lower MIC of teicoplanin for the revertants of these strains than we did in the respective parent. For the vancomycin-susceptible class C isolate, Mu3, the MIC of vancomycin did not change with serial passaging; however, the MIC of teicoplanin decreased. The MICs of vancomycin and teicoplanin did not decrease

TABLE 2. MICs of glycopeptides and capsule type[a]

Strain	MIC (μg/ml)		Capsule type[b]
	Vancomycin	Teicoplanin	
MI	6 (I)	8 (S)	5
MI[P15]	4 (S)	5 (S)	N
MI[P25]	4 (S)	ND	ND
MI[P60]	2 (S)	3 (S)	N
MI[VP15]	6 (I)	ND	5*
MI[VP60]	6 (I)	ND	5*
NJ	5 (I)	5 (S)	5
NJ[P15]	2–3 (S)	4 (S)	N
NJ[P60]	2–3 (S)		
NJ[VP15]	5 (I)	ND	5*
PC	5 (I)	5 (S)	5
PC[P15]	2 (S)	3 (S)	N
PC[P30]	ND	ND	N
PC[VP15]	5 (I)	ND	5
PC[VP30]	ND	ND	5
Mu50	5 (I)	5 (S)	5
Mu50[P15]	2 (S)	2 (S)	5
Mu50[VP15]	5 (I)	ND	5
Mu3	2 (S)	9 (I)	5
Mu3[P15]	2 (S)	6 (S)	ND
Mu3[VP15]	2 (S)	ND	ND

[a] [P15], passaged on plain medium for 15 days; [P60], passaged on plain medium for 60 days; [VP15], passaged on vancomycin containing medium for 15 days; [VP60], passaged on vancomycin containing medium for 60 days. Broth dilution MICs were determined as described in Materials and Methods with drug concentrations diluted in arithmetic increments. According to NCCLS criteria (17) isolates are susceptible (S) to vancomycin if their MIC of vancomycin is ≤4 μg/ml. Isolates are susceptible to teicoplanin if their MIC of teicoplanin is ≤8 μg/ml. Since arithmetic dilutions of glycopeptide were used, MICs of vancomycin of >4 and <32 μg/ml and MICs of teicoplanin of >8 and <32 μg/ml are intermediate (I). No clinical *S. aureus* isolate has been reported to have a resistant MIC of vancomycin or teicoplanin (≥32 μg/ml).

[b] The capsule serotyping was performed as described in Materials and Methods. N, nontypeable; ND, not determined. An asterisk indicates a weaker reaction with capsular antiserum compared with the control strain.

when the GISA isolates were serially passaged on medium containing vancomycin.

All parent and revertant isolates had an MIC of oxacillin ranging from 16 to 1,024 μg/ml and were thus classifiable as MRSA by NCCLS criteria (17).

Vancomycin heteroresistance profiles. Figure 1 shows the population analyses for MI, NJ, PC, and Mu50, their respective revertants and RN4220 (Fig. 1A), a methicillin-susceptible *S. aureus* control strain. Countable subpopulations that grew on medium containing >4 μg vancomycin/ml were no longer detected in the revertant isolates of MI[P60], NJ[P15], and PC[P15], and the heteroresistance profile curves were therefore "shifted to the left" (Fig. 1A to C). A similar shift was observed for the MI[P15] and Mu50[P15] revertants compared with the respective parent (Fig. 1A and 1D); however, these revertants maintained a subpopulation with intermediate (>4 μg/ml) vancomycin resistance. For the Mu3 revertant which had no decrease in the MIC of vancomycin but did have a decrease in the MIC of teicoplanin, the shape of the vancomycin heteroresistance profile curve was unchanged (data not shown). Thus, the decrease in the MIC of vancomycin was associated with a left shift in the heteroresistance curve and, with the exception of MI[P15] and Mu50[P15], apparent elimination of the subpopulation with intermediate glycopeptide resistance. It should be emphasized, however, that all revertant isolates were considerably more resistant than RN4220 (Fig. 1A) to vancomycin by population analysis.

Capsule serotyping and exoprotein phenotypic characterization. Previous studies indicated that a number of *S. aureus* exoproteins are phenotypically altered in *S. aureus* strains with intermediate vancomycin or teicoplanin resistance (4–6, 15, 18, 20, 23). The revertants described in this study provided us with a related susceptible clinical isolate for each GISA strain which could be used to evaluate differences in exoprotein phenotypic expression associated with the glycopeptide resistance phenotype. Results from capsule serotyping are shown in Table 2. All parent GISA strains tested (Mu50, Mu3, MI, NJ, and PC) produced CP5. Interestingly, the revertant isolates MI[P15], MI[P60], NJ[P15], and PC[P15] were nontypeable. Isolate Mu50[P15] was the only revertant tested which still produced CP5. In contrast, when the GISA parents were passaged on medium containing vancomycin for the same number of days as the revertants, they still produced detectable quantities of CP5, although the reaction with CP5 antiserum compared with the GISA parent isolates was weak for vancomycin-passaged isolates obtained from MI, NJ, and PC strains. No other phenotypic differences between parents and revertants were documented in hemolysis phenotype, coagulase activity, or lysostaphin sensitivity (data not shown). It is interesting that although the colony pigmentation of the MI GISA strain changed from yellow to cream between 5 and 10 days of passage, isolates at 35 and 40 days of passage produced colonies with yellow pigmentation similar to that of the parent MI strain, and isolates from day 45 through 60 produced cream-colored colonies.

DISCUSSION

This report clearly shows that reversion of glycopeptide resistance can occur in all types of GISA clinical isolates described to date. Decreases in MICs of vancomycin and teicoplanin uniformly occurred after 15 days of serial passage on nonselective medium; the presence of vancomycin in the medium prevented such reversion. It is unclear why others did not observe reversion of GISA isolates (6, 21, 25). We can speculate that an insufficient number of generations of growth on nonselective medium were allowed before reversion was evaluated. The number of passages required to decrease the MIC of vancomycin for the MI GISA isolate to 2 μg/ml was higher than that required for the other GISA strains, an observation suggesting that the stability of the glycopeptide-resistance phenotype depends to some extent on the genetic background of the bacterial strain.

We found two classes of revertant phenotypes distinguishable by population analysis. Three revertants (MI[P60], NJ[P15], and PC[15]) no longer had countable subpopulations recoverable from media containing >4 μg vancomycin/ml. The second class was represented by Mu50[P15] and MI[P15] revertants which still produced subpopulations with intermediate resistance (>4 μg/ml) to vancomycin that was detectable on agar medium. It should be emphasized, however, that population analysis indicated that none of the revertant isolates appeared to be as vancomycin susceptible as RN4220. These data suggest that the revertants could have a greater potential for "converting" to the resistance phenotype than would a typical vancomycin-susceptible *S. aureus* isolate. Accordingly, it has been proposed that class C GISA isolates such as Mu3 (Table 1) represent a pre-GISA evolutionary state (6). Conversely, our data raise the possibility that isolates from GISA classes B

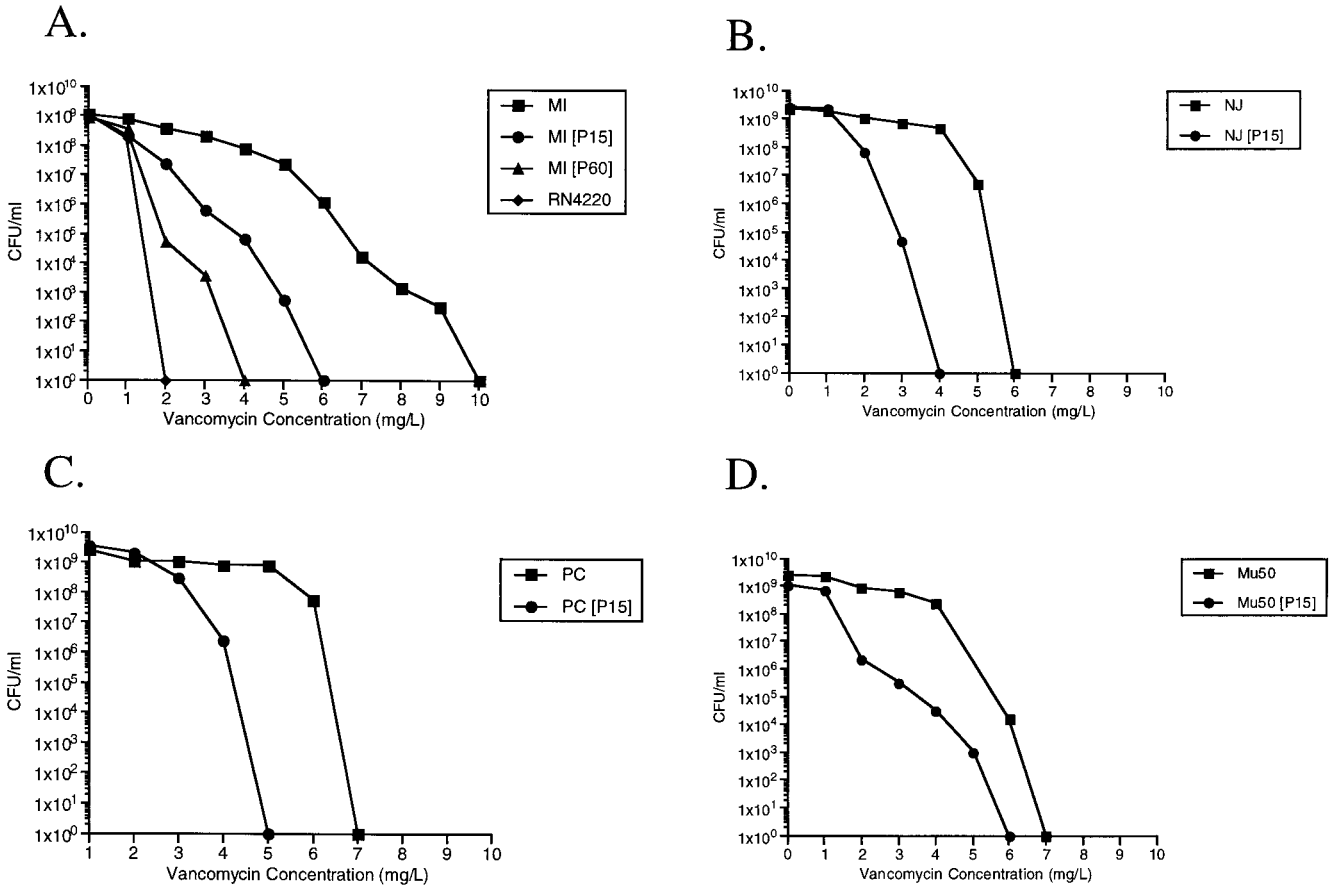

FIG. 1. Population analysis of parent GISA isolates MI, NJ, PC, and Mu50 and revertants. Strains grown overnight were serially diluted and plated on various concentrations of vancomycin-containing agar medium as described in Materials and Methods. "[P60]" refers to the revertant isolate obtained by passaging the MI parent strain for 60 days. "[P15]" refers to the revertant isolate obtained by passaging the indicated parent strain for 15 days. Points appearing on the abscissa refer to zero CFUs.

and C could represent revertants of isolates from GISA class A (vancomycin intermediate, teicoplanin intermediate).

All parent and revertant isolates had an MIC of oxacillin ranging from 16 to 1,024 μg/ml and were thus methicillin-resistant by NCCLS criteria (17). Thus, although Sieradzki and colleagues (21–23) previously observed an inverse relationship between the MICs of oxacillin and vancomycin, our data do not support such a view. We did not document an increase in the MIC of oxacillin with a decrease in the MIC of vancomycin in the revertants. Rather, the MICs of oxacillin for the revertant isolates tended to be one or two dilutions lower than those of the respective parent GISA strains (data not shown). Moreover, the MIC of oxacillin we measured for the PC isolate was 16 μg/ml, a value that was higher than the MIC of oxacillin of 0.8 μg/ml reported by Sieradzki et al. (21). We cannot explain this discrepancy; however, the MICs of oxacillin we documented for all our GISA isolates are consistent with other reports (1–3, 6, 7, 18, 25, 29) which documented the GISA isolates as MRSA.

The revertant derived from Mu3 (Mu3[P15]) was associated with a decrease in the teicoplanin broth dilution MIC with no change in the broth dilution MIC of vancomycin or vancomycin population analysis. These data may indicate there is a baseline MIC of vancomycin which cannot further decrease for each bacterial strain, a feature likely to be dictated by the strain-specific cell wall milieu.

It is unclear why CP5 was no longer produced in all revertant isolates except Mu50[P15]. Based on the observation that the U.S. isolates appear to be more closely related to each other than Mu50, we can speculate that the tendency to lose capsule production in GISA isolates depends to some extent on the genetic background. We recognize the possibility that the loss of CP5 production in the revertants could be due to in vitro passaging and that concomitant loss of capsule production and vancomycin resistance may be unrelated. However, historical evidence suggests that capsule production is not lost during in vitro passage (26). Moreover, in this study, in vitro-passaged GISA strains did not lose their ability to produce CP5 when vancomycin-selective pressure was present during the passaging. Additionally, isolate 523, a previously described vancomycin-susceptible clinical isolate (4), continued to produce CP8 after 15 days of serial passage on nonselective medium. Further studies are warranted to determine whether type 5 capsule is a cofactor in vancomycin resistance.

The reversion phenomenon we observed may explain why vancomycin-resistant *S. aureus* isolates are not usually isolated from the blood of patients with sustained *S. aureus* bacteremia even though they are receiving vancomycin therapy. We speculate that most *S. aureus* isolates revert rapidly in the absence of glycopeptides but that the identified GISA isolates may have developed a mechanism to stabilize the glycopeptide resistance phenotype for a longer period. Clinical microbiology labora-

tories routinely incubate isolates on nonselective media before susceptibility testing is performed, a situation which provides an opportunity for reversion to occur. Despite this apparent stability of resistance in the identified GISA isolates, susceptibility testing by disk diffusion and the Vitek System has proven unreliable in detecting glycopeptide resistance (29). Even MIC testing by broth dilution is not able to detect the vancomycin-intermediate bacteria in class C GISA isolates such as Mu3 and the Mu50 revertant. It is possible the insensitivity of these techniques in detecting GISA isolates is related to the decreased resistant subpopulation remaining after reversion.

Considering the tendency to revert, identified GISA isolates should be maintained on vancomycin-containing media to prevent reversion. Indeed, since we received all isolates growing on nonselective media, reversion of the teicoplanin resistance phenotype during transit may explain why the MICs of teicoplanin we documented are lower than were previously published for most isolates. Accordingly, reversion should be considered as a possible explanation when decreased glycopeptide resistance is observed in *S. aureus* mutants produced in the laboratory (24), especially when the procedures used to produce mutations involve incubations in nonselective medium for prolonged periods. Additionally, it might be recommended that *S. aureus* isolates should be cultured in clinical microbiology laboratories on medium containing a low level of vancomycin as well as on routine nonselective medium to ensure that glycopeptide-resistant isolates can be identified.

A further understanding of the cause of reversion and the revertant phenotype could lead to an understanding of the mechanism of glycopeptide resistance and possibly of the factors affecting the regulation of resistance. For instance, why did reversion occur in the absence of selective antimicrobial pressure? First, it is likely that the mutation(s) responsible for the glycopeptide-resistant phenotype may be relatively unfit and maintained only under continued selective glycopeptide pressure. Alternately, a genetic element mediating resistance in the clinical GISA isolates might be lost during serial passage. This latter possibility seems unlikely because glycopeptide MICs decreased in stepwise fashion in the serially passaged isolates and resistance from the GISA isolates has not been shown to be transferrable to date (unpublished data). Thus, since the MIC decreased in stepwise fashion (e.g., the MI revertants on day 15 compared with those on day 60) the reversion phenomenon observed here likely involves multiple stepwise chromosomal mutations that together conserve energy in the bacterial population in the absence of glycopeptides. The stepwise nature of reversion also suggests that in vivo, the development of resistance occurred by stepwise mutations. Identification of the genetic loci that are altered in each revertant could lead to an understanding of the mechanism(s) of resistance.

ACKNOWLEDGMENTS

We are grateful to Christine C. Ebert and Vasanthi Pallinti for technical assistance with the PFGE and MIC determinations and to Jessica Lam for performing the capsular serotyping. We are also grateful to Keichii Hiramatsu for supplying GISA strains Mu50 and Mu3 and to Fred Tenover for supplying GISA strains MI, NJ, and PC. Teicoplanin was a gift of Hoechst Marion Roussel, Milano, Italy.

This work was supported by NIAID grant RO1 AI40481-01 BM (R.S.D. and S.B.-V.), the Grant Healthcare Foundation (R.S.D. and S.B.-V.) and the Richter Foundation (S.K.B.).

ADDENDUM IN PROOF

During the revision of the manuscript, a decrease in vancomycin MIC was demonstrated after serial passage of three clinical *S. aureus* isolates with decreased susceptibility to vancomycin, including Mu50 (Aeschlimann et al., Antimicrob. Agents Chemother. **43:**1914–1918, 1999).

REFERENCES

1. **Centers for Disease Control and Prevention.** 1997. Reduced susceptibility of *Staphylococcus aureus* to vancomycin—Japan, 1996. Morbid. Mortal. Weekly Rep. **46:**624.
2. **Centers for Disease Control and Prevention.** 1997. *Staphylococcus aureus* with reduced susceptibility to vancomycin—United States, 1997. Morbid. Mortal. Weekly Rep. **46:**765–766.
3. **Centers for Disease Control and Prevention.** 1997. Update: *Staphylococcus aureus* with reduced susceptibility to vancomycin—United States, Sept. 1997. Morbid. Mortal. Weekly Rep. **46:**813–815.
4. **Daum, R. S., S. Gupta, R. Sabbagh, and W. M. Milewski.** 1992. Characterization of *Staphylococcus aureus* isolates with decreased susceptibility to vancomycin and teicoplanin: isolation and purification of a constitutively produced protein associated with decreased susceptibility. J. Infect. Dis. **166:**1066–1072.
5. **Hanaki, H., K. Kuwahara-Arai, S. Boyle-Vavra, R. S. Daum, H. Labischinski, and K. Hiramatsu.** 1998. Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50. J. Antimicrob. Chemother. **42:**199–209.
6. **Hiramatsu, K., N. Aritaka, H. Hanaki, S. Kawasaki, Y. Hosoda, S. Hori, Y. Fukuchi, and I. Kobayashi.** 1997. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet **350:**1670–1673.
7. **Hiramatsu, K., H. Hanaki, T. Ino, K. Yabuta, T. Oguri, and F. C. Tenover.** 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. **40:**135–136.
8. **Kaatz, G. W., S. M. Seo, N. J. Dorman, and S. A. Lerner.** 1990. Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. J. Infect. Dis. **162:**103–108.
9. **Lee, J. C., M. J. Liu, J. Parsonnet, and R. D. Arbeit.** 1990. Expression of type-8 capsular polysaccharide and production of toxic shock syndrome toxin-1 are associated among vaginal isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **28:**2612–2615.
10. **Lee, J. C., S. Takeda, P. J. Livolsi, and L. C. Paoletti.** 1993. Effects of in vitro and in vivo growth conditions on expression of type 8 capsular polysaccharide by *Staphylococcus aureus*. Infect. Immun. **61:**1853–1858.
11. **Levine, D. P., B. S. Fromm, and B. R. Reddy.** 1991. Slow response to vancomycin or vancomycin plus rifampin in methicillin-resistant *Staphylococcus aureus* endocarditis. Ann. Intern. Med. **115:**674–680.
12. **Mainardi, J. L., D. M. Shlaes, R. V. Goering, J. Shlaes, J. F. Acar, and F. W. Goldstein.** 1995. Decreased teicoplanin susceptibility of methicillin-resistant strains of *Staphylococcus aureus*. J. Infect. Dis. **171:**1646–1650.
13. **Maranan, M. C., B. Moreira, S. Boyle-Vavra, and R. S. Daum.** 1997. Antimicrobial resistance in staphylococci. Infect. Dis. Clin. North Am. **11:**813–849.
14. **Maslow, J., A. Slutsky, and R. Arbeit.** 1993. The application of pulsed field gel electrophoresis to molecular epidemiology, p. 563–572. *In* H. Persing, T. Smith, F. Tenover, and T. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
15. **Moreira, B., S. Boyle-Vavra, B. L. M. De Jonge, and R. S. Daum.** 1997. Increased production of penicillin-binding protein 2, increased detection of other penicillin-binding proteins and decreased coagulase activity associated with glycopeptide resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **41:**1788–1793.
16. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed., vol. 17, no. 2. Approved standard M7-A4. National Committee for Clinical Laboratory Standards, Villanova, Pa.
17. **National Committee for Clinical Laboratory Standards.** 1999. Performance standards for antimicrobial susceptibility testing: ninth informational supplement M100-S9, vol. 19. National Committee for Clinical Laboratory Standards, Wayne, Pa.
18. **Rotun, S. S., V. McMath, D. J. Schoonmaker, P. S. Maupin, F. C. Tenover, B. C. Hill, and D. M. Ackman.** 1999. *Staphylococcus aureus* with reduced susceptibility to vancomycin isolated from a patient with fatal bacteremia. Emerg. Infect. Dis. **5:**147–149.
19. **Shimada, K., H. Kobayashi, K. Sunagawa, T. Inamatsu, and K. Yamaguchi.** 1995. Clinical summary of intravenous use of vancomycin hydrochloride for severe infection caused by methicillin-resistant *Staphylococcus aureus*. Japan. J. Chemother. **43:**1048–1061.
20. **Shlaes, D. M., J. H. Shlaes, S. Vincent, L. Etter, P. D. Fey, and R. V. Goering.** 1993. Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. Antimicrob. Agents Chemother. **37:**2432–2437.
21. **Sieradzki, K., R. B. Roberts, S. W. Haber, and A. Tomasz.** 1999. The development of vancomycin resistance in a patient with methicillin-resistant

Staphylococcus aureus infection. N. Engl. J. Med. **340:**517–523.
22. **Sieradzki, K., and A. Tomasz.** 1996. A highly vancomycin-resistant laboratory mutant of *Staphylococcus aureus*. FEMS Microbiol. Lett. **142:**161–166.
23. **Sieradzki, K., and A. Tomasz.** 1997. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*. J. Bacteriol. **179:**2557–2566.
24. **Sieradzki, K., and A. Tomasz.** 1998. Suppression of glycopeptide resistance in a highly teicoplanin-resistant mutant of *Staphylococcus aureus* by transposon inactivation of genes involved in cell wall synthesis. Microb. Drug Resist. **4:**159–168.
25. **Smith, T. L., M. L. Pearson, K. R. Wilcox, C. Cruz, M. V. Lancaster, B. Robinson-Dunn, F. Tenover, M. J. Zervos, J. D. Band, E. White, and W. R. Jarvis.** 1999. Emergence of vancomycin resistance in *Staphylococcus aureus*. N. Engl. J. Med. **340:**493–501.
26. **Sompolinsky, D., Z. Samra, W. W. Karakawa, W. F. Vann, R. Schneerson, and Z. Malik.** 1985. Encapsulation and capsular types in isolates of *Staphylococcus aureus* from different sources and relationship to phage types. J. Clin. Microbiol. **22:**828–834.
27. **Tan, T., E. J. Mason, C. Ou, and S. Kaplan.** 1993. Use of intravenous rifampin in neonates with persistent staphylococcal bacteremia. Antimicrob. Agents Chemother. **37:**2401–2406.
28. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelson, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
29. **Tenover, F. C., M. V. Lancaster, B. C. Hill, C. D. Steward, S. A. Stocker, G. A. Hancock, C. M. O'Hara, N. C. Clark, and K. Hiramatsu.** 1998. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J. Clin. Microbiol. **36:**1020–1027.
30. **Tomasz, A., S. Nachman, and H. Leaf.** 1991. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob. Agents Chemother. **35:**124–129.