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Development of vancomycin and lysostaphin resistance in a methicillin-resistant *Staphylococcus aureus* isolate

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Glycopeptide resistance in *Staphylococcus aureus* is poorly understood. The diversity of change documented in cell walls of clinical glycopeptide-intermediate *S. aureus* (GISA) isolates is evidence that a single genetic or biochemical change cannot account for resistance in all isolates described to date. Therefore, identification of new GISA clinical isolates provides an opportunity to gain insight into the range of adaptive strategies employed by staphylococci to survive in the presence of glycopeptides. In April 1999, a GISA isolate was obtained from the blood of a 63-year-old dialysis patient in Illinois. This isolate was one of six clonally identical MRSA isolates (A–F) serially obtained from the blood of this patient who was receiving vancomycin therapy. All isolates were resistant to oxacillin (MIC > 256 mg/L). The initial isolate had an MIC of vancomycin of 1 mg/L. However, the presence of a subpopulation that could grow in the presence of 5 mg/L of vancomycin indicated that this isolate was predisposed to the acquisition of the GISA phenotype (MIC of vancomycin 10–12 mg/L), which occurred 13 days later, associated with an increased MIC of the endopeptidase lysostaphin and slightly increased cell wall thickness. The first and last isolates in the series, A and F, resisted killing when incubated in vancomycin 2 mg/L, resisted autolysis when incubated in Triton X-100 and had a decreased expression of a *c.* 116 kDa autolytic band, properties that were different from glycopeptide-susceptible control isolates. Lysostaphin resistance was not accompanied by alterations in the peptidoglycan pentaglycine cross-bridge or a decrease in oxacillin MIC. These data, when taken together with the demonstration of increased cross-linking in isolate F compared with isolate A, demonstrate that vancomycin resistance in these isolates probably occurred by a mechanism different from that of other GISA isolates described to date.

Introduction

Staphylococcus aureus clinical isolates with intermediate resistance to the glycopeptides vancomycin and/or teicoplanin, so-called GISA isolates, have recently been recognized in Japan, the USA and elsewhere.[1,2] The low-level resistance of these isolates and the lack of hybridization with enterococcal glycopeptide resistance genes indicate that the glycopeptide resistance mechanisms in staphylococci are distinct from those mediating glycopeptide resistance in *Enterococcus* spp.

With a single exception,[3] GISA isolates described to date have been uniformly resistant to methicillin and have been sorted into three phenotypic classes designated A–C.[4] All are heteroresistant in that only a subpopulation of cells express the resistant phenotype. Additionally, isolates have been described that are susceptible to both glycopeptides by MIC testing (MIC of vancomycin <4 mg/L) but contain a subpopulation that can survive on vancomycin >4 mg/L. These *S. aureus* isolates have been variously termed heterogeneous vancomycin resistant,[5] heterogeneously resistant to vancomycin,[6–8] hetero-VISA,[9,10] heterogeneously vancomycin resistant[9] and heteroresistant.

The mechanism of glycopeptide resistance in *S. aureus* is not known with certainty, although various changes in cell surface phenotypes have been described in the clinical GISA isolates studied to date.[11–14] Demonstration of substitution of D-glutamate for D-glutamine in the peptidoglycan stem peptide in a GISA isolate from Japan has indicated that the GISA phenotype may be associated with structural changes in peptidoglycan. However, only one of four clinical GISA isolates we examined from the USA

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had a similar D-glutamate substitution, the others had no substantive changes in peptidoglycan composition other than increased cross-linking.[15] The diversity of change documented in the cell walls of clinical GISA isolates demonstrates that a single genetic or biochemical event cannot account for all resistance observed to date.[15] Therefore, it is instructive to continue to characterize newly recognized GISA isolates to gain further insight into the range of strategies employed by staphylococci to adapt to glycopeptide antimicrobials.

In April 1999, a GISA isolate was obtained from the blood of a 63-year-old female dialysis recipient in Illinois (patient IL).[16] This isolate was one of a series of MRSA isolates from the blood of patient IL, who was receiving vancomycin therapy but ultimately succumbed to this infection. The initial isolate was susceptible to glycopeptide antimicrobials by routine susceptibility testing. As bacteraemia continued, the MICs of vancomycin and teicoplanin among these clonally related blood isolates increased. The recognition that peptidoglycan from these isolates did not have increased glutamine non-amidated stem peptide substitutions[15] prompted a phenotypic analysis of the series of isolates to gain insight into possible novel resistance mechanisms. Moreover, the availability of these MRSA and MRSA/GISA isolates from the same patient provided a unique opportunity to study a variety of susceptibility testing parameters, population dynamics and cell surface characteristics of this strain as it became increasingly resistant to glycopeptides.

Materials and methods

S. aureus isolates

Six *S. aureus* isolates were obtained from blood cultures drawn from patient IL over a 16 day period (Table 1). Other GISA isolates used in this study were obtained from patients in the USA and Japan (Table 1). We also employed several prototype *S. aureus* isolates from the ATCC including vancomycin- and methicillin-susceptible isolate ATCC 29213, vancomycin-susceptible MRSA isolate ATCC 43300, vancomycin-susceptible strain NCTC 8325 (ATCC 35556), a community-acquired MRSA isolate 4/16-6N described previously,[17] strain 523, a previously described methicillin-susceptible *S. aureus* (MSSA) clinical isolate[18] and RN4220, a broadly susceptible host strain derived from isolate NCTC 8325.[19] Speciation of all *S. aureus* isolates was confirmed with the Staphaurex latex agglutination test (Abbott Laboratories, Chicago, IL, USA) and a tube coagulase test as described previously.[18] Haemolysis was assessed by plating fresh broth cultures on Columbia sheep blood agar plates (BBL, Cockeysville, MD, USA). All *S. aureus* isolates were routinely cultured at 37°C and stored as frozen stocks in skimmed milk (Difco Laboratories, Detroit, MI, USA) at −70°C as described previously.[18]

Antimicrobial susceptibility testing

Broth dilution MIC testing for vancomycin, teicoplanin and oxacillin was performed as described previously[4] except that brain–heart infusion (BHI) broth was sometimes substituted for cation-supplemented Mueller–Hinton broth (CAMHB). NaCl (2%) was included in the medium when assaying the MIC of oxacillin as recommended by NCCLS. To discern small changes in resistance phenotype, our procedure differed from the NCCLS protocol in the testing of the MICs of glycopeptides in that we used arithmetic instead of two-fold dilutions. Etest MIC testing (AB Biodisk, Piscataway, NJ, USA) for vancomycin was performed according to the manufacturer's

Table 1. Bacterial strains

Strain	GISA phenotype class[a]	Specimen collection date	Origin
A	'pre'	11 April	initial IL isolate
B	'pre'	15 April	IL isolate
C	C	24 April	IL isolate
D	A	24 April	IL isolate
E	A	24 April	IL isolate
F	A	27 April	IL isolate
MI	A		CDC[b] isolate HIP5827; F. Tenover (CDC, Atlanta, GA)
NJ	B		CDC isolate HIP5836; F. Tenover (CDC)
PC	B		CDC isolate HIP6297; F. Tenover (CDC)
Mu50	B		K. Hiramatsu (Juntendo University, Tokyo, Japan)

All isolates are MRSA.
[a]Classes A, B and C refer to glycopeptide resistance (see text).
[b]CDC, Centers for Disease Control and Prevention.

instructions with an inoculum equivalent to a 0.5 McFarland standard.

Lysostaphin susceptibility testing

The MIC of lysostaphin was determined by macrobroth dilution in Mueller–Hinton broth using lysostaphin (Sigma, St Louis, MO, USA) suspended in buffer (pH 7.6, 0.15 M NaCl, 50 mM Tris). Lysostaphin was tested in two-fold dilutions ranging from 32 to 0.125 mg/L with an inoculum of 10^5 cfu and incubation at 35°C. The lysostaphin MIC was defined as the lowest concentration that produced a clear well after an incubation of 48 h.

Population analysis

Quantification of vancomycin heteroresistance was performed using population analysis or 'efficiency of plating' analysis on medium containing vancomycin as described previously.[4] Briefly, strains were grown overnight in trypticase soy broth (TSB) (Difco), serially diluted in BHI broth and plated on BHI agar containing vancomycin.

Vancomycin kill curves

Mid-logarithmic phase broth culture (BHI) (0.15–0.3 mL) was inoculated into 15 mL fresh BHI broth containing vancomycin at various concentrations ≤6 mg/L. Flasks were sampled hourly for 6 h; the sample obtained was diluted and plated so that the bacterial densities in the original suspension could be calculated.

Pulsed-field gel electrophoresis (PFGE)

Whole cell DNA was prepared and digested in agarose plugs with *Sma*I as described previously[4,20] and restriction fragments were resolved using a CHEF DRII apparatus (Bio-Rad, Hercules, CA, USA) as described previously.[4]

Electron microscopy

Transmission electron microscopy (TEM) was performed as described previously[18] except that strains were grown overnight in trypticase soy broth, fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and the specimens were embedded in Spurr's resin according to the manufacturer's instructions.

Susceptibility to Triton X-100 autolysis

This was studied because laboratory-derived GISA isolates have been resistant to the lytic effect of this detergent[21,22] and was assessed as described previously.[23] Briefly, mid-logarithmic phase cultures grown in BHI were assayed by rinsing bacterial cell pellets twice in ice-cold water followed by resuspension in lysis buffer [0.05 M Tris–HCl pH 7.2, 0.05% Triton X-100 (Sigma)]. The decrease in absorbance (A_{620}) was monitored at 30 min intervals for 4 h.

Zymography

Cultures were grown at 35°C in BHI broth (Difco) to a measured density (A_{620}) of 0.3. Murein hydrolysates were obtained by pelleting cells from each culture, rinsing in ice-cold water and resuspending in 15 mL of 4% SDS.[24] Mechanical disruption of cells was performed with the use of 0.1 mm zirconium/glass beads (Biospec Products, Bartlesville, OK, USA) and a Biospec beadbeater (Biospec Products) pulsed for 10–15 s alternating with cooling on ice for 5 min. This procedure resulted in lysis of *c.* 90% of the cells as estimated by performing a Gram's stain on the lysate. The resulting supernatant, containing both intracellular and cell wall murein hydrolases, was stored at −70°C and retained as the source of lytic enzymes. The protein concentration in the extract was determined with a modified precipitation Lowry assay.[25] Extracts containing 150 μg protein were resolved by electrophoresis with the use of a maxigel apparatus (Bio-Rad) using SDS–PAGE gels (7.5%) containing heat-killed *Micrococcus luteus* (1 mg/L; Sigma) as the substrate for the lytic enzymes. Electrophoresis, renaturation of murein hydrolases in the gel[26] and visualization of clear zones in the gel with methylene blue staining were performed as described previously.[27] Equivalence of loading between lanes was assessed by Coomassie Blue staining of SDS–PAGE gels run in parallel.

Results

S. aureus isolates

Tube coagulase testing revealed that all six IL isolates (Table 1) had a positive test that required 24 h for clot formation; none were positive at 4 h. β-Haemolytic activity surrounding individual colonies was of non-uniform intensity amongst the isolates in the series. Only colonies formed by isolates B and D had consistent, complete β-haemolysis. All other isolates had weak or incomplete zones of haemolysis.

Glycopeptide and oxacillin susceptibility testing

The MICs of both vancomycin and teicoplanin were generally higher in BHI than in CAMHB (Table 2), consistent with results for the isolate Mu50 from Japan.[7] Also, the MIC usually increased by one to two dilutions between 24 and 48 h of incubation.

The first two MRSA isolates, A and B, were susceptible to vancomycin by Vitek (Hazelwood, MO, USA) and Etest MIC. Intermediate resistance to vancomycin was first documented in all three isolates obtained on 24 April and

Table 2. Glycopeptide and lysostaphin susceptibility of IL isolates

Isolate	Site[a]	Vitek MIC[b] (mg/L)	Etest VAN MIC (mg/L)	VAN MIC (mg/L)				TEC MIC (mg/L)				Lysostaphin broth MIC (mg/L)
				CAMHB		BHI broth		CAMHB		BHI broth		
				24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	
A	p[b]	1	3	2	3	3	4	4	8	4	8	0.1
B	p	1	3	2	2	4	4	8	8	16	16	0.1
C	CL1	1	3	6	6	7	8	8	16	16	32	>32.0
D	CL2	4	4	7	9	9	12	16	16	16	32	>32.0
E	p	4	6	6	8	8	12	16	16	16	32	>32.0
F	p	ND	6	7	10	9	12	16	16	16	32	>32.0

[a]p, peripheral blood; CL, blood drawn through a central line. Two CL cultures were obtained on 24 April.
[b]Data from CDC.[16]
VAN, vancomycin; TEC, teicoplanin; CAMHB, cation adjusted Mueller–Hinton broth; BHI, brain–heart infusion.

those thereafter as assessed by broth dilution MIC testing performed in BHI. One of the three 24 April isolates, C, had an MIC of vancomycin of 1 mg/L when evaluated by Vitek testing, identical to that of the earlier A and B isolates. However, when isolate C was tested by broth MIC analysis in CAMHB, the NCCLS recommended medium, the MIC of vancomycin was 6 mg/L after 24 and 48 h incubation, a value that would correspond to an MIC of 8 mg/L if conventional two-fold dilution MIC testing were performed. Thus, vancomycin resistance in isolate C would have been undetected by the routinely employed Vitek modality.

Intermediate resistance to teicoplanin (⩾16 mg/L) was documented in the second isolate, B, and those obtained thereafter by broth dilution MIC testing performed in BHI after 24 h. However, when this isolate was tested in NCCLS-recommended CAMHB after a 48 h incubation, the MIC of teicoplanin was 8 mg/L, a value in the susceptible range. Thus, in retrospect, it was the MIC of teicoplanin, not vancomycin, determined in BHI, that was the first broth MIC test indicating that the more recognizable GISA phenotype was to come.

The MICs of oxacillin did not decrease as the glycopeptide MICs increased. The values ranged from 128 to >256 mg/L and did not decrease with the acquisition of the GISA phenotype.

Lysostaphin MIC

The MIC of lysostaphin increased substantially in isolate C, the same isolate whose MIC of vancomycin in BHI broth first indicated intermediate resistance (Table 2). All subsequent isolates in the series had identical high MICs of lysostaphin. Importantly, MIC values for MI, NJ, PC and Mu50, the other GISA isolates we tested, ranged from 0.13 to 2.0 mg/L after 48 h incubation. Thus, this high lysostaphin MIC was unique to the IL GISA isolates.

Vancomycin population analysis

Vancomycin population analysis (Figure 1) revealed two distinct patterns of heteroresistance among the six GISA isolates from patient IL. The glycopeptide-susceptible isolates A and B contained subpopulations that grew on medium containing 3–5 mg/L vancomycin. Thus, even these 'susceptible' isolates were more 'heteroresistant' than, for example, the susceptible control isolate RN4220 that had only a small, minority population that grew on media containing vancomycin at 2 mg/L.

The subsequent isolates in the series contained large subpopulations that grew on medium containing ⩾9 mg/L vancomycin. The shape of the curves (Figure 1) for these

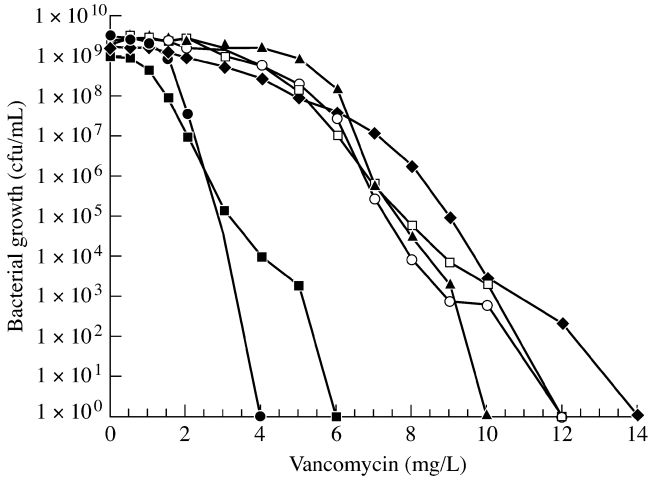

Figure 1. Population analysis of isolates from patient IL. Strains grown overnight were serially diluted and plated on varying concentrations of vancomycin-containing BHI medium as described in Materials and methods. Points on the abscissa refer to zero cfu values. The data shown are representative of experiments repeated on three occasions. Symbols used for isolates are as follows: A, ■; B, ●; C, ▲; D, ◆; E, □; F, ○.

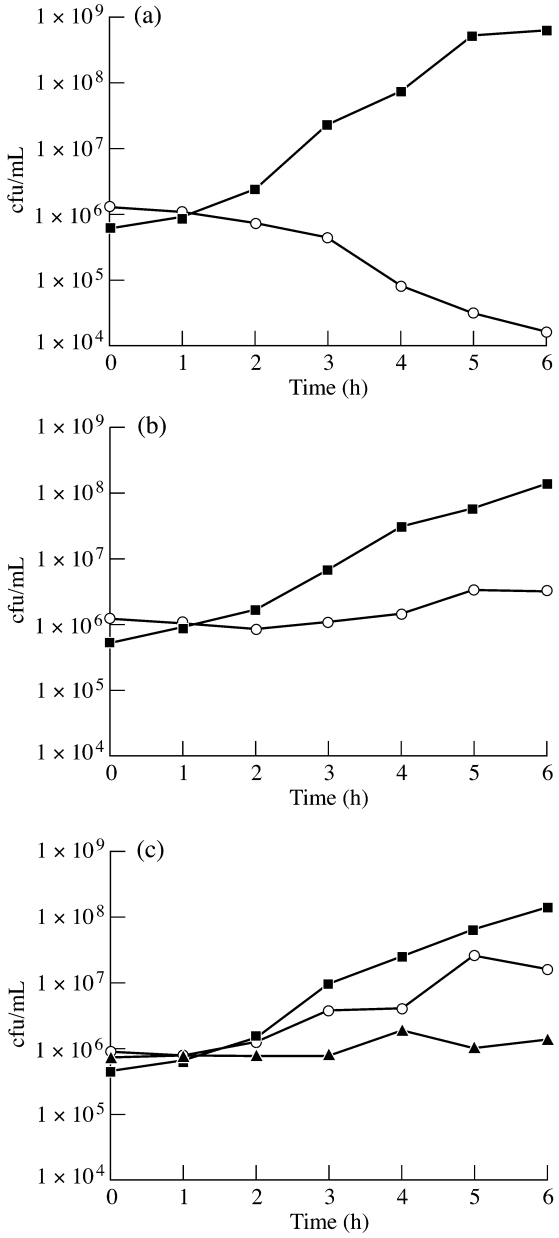

Figure 2. Vancomycin kill curves of isolates from patient IL. Samples of mid-logarithmic phase broth cultures were inoculated into fresh broth containing vancomycin at various concentrations ⩽6 mg/L. Flasks were sampled hourly for 6 h for viable cfu as described in Materials and methods. The concentrations of vancomycin used are as follows. (a) Isolate ATCC MRSA 43300: ■, 0 mg/L; ○, 2 mg/L. (b) Isolate A: ■, 0 mg/L; ○, 2 mg/L. (c) Isolate F: ■, 0 mg/L; ○, 4 mg/L; ▲, 6 mg/L.

more resistant isolates (C–F) indicates that a higher proportion of the bacterial population was able to grow on media containing vancomycin than of isolates A or B.

PFGE

The IL isolates in Table 1 were previously reported to have identical *Sma*I restriction enzyme patterns resolved by PFGE.[16] Analysis performed in our laboratory confirmed this clonal relationship among isolates A–F. A single exception was one variant of GISA isolate F that had one band missing from each of two doublets running at *c.* 50 and 100 kb, respectively, but was otherwise identical to isolates A–E.

We compared the *Sma*I restriction enzyme patterns of the IL isolates with those of GISA isolates from the USA (MI, NJ, PC) and from Japan (Mu50). The restriction patterns indicate that GISA isolate IL is clonally identical to MI and PC (except the variant of isolate F) as assessed by this technique.

Kill curve data

Vancomycin kill curve data (Figure 2) indicated that vancomycin at 2 mg/L produced a 2 log kill in the 6 h test period for the ATCC MRSA isolate (Figure 2a). In contrast, vancomycin at 2 mg/L did not affect killing of the initial MRSA isolate A (Figure 2b). Not surprisingly, vancomycin at a concentration ⩽6 mg/L allowed survival of GISA isolate F even at the highest concentration tested (Figure 2c) but not of isolate A (data not shown).

Susceptibility to Triton X-100-induced autolysis

To further characterize the autolytic phenotype of the GISA clinical isolate, susceptibility to Triton X-100, an agent thought to remove inhibitors of endogenous autolysins, was assessed on cells grown in the absence of vancomycin. The initial isolate (A) and last GISA isolate (F) were both relatively resistant to autolysis in the presence of Triton X-100 compared with the ATCC MRSA isolate, the ATCC MSSA isolate, a representative community-acquired MRSA isolate 4/16-6N[17] and NCTC 8325 (ATCC 35556) (Figure 3). Thus, IL isolates A and F are the first reported clinical heteroresistant (A) and GISA (F) isolates with resistance to Triton X-100-induced autolysis, demonstrable when grown in the absence of vancomycin, although similar resistance was found in laboratory-derived GISA isolates.[21,22]

Expression of autolytic proteins as assessed by zymography

To assess whether resistance to autolysis was related to changes in endogenous autolysin expression, zymographic analysis was performed using SDS–PAGE. The autolytic profiles of cellular extracts from isolates A and F were similar. However, a 116 kDa autolytic band (Figure 4) was present in lower abundance in both isolates compared with a susceptible, vancomycin-naive control isolate 523. This band has been said to arise from proteolytic processing of Atl, the major autolysin encoded from the *atl* gene.[28,29] The 51 kDa *N*-acetylglucosaminidase and 62 kDa *N*-acetylmuramyl-L-alanine-amidase processed forms of

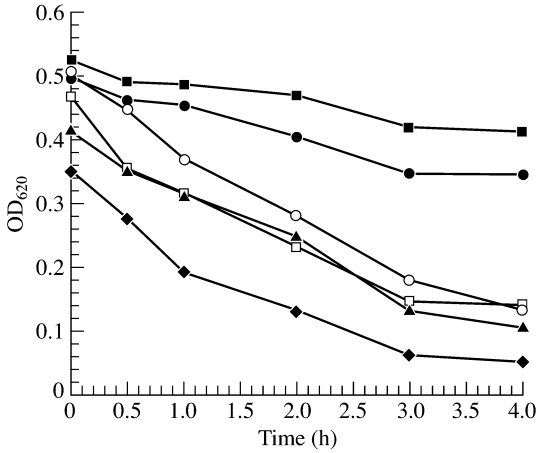

Figure 3. Autolysis assay. Resistance to autolysis in the presence of Triton X-100 of heteroresistant isolate A (■) and the related GISA isolate F (●) compared with several control strains: MSSA isolate ATCC 29213 (▲), MRSA isolate ATCC 43300 (◆), MSSA isolate NCTC 8325 (ATCC 35556) (□) and MRSA isolate 4/16-6N (○). Cultures were grown to mid-logarithmic phase in BHI and were assayed as described in Materials and methods.

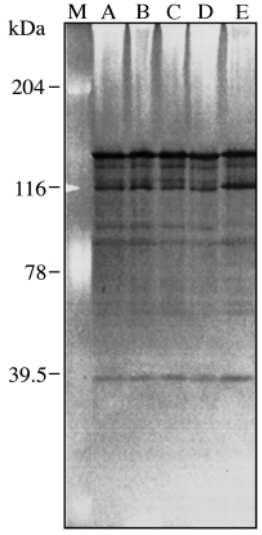

Figure 4. Autolytic activity assayed by zymography. Lane A, isolate A grown in the absence of vancomycin; lane B, isolate A grown in the presence of vancomycin 1 mg/L; lane C, isolate F grown in the absence of vancomycin; lane D, isolate F grown in the presence of vancomycin 4 mg/L; lane E: 523 grown in the absence of vancomycin.

Atl and a *c.* 39 kDa band appeared to have similar activity in the two strains. Incubation of isolate A or F in vancomycin did not influence the activity of the 116 kDa autolytic protein (Figure 4, lanes B and D).

Transmission electron microscopy

A modest increase in thickness of the peripheral cell wall was observed in isolate F ($n = 18$, mean 40 ± 12 nm) compared with isolate A ($n = 21$, mean 30 ± 12 nm, $P = 0.009$, unpaired *t*-test).

Discussion

Among the isolates from patient IL, the MIC of vancomycin changed from a susceptible to intermediate phenotype in the same organism (isolate C) in which the MIC of lysostaphin increased (Table 2). Thus, lysostaphin resistance was the most distinctive change associated with the vancomycin-resistant phenotype.

Our data indicate that the first two IL MRSA isolates, declared glycopeptide susceptible by Vitek and Etest susceptibility testing, had a 'pre-GISA' phenotype that caused an infection unsuccessfully treated with vancomycin. Decreased vancomycin kill rates, resistance to Triton X-100-induced autolysis in the absence of vancomycin, decreased expression of the *c.* 116 kDa autolysin band (Atl) and the 24 h period required for a positive tube coagulase test were phenotypes all observed even in the earliest isolates in the series. These phenotypes are consistent with the presence of a substantial subpopulation already resistant to vancomycin in these early isolates that could also survive the presence of low-level vancomycin. How frequent such pre-GISA isolates are among all *S. aureus* clinical isolates remains to be determined. Their earlier recognition would be useful in determining therapeutic options.

Additional phenotypic changes occurred with continued vancomycin exposure in association with acquisition of vancomycin resistance, most notably the increase in lysostaphin resistance. Lysostaphin is a 27 kDa endopeptidase produced by *Staphylococcus simulans* biovar *staphylolyticus* that lyses staphylococcal cells by hydrolysing the pentaglycine bridges that cross-link the peptidoglycan of several members of this genus,[30] including *S. aureus*. It is possible that the increase in cross-linking we observed[15] prevented lysostaphin access to all pentaglycine targets or increased the number of cross-linkers requiring cleavage before the strain could lyse. However, the MIC of lysostaphin in the highly cross-linked GISA isolate PC was much lower than that of the last four IL isolates, an observation that indicates that a highly cross-linked peptidoglycan is insufficient to produce lysostaphin resistance.

Lysostaphin resistance has not been reported in clinical GISA isolates to date but has been reported in the majority of laboratory-selected GISA isolates.[18,22] The identification of a clinical GISA isolate that is also resistant to lysostaphin has important implications since lysostaphin is currently under consideration as a therapeutic agent;[31–33] experimental endocarditis in rabbits, caused by the GISA isolate MI (Table 1) was successfully treated with lysostaphin.[32] It seems likely, but remains to be proven, that the high MICs

of lysostaphin we have documented would be associated with lysostaphin treatment failure.

The mechanism of lysostaphin resistance and its relationship to vancomycin resistance remains unclear. We considered whether lysostaphin resistance could be associated with altered cross-bridge structure. For example, substitution of one or more sites in the pentaglycine cross-bridge by a non-glycine moiety, e.g. serine, with or without acquisition of the lysostaphin resistance gene, *epr*[34] or *lif*,[35] produced isolates resistant to lysostaphin. Similarly, lysostaphin-resistant *S. aureus femAB* null mutants with monoglycine cross-bridges have also been described.[36] However, we found no evidence for such a serine substitution or a monoglycine bridge by HPLC analysis of mutanolysin-digested peptidoglycan since the A and F isolates have intact pentaglycine cross-bridges.[15]

Data from the serial MRSA isolates obtained from patient IL underscore several issues relevant to the early clinical detection of GISA isolates. Population analysis indicated that the first isolate in the series, A, contained a subpopulation that could grow on BHI medium containing 5 mg/L of vancomycin. For unknown reasons, isolate B was slightly more susceptible than isolate A. However, isolates A and B both contained substantial subpopulations that were able to survive on medium containing 2 mg/L of vancomycin; the survival frequency was *c.* 10^{-2} cfu/mL. The survival frequency for isolates A and B was greater than for isolate RN4420 where only *c.* 10^{-6} of the bacterial population could survive on medium containing 2 mg/L of vancomycin. This rate for RN4220 was more typical of a glycopeptide-susceptible *S. aureus* isolate.

The MIC of vancomycin is most often determined in clinical microbiology laboratories in the USA by an automated technique such as Vitek testing. Confirmatory testing is performed when a Vitek result such as 4 mg/L prompts other tests such as a broth MIC. For our isolates, this trigger did not occur until 24 April, and even then in only two of the isolates obtained on that day, thus indicating that Vitek testing is an insensitive barometer of early changes producing the GISA phenotype. Broth MICs, when performed by clinical microbiology laboratories, are usually performed in Mueller–Hinton medium to conform to NCCLS standards. However, as demonstrated by our data and those of others,[7] GISA isolates generally test as 'more resistant' in BHI broth and more resistant when read after a 48 h incubation. Thus, our series of related isolates allowed us to ascertain whether the use of BHI would have allowed resistance to have been detected earlier. Indeed, the MIC of teicoplanin performed in BHI broth would have allowed the recognition of the GISA phenotype in the B isolate 9 days before the currently recommended NCCLS practice did. Population analysis was even more sensitive and, were it performed on isolate A, the results may have been predictive of treatment failure.

With the use of TEM, we demonstrated modestly increased cell wall thickness in GISA isolate F even when grown in the absence of vancomycin; a similar increased thickness has characterized laboratory-derived GISA isolates such as 523k[18] and clinical GISA isolates such as Mu50.[37] These data document for the first time an isogenic clinical isolate from the same patient in which an increase in cell wall thickness occurred in association with the acquisition of the GISA phenotype in the absence of vancomycin. In contrast, in isolate PC-3, a well-studied GISA isolate documented to have been acquired *in vivo*,[14] cell wall thickening did not occur in the absence of vancomycin. It has been proposed that such increased cell wall thickness may restrict access of vancomycin to D-Ala-D-Ala targets of the peptidoglycan precursors at the cell membrane.

By HPLC analysis, we were unable to demonstrate a high proportion of non-amidated muropeptides in the cell wall of the IL isolates[15] such as is present in Mu50, Mu3 and NJ.[11,15] Such an increase in non-amidated muropeptides was interpreted in those strains to cause decreased cross-linking and therefore an increase in the number of D-Ala-D-Ala binding sites that could bind vancomycin and render it unavailable for binding to the D-Ala-D-Ala binding sites on the peptidoglycan precursors. However, IL GISA isolate F actually had increased cross-linking compared with isolate A obtained earlier from this patient, an observation indicating a decrease in D-Ala-D-Ala termini in peptidoglycan in the cell wall of GISA isolate F, and therefore a decrease in the number of 'false targets' available for vancomycin binding. Thus, although our isolates share a modest increase in cell wall thickness consistent with GISA isolates from Japan, the data regarding lysostaphin resistance, taken together with HPLC data, indicate that the cell wall changes in the IL GISA strain are distinct from those of other GISA isolates. Therefore, resistance in our isolates is probably mediated by different biochemical and genetic events.

PFGE analysis of the IL isolates documented an identical *Sma*I restriction pattern and, thus, a close clonal relatedness to the clinical GISA strains MI and PC. We had previously shown that the *Sma*I restriction digestion patterns of GISA isolates MI and PC were identical,[4] and that only a few bands distinguished the pattern of MI and PC from another USA GISA isolate, NJ. Thus, the USA GISA isolates we studied are all closely related. The importance of this observation lies in the realization that while all these GISA isolates have the same genetic background, important differences exist in their peptidoglycan composition and cross-linking and in their resistance to lysostaphin. Whether this genetic background itself is relevant to the GISA phenotype or merely a chance association remains to be determined.

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