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Exposure of *Staphylococcus aureus* to Subinhibitory Concentrations of β -Lactam Antibiotics Induces Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus*

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Glycopeptides are known to select for heterogeneous vancomycin-intermediate *Staphylococcus aureus* (h-VISA) from susceptible strains. In certain clinical situations, h-VISA strains have been isolated from patients without previous exposure to glycopeptides, such as cystic fibrosis patients, who frequently receive repeated treatments with beta-lactam antibiotics. Our objective was to determine whether prolonged exposure to beta-lactam antibiotics can induce h-VISA. We exposed 3 clinical vancomycin-susceptible methicillin-resistant *Staphylococcus aureus* (MRSA) strains to ceftazidime, ceftriaxone, imipenem, and vancomycin (as a control) at subinhibitory concentrations for 18 days *in vitro*. Population analyses showed progressive increases in vancomycin resistance; seven of the 12 derived strains obtained after induction were classified as h-VISA according to the following criteria: area under the curve (AUC) on day 18/AUC of Mu3 of $\geq 90\%$ and/or growth on brain heart infusion (BHI) agar with 4 mg/liter vancomycin. The derived isolates had thickened cell walls proportional to the level of glycopeptide resistance. Genes known to be associated with glycopeptide resistance (*vraSR*, *yyqF*, SA1703, *graRS*, *walkR*, and *rpoB*) were PCR sequenced; no *de novo* mutations were observed upon beta-lactam exposure. To determine whether *trfA*, a gene encoding a glycopeptide resistance factor, was essential in the selection of h-VISA upon beta-lactam pressure, a *trfA*-knockout strain was generated by allelic replacement. Indeed, beta-lactam exposure of this mutated strain showed no capacity to induce vancomycin resistance. In conclusion, these results showed that beta-lactam antibiotics at subinhibitory concentrations can induce intermediate vancomycin resistance *in vitro*. This induction required an intact *trfA* locus. Our results suggest that prior use of beta-lactam antibiotics can compromise vancomycin efficacy in the treatment of MRSA infections.

Since the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin (VAN) has become the treatment of choice for MRSA infections. The first *S. aureus* strains with reduced susceptibility to vancomycin (vancomycin-intermediate *S. aureus* [VISA]) were reported in Japan in 1997 (1), but these strains remain rare. Heterogeneous vancomycin-intermediate *S. aureus* (h-VISA) strains were also reported in Japan in 1997 (2) and are much more frequent. The h-VISA strains contain subpopulations with reduced vancomycin susceptibility but remain undetectable by standard MIC determinations. h-VISA strains have been reported worldwide (3, 4), mostly of MRSA (5) and occasionally of methicillin-susceptible *S. aureus* (MSSA) (6). Today, the prevalence of h-VISA among MRSA strains is highly variable (0.7 to 50%, with a median prevalence of approximately 10%) (4), depending on both the country and the detection methods.

The clinical significance of h-VISA is difficult to determine definitively, but many studies suggest that h-VISA may be at the origin of treatment failures (7, 8). In addition, repeated exposure of h-VISA to vancomycin can select for variants reaching the homogeneous VISA resistance level (2, 5). Several reports have shown a linear relationship between MIC values and treatment failure, even for strains with MICs remaining in the susceptible range (9).

Regarding the ontology of h-VISA strains, the most plausible scenario is that these strains emerged from vancomycin-susceptible *S. aureus* (VSSA) strains exposed to vancomycin, especially in

patients (infected or colonized with *S. aureus*) receiving long-term treatment with this antibiotic (10). In 2009, Katayama et al. showed that imipenem (IPM), a beta-lactam antibiotic largely used in Japan, can also select for a vancomycin-resistant population from a laboratory VSSA strain (11). Although it is based on a single laboratory strain, this result suggests that beta-lactam exposure can be a risk factor for the development of h-VISA strains. In clinical situations, certain patient populations, such as cystic fibrosis patients, are more chronically exposed to beta-lactam antibiotics than others. In this context, h-VISA strains have been isolated from patients who were not previously exposed to glycopeptides (12, 13). A retrospective study of cystic fibrosis patients' clinical characteristics that was conducted at our hospital suggested that ceftazidime (CAZ), a beta-lactam largely used to

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TABLE 1 Characteristics of tested strains

Strain	Origin	Resistance	agr type/ CC ^a	Associated resistances	VAN MIC (mg/liter)	Growth on BHI agar with 4 mg/ liter VAN	AUC/Mu3 AUC (%)
FOU	Isolated from patient with cystic fibrosis	MRSA, <i>mecA</i> positive	II/CC5	Kanamycin, tobramycin, erythromycin, lincomycin, fusidic acid, rifampin	1.5	No	51
NAR	Isolated from patient with cystic fibrosis	MRSA, <i>mecA</i> positive	I/CC8	Kanamycin, tobramycin, levofloxacin	1.5	No	41
GUA	Isolated from patient with cystic fibrosis	MRSA, <i>mecA</i> positive	II/CC5	Kanamycin, tobramycin, fusidic acid	2	No	58

^a CC, clonal complex, determined from DNA microarray.

treat *Pseudomonas aeruginosa* infections in such patients, may be a risk factor for the development of h-VISA (14).

Taken together, these observations raise the question of whether prolonged exposure to beta-lactam antibiotics can induce reduced susceptibility to glycopeptides, thus impairing their efficiency if they are actually required for the treatment of *S. aureus* infections. We performed *in vitro* experiments to determine whether prolonged exposure of *S. aureus* to subinhibitory concentrations of beta-lactams can lead to the emergence of h-VISA. To this end, we exposed three clinical vancomycin-susceptible MRSA strains to 3 beta-lactams (CAZ, ceftriaxone [CRO], and IPM) and to vancomycin (as a control) for 18 days. We then examined the strains' vancomycin resistance levels by population analyses. Confirming our hypothesis, we observed a number of h-VISA strains among our strains. We then investigated the possible molecular mechanisms underlying this phenomenon.

MATERIALS AND METHODS

Definition of h-VISA. Because there is no consensus definition of h-VISA, we used two definitions. (i) h-VISA was defined as an *S. aureus* strain with a vancomycin MIC of ≤ 4 mg/liter by standard Etest on Mueller-Hinton (MH) agar and a vancomycin population analysis profile similar to that of Mu3, with a subpopulation growing with 4 mg/liter vancomycin (15). (ii) h-VISA was also defined based on an area under the curve (AUC) ratio for the strain versus Mu3, as determined by population analyses, that was higher than 0.90 (90%) (16); this definition has recently been reported to be one of the most accurate (4).

Bacterial strains. The *S. aureus* strains used in the present study were three randomly selected MRSA strains isolated from cystic fibrosis patients. These strains were classified as VSSA according to the definitions presented above (Table 1). Mu3 was used as a control strain for heterogeneous vancomycin resistance in the population analyses.

Induction phase. The strains were induced in microplates using the following ranges of antibiotic concentrations (dilutions in MH broth): ceftazidime (CAZ) (GlaxoSmithKline, Brentford, England), 4 to 8,200 mg/liter; ceftriaxone (CRO) (Mylan, Pittsburgh, PA), 2 to 12,500 mg/liter; imipenem (IPM)-cilastatin (Fresenius Kabi, Bad Homburg, Germany), 0.0625 to 2,048 mg/liter; vancomycin (VAN) (Mylan, Pittsburgh, PA), 0.03125 to 64 mg/liter. Initial inocula (100 μ l) were made from a 0.5 McFarland standard suspension diluted 1:50, which corresponded to a final microplate concentration of 10^6 CFU/ml. The strains were subcultured daily for 18 days by sampling the well with the highest antibiotic concentration at which growth was visually observed. The bacteria from this well were diluted (1:100) in MH broth and inoculated in each well of a new microplate at a 1:200 final dilution. For each strain, a blank was established by serial passaging in drug-free MH broth. Every 6 days, a vancomycin population analysis was performed for each strain exposed to each antibiotic.

h-VISA stability testing. The stability of the antibiotic-induced strains (AB strains), defined as h-VISA according to one or both of the

definitions presented above, was tested by nonselective serial passaging in drug-free MH broth for 18 days. The stability of a homogenous resistant subpopulation selected from the NAR strain after exposure to ceftazidime and growth on brain heart infusion (BHI) agar with vancomycin at 6 mg/liter was tested for 36 days.

Vancomycin population analysis. Population analyses were performed as described by Wootton et al. (16). After 24 h of culture in liquid BHI medium (Bacto BHI; Becton, Dickinson, Heidelberg, Germany) at 37°C, the bacterial suspension was adjusted to a 2 McFarland standard. This suspension (50 μ l) was spiral plated on BHI agar (Difco BHI agar; Becton, Dickinson, Heidelberg, Germany) containing 0, 1, 2, 3, 4, 5, or 6 mg/liter vancomycin. To control the inoculum, the suspension was diluted 1:10⁴ and spiral plated on BHI agar without vancomycin. Colonies were counted after 48 h of incubation at 37°C and after 72 h to take into account small resistant colonies, which often grow slowly.

Statistics and mathematical interpretation of population analysis data. The Mu3 AUC was used as a reference and was calculated for each series of BHI-vancomycin agars. To check the reproducibility of the population analyses, a statistical Shapiro-Wilk normality test was performed on 15 different data sets obtained with Mu3, with a confidence interval of 95%. This test showed no significant differences ($P = 0.7956$) among the sets of analyses. The AUC values of the different AB strains were calculated, and the ratios of the AUCs for the tested strains to the corresponding AUC for Mu3 were determined. In parallel with that calculation, the ratio of the final AUC (day 18) to the initial value (day 0) was calculated for each tested strain. All analyses were performed using Prism software (GraphPad, San Diego, CA).

Vancomycin and daptomycin MICs. A 0.5 McFarland standard suspension was prepared in 0.85% NaCl using colonies sampled from a 24-h blood agar plate. The suspension was evenly streaked on MH agar using a cotton swab. Etest strips (bioMérieux, Marcy l'Etoile, France) for vancomycin or daptomycin were applied. The plates were incubated for 48 h at 37°C before reading.

Strain monitoring. Antibiotic susceptibility testing was performed every 6 days by the standardized disc diffusion method, to monitor the strains and to check for cross-contamination. The strains were also checked by DNA microarray analysis at day 0 and day 18 of the induction phase. Bacterial DNA was extracted using a commercial extraction kit according to the manufacturer's recommended protocol (DNA minikit; Qiagen, Venlo, Netherlands). The diagnostic DNA microarray used in this study (Identibac *S. aureus* genotyping kit; Alere Technologies, Jena, Germany) and the related procedures and protocols have been described in detail previously (17). This microarray covers 332 different target sequences, corresponding to approximately 185 distinct genes and their allelic variants.

Sequencing of candidate loci involved in h-VISA. The most common loci known to be associated with h-VISA, namely, *vraS*, *vraR*, *vyqF*, SA1703, *graR*, *graS*, *walR*, *walK*, and *rpoB*, were sequenced in parental strains and in h-VISA obtained upon beta-lactam and vancomycin induction. PCR fragments were amplified using the primers described by Hafer et al. (18). Sequences were aligned and compared with the reference strain

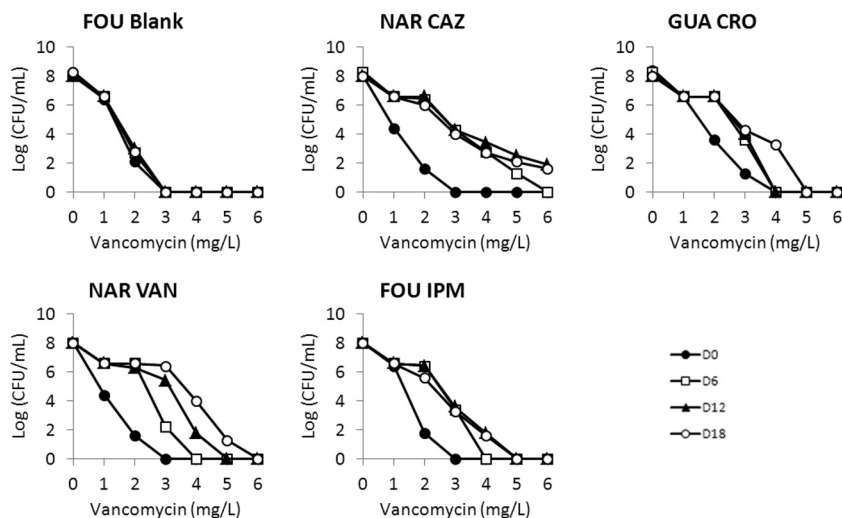


FIG 1 Representative population analyses profiles of the induction phase. Population analyses are shown for selected isolates before beta-lactam induction (D0) and during the induction phase on day 6 (D6), day 12 (D12), and day 18 (D18). The numbers of cells growing on BHI agar containing vancomycin are shown on the y axis, and the vancomycin concentrations are shown on the x axis.

S. aureus subsp. *aureus* N315 (taxid, 158879) using BLAST (19). Only nonsynonymous mutations were reported.

Transmission electron microscopy. *S. aureus* samples were prepared for transmission electron microscopy after overnight culture in BHI liquid medium, as described previously (6). Observations were made on a transmission electron microscope (1400 JEM; Jeol, Tokyo, Japan) equipped with a Gatan camera (Orius 600) and digital micrograph software. Images were analyzed further using ImageJ software (National Institutes of Health, Bethesda, MD). Cell wall thickness was determined by measuring at least 30 bacteria of each strain at 6 different points. Results were expressed in nanometers.

Targeted *trfA* gene disruption mutagenesis. A chromosomal *trfA* disruption mutant was constructed by deletion of the *trfA* gene and insertion of a tetracycline resistance gene, resulting in strain AR612, as described previously (20). The $\Delta trfA$ disruption mutant in strain NAR was obtained by transduction from AR612 using bacteriophage $\Phi 80\alpha$ and selection for tetracycline resistance. Two colonies, designated AR1453 and AR1454, were selected, and deletion of the *trfA* gene was confirmed by PCR and reverse transcription-PCR. The AR1454 strain, designated NAR $\Delta trfA$, was then induced by beta-lactam antibiotics and vancomycin as described above for the wild-type NAR strain.

RESULTS

Induction phase. Three MRSA clinical isolates, initially classified as VSSA, were subcultured daily for 18 days in the presence of 4 different antibiotics, i.e., 3 beta-lactams (IPM, CAZ, and CRO) and vancomycin. Population analyses were performed every 6 days. Representative curves obtained for the induction phase are shown in Fig. 1. The results of the population analyses showed progressive increases in vancomycin resistance for both beta-lactam- and vancomycin-induced strains, as assessed by the day 18/day 0 AUC ratio. Variations were from 12% to 187% for the antibiotic-induced strains (AB strains), depending on both the antibiotic used and the initial strains (Table 2). A control experiment using the same isolates cultured without antibiotic (blank) showed no significant variation (from -2% to 8%) in the AUC ratio over the 18 days (Table 2).

In considering the two definitions of h-VISA, six AB strains complied with both definitions, namely, FOU with VAN, NAR

with CAZ and VAN, and GUA with IPM, CRO, and VAN. In addition, FOU with IPM conformed to only the first definition; this strain grew on BHI agar with 4 mg/liter vancomycin, but its AUC ratio with Mu3 was immediately below the 90% threshold. For each strain, vancomycin induced the maximum increase in resistance, as assessed by the day 18/day 0 AUC ratio; however, the best inducer among the beta-lactams varied among the 3 strains.

Although the population analyses revealed an increase in vancomycin resistance after beta-lactam exposure, the resistance remained undetectable in standard MIC determinations by Etest with a 0.5 McFarland standard inoculum (Table 3) and by antibiotic susceptibility testing by the disc diffusion method. Altogether, these results classify our AB strains as h-VISA and not VISA.

A genome-based comparison of the AB strains with their parental counterparts showed no modification in the DNA microarray profiles, confirming the lack of major genomic variations and of contamination by another strain. Similarly, the antibiotic susceptibility testing results for the wild-type and AB strains were superimposable.

h-VISA stability testing. To assess the stability of the observed increases in resistance to vancomycin, the 7 antibiotic-induced h-VISA strains were tested by serial passages in drug-free MH broth for 18 days (after 3 weeks of storage at -20°C). The results are designated as being collected on day 36 (18 days of induction and 18 days of decrease). The population analyses performed every 6 days revealed progressive decreases in the resistance to vancomycin for most of the strains with all antibiotics, including vancomycin (Table 4; see Fig. S1 in the supplemental material). However, none of the strains reached its initial level of vancomycin susceptibility, and several strains remained classified as h-VISA. In addition, the stability of a homogeneous resistant subpopulation selected from the NAR strain after exposure to ceftazidime and growth on BHI agar with 6 mg/liter vancomycin was tested for 36 days. This subpopulation also showed a slight AUC decrease of 9% after 18 days of passaging without antibiotics, as found for the NAR strain with CAZ. During this phase, we also tested the stability of Mu3. In contrast to what we observed for

TABLE 2 Vancomycin population analysis during induction phase for clinical strains

Strain	No. of days	Antibiotic exposure	AUC (log ₁₀ [CFU/ml] × mg/liter)	Day 18 AUC/day 0 AUC (%)	Growth on 4 mg/liter VAN	AUC/Mu3 AUC (%)
FOU	0		12.51		No	51
	18	Blank	12.75	2	No	52
	18^a	VAN	23.61	89	Yes	97
	18	IPM	20.99	68	Yes	86
	18	CAZ	14.04	12	No	58
	18	CRO	16.32	30	No	67
NAR	0		10.03		No	41
	18	Blank	9.87	-2	No	39
	18	VAN	28.82	187	Yes	115
	18	IPM	16.39	63	No	66
	18	CAZ	26.18	161	Yes	105
	18	CRO	17.74	77	No	71
GUA	0		14.1		No	58
	18	Blank	15.21	8	No	61
	18	VAN	32.71	132	Yes	131
	18	IPM	23.98	70	Yes	98
	18	CAZ	17.15	22	No	69
	18	CRO	24.69	75	Yes	10

^a Data for antibiotic-induced strains classified as h-VISA are in bold type.

most of our AB strains, the vancomycin resistance of Mu3 remained stable during the entire stability testing phase.

Daptomycin resistance. Daptomycin is a lipopeptide antibiotic that can be used to treat MRSA infections if vancomycin treatment has failed or if the strains have been classified as h-VISA (21). Recent reports have indicated that daptomycin susceptibility may be reduced in h-VISA and VISA strains. We thus compared the daptomycin MICs in our strains before and after 18 days of

exposure to beta-lactam antibiotics or to vancomycin. The results revealed moderate increases in the daptomycin MICs for strains passaged with vancomycin (day 18/day 0 MIC ratios of 3.0 to 21.3, depending on the isolate), with two of the strains reaching the breakpoint value of 1 mg/liter. The other antibiotics induced only slight reductions in daptomycin susceptibility (day 18/day 0 MIC ratios of 1.0 to 5.3), without reaching the breakpoint value (Table 3).

TABLE 3 Vancomycin and daptomycin MICs determined initially and after induction phase

Strain	No. of days	Antibiotic exposure	Vancomycin		Daptomycin	
			MIC (mg/liter) ^a	Day 18 MIC/day 0 MIC (mg/liter)	MIC ^a (mg/liter)	Day 18 MIC/day 0 MIC (mg/liter)
FOU	0		1.5		0.064	
	18	Blank	1.5	1	0.064	1.0
	18	VAN^b	3	2	0.19	3.0
	18	IPM	1.5	1	0.094	1.5
	18	CAZ	1.5	1	0.125	2.0
	18	CRO	2	1.3	0.19	3.0
NAR	0		1.5		0.047	
	18	Blank	1.5	1	0.064	1.4
	18	VAN	4	2.7	1	21.3
	18	IPM	1.5	1	0.094	2.0
	18	CAZ	3	2	0.25	5.3
	18	CRO	2	1.3	0.19	4.0
GUA	0		2		0.094	
	18	Blank	1.5	0.75	0.094	1.0
	18	VAN	4	2	1	10.6
	18	IPM	2	1	0.094	1.0
	18	CAZ	2	1	0.094	1.0
	18	CRO	2	1	0.19	2.0

^a MICs were determined using Etest.

^b Data for antibiotic-induced strains classified as h-VISA are in bold type.

TABLE 4 Vancomycin population analysis at end of stability testing phase

Strain	No. of days	Antibiotic exposure	Day 36 AUC (\log_{10} [CFU/ml] \times mg/liter)	Day 36 AUC/day 18 AUC (%)	Day 36 AUC/day 0 AUC (%)	Growth on 4 mg/liter VAN	AUC/Mu3 AUC (%)
FOU	36	IPM	16.75	-20	34	No	72
	36	VAN	16.74	-29	34	No	72
NAR	36	CAZ^a	26.67	2	166	Yes	114
	36	CAZ V6^b	34.14	-8		Yes	146
	36	VAN	22.66	-21	126	Yes	97
GUA	36	IPM	20.85	-13	48	Yes	89
	36	CRO	23.38	-5	66	Yes	100
	36	VAN	20.52	-37	46	No	88
Mu3			24.76	5		Yes	106

^a Data for antibiotic-induced strains classified as h-VISA are in bold type.

^b Homogeneous VAN-resistant subpopulation growing on BHI agar with 6 mg/liter VAN.

Cell wall thickness. h-VISA strains are known to have thickened cell walls. Transmission electron microscopy was performed on some of our h-VISA strains to check for this phenotype (Fig. 2). h-VISA strains obtained by induction had thickened cell walls, compared to their VSSA counterparts ($P < 0.001$). A significant

correlation between cell wall thickness and vancomycin resistance level was found, with a Pearson correlation coefficient of 0.8954 (Fig. 2C). These results confirmed that cell wall thickening is a common feature of h-VISA strains, including those induced by beta-lactams.

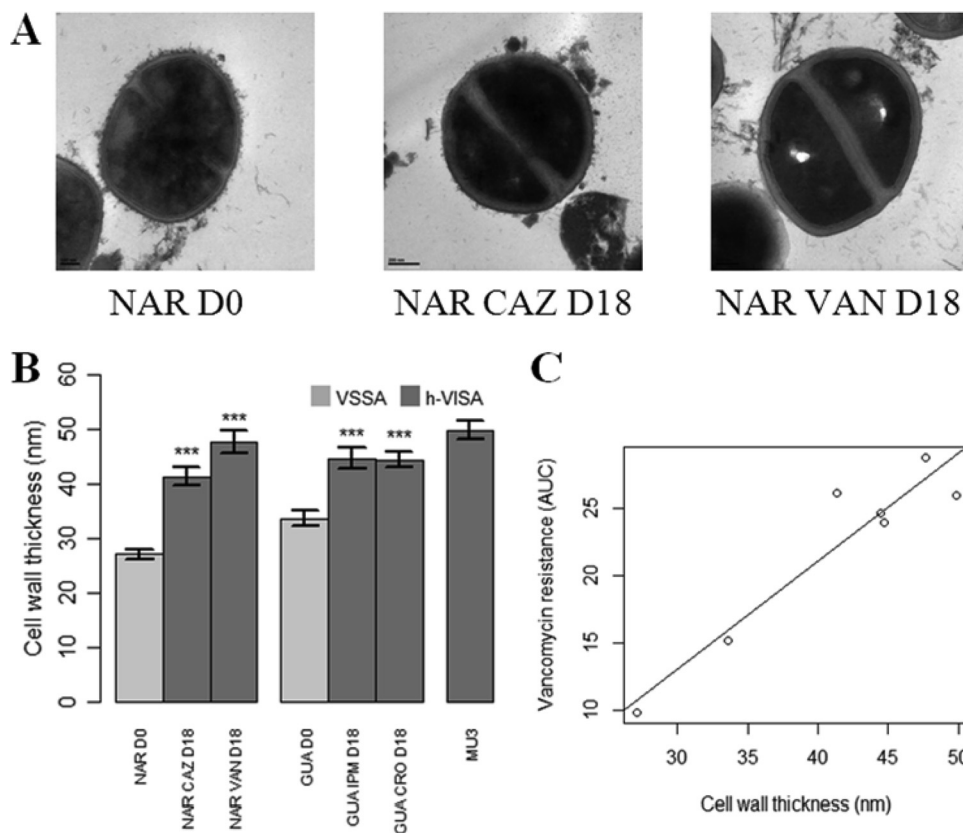


FIG 2 Variation of cell wall thickness upon induction. (A) *S. aureus* images obtained with a transmission electron microscope (1400 JEM; Jeol, Tokyo, Japan) equipped with a Gatan camera (Orius 600). The NAR strain is shown before beta-lactam induction (D0) and at day 18 (D18) of induction with ceftazidime (CAZ) or vancomycin (VAN). (B) Analysis of the *S. aureus* cell wall thickness measured by transmission electron microscopy. The values represent the means of 30 independent bacterial cells with 95% confidence intervals. h-VISA strains obtained at the end of the induction phase showed thickened cell walls, compared to their VSSA counterparts, using a paired Student *t* test ($P < 0.001$). (C) Correlation between cell wall thicknesses and vancomycin resistance levels, expressed as AUC ($r^2 = 0.8954$, $P < 0.01$).

TABLE 5 Nonsynonymous mutations of genes involved in h-VISA resistance

Strain	No. of days of treatment	Mutation(s) ^a								
		<i>vraS</i>	<i>vraR</i>	<i>yvqF</i>	SA17003	<i>graR</i>	<i>graS</i>	<i>rpoB</i>	<i>walk</i>	<i>walR</i>
NAR	0	None	E59D	None	None	D148Q	L26F, I59L, T224I	None	None	None
NAR CAZ (h-VISA)	18	None	E59D	None	None	D148Q	L26F, I59L, T224I	None	None	None
NAR VAN (h-VISA)	18	None	E59D	None	None	D148Q	L26F, I59L, T224I	None	R282C ^b	None
GUA	0	None	None	Y128C	None	None	None	A477D, G171D	None	None
GUA CRO (h-VISA)	18	None	None	Y128C	None	None	None	A477D, G171D	None	None
GUA VAN (h-VISA)	18	None	None	Y128C	None	None	None	A477D, G171D	T279I, ^b S221P ^b	None
FOU	0	None	None	None	None	None	None	D471Y	None	None
FOU IPM (h-VISA)	18	None	None	None	None	None	None	D471Y	None	None
FOU VAN (h-VISA)	18	None	None	None	None	None	None	D471Y	K13R ^b	None

^a Amino acid substitutions are indicated using N315 as a reference.

^b New mutations, which appeared in the course of antibiotic induction.

Sequencing of candidate loci involved in h-VISA resistance.

vraS, *vraR*, *yvqF*, SA1703, *graR*, *graS*, *walR*, *walk*, and *rpoB* were sequenced, and nonsynonymous mutations were compared to N315 (Table 5). No *de novo* mutations were observed upon beta-lactam exposure, while the true h-VISA strains obtained by vancomycin induction showed new mutations in *walk*.

Induction of Δ*trfA* NAR strain. To determine whether *trfA* was required for the induction of h-VISA, a *trfA*-knockout strain was generated in the clinical strain NAR and the resulting mutant was induced by a beta-lactam or vancomycin for 18 days. Beta-lactam induction of the Δ*trfA* NAR strain showed no capacity to induce vancomycin resistance, with variation in AUC values of -30% to 12% (Fig. 3). For the vancomycin induction, the variation rate was 39% but the strain was not classified as h-VISA with a day 18 AUC/Mu3 AUC value of 61% and no growth on BHI agar with 4 mg/liter vancomycin. A duplicate of this experiment showed the same results (data not shown).

DISCUSSION

The induction phase showed that exposure of three clinical vancomycin-susceptible MRSA strains to 3 different beta-lactams

(imipenem, ceftazidime, and ceftriaxone) can select for h-VISA. This finding partly matches the observation of Katayama et al. (11), who demonstrated the selection of h-VISA by imipenem. In contrast to our results, however, ceftriaxone did not select for h-VISA strains in their experiment. This result may be explained by the fact that the researchers used only one laboratory strain (ΔIP). As shown in the present study, the level of vancomycin resistance induced by beta-lactams varied greatly between the strains. Because each of the three beta-lactams tested here can induce h-VISA, all beta-lactams may be capable of inducing increased vancomycin resistance. Due to our small strain number, we were unable to perform statistical analyses to test whether the increased resistance depended on the beta-lactam antibiotic used or on the genetic characteristics/backgrounds of the strains.

The results of the stability testing phase showed that h-VISA strains induced by beta-lactams were apparently unstable. Accordingly, it has already been demonstrated that the vancomycin resistance of clinical isolates reverts during serial passages on non-selective medium (22, 23). In addition, this reversion occurs in patients; when vancomycin treatment of a resistant strain is sus-

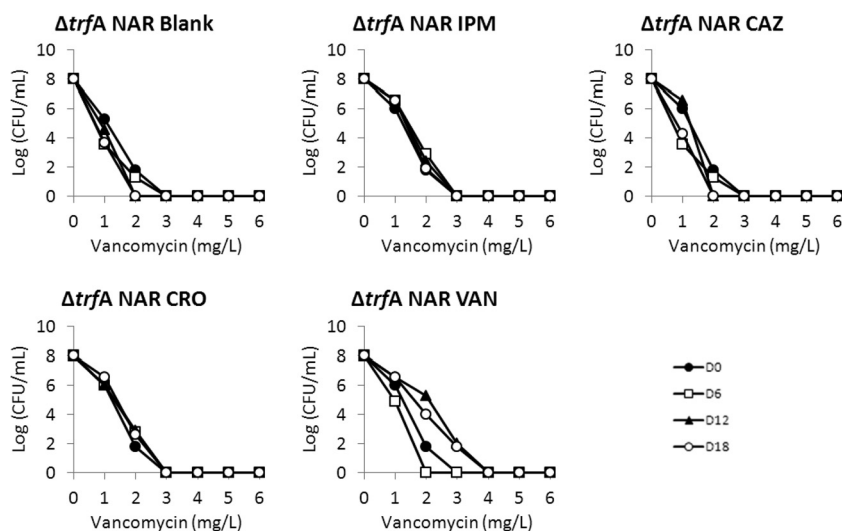


FIG 3 Population analysis profiles of the Δ*trfA* NAR strain. Population analyses are shown for selected isolates before beta-lactam induction (D0) and during the induction phase on day 6 (D6), day 12 (D12), and day 18 (D18). The numbers of cells growing on BHI agar containing vancomycin are shown on the y axis, and the vancomycin concentrations are shown on the x axis.

pended, the h-VISA or VISA strain reverts to a sensitive status after several months (24). Our results are in agreement with these observations, although we did not observe complete reversion to the initial level of sensitivity to vancomycin after 18 days of subculture in antibiotic-free medium. Complete reversion might be achieved only after extended subculture in antibiotic-free medium (22, 25). This reversibility suggests that the mechanism underlying such resistance is counterselective in the absence of antibiotic selective pressure. Genetic support for this reversible phenotype might be either epigenetic or caused by a mutated subpopulation that is purged in the absence of selective antibiotic pressure. The fact that even the homogeneous resistant population resulting from exposure to vancomycin at 6 mg/liter showed decreased resistance in the absence of antibiotics does not support the mutation hypothesis. In addition, the epigenetic hypothesis is supported by the results of PCR sequencing of the candidate loci commonly associated with glycopeptide resistance, which showed no *de novo* mutations upon beta-lactam pressure (Table 5).

Modification of the cell wall seems to be involved in the mechanism of intermediate resistance to vancomycin. Compared with their VSSA counterparts, our h-VISA strains showed a thickened cell wall (Fig. 2), which is most probably due to activated cell wall synthesis (26–28). It has been reported that the simultaneous exposure of *S. aureus* to vancomycin and beta-lactams *in vitro* can lead to vancomycin resistance in certain strains, designated beta-lactam-induced vancomycin-resistant MRSA (BIVR) (29). The proposed mechanism was the linkage of the increase in cell wall precursors observed in the presence of beta-lactams to the vancomycin present in the medium, thus limiting vancomycin's availability for target inhibition (26). The persistence of such a mechanism in the absence of beta-lactams was not evaluated. We can speculate that this mechanism may have been at work in our study, with the decreased resistance in the absence of antibiotics caused by a progressive return to the wild-type expression of cell wall precursors. A very common feature in VISA strains is modification of the expression of genes that regulate the *S. aureus* cell wall biosynthesis pathway (30). Recently, beta-lactams have been shown to be able to induce similar transcriptomic changes (31, 32), supporting the possibility of beta-lactam-induced reduced susceptibility to vancomycin. These transcriptomic modifications involve several two-component systems, i.e., *vraRS*, *graRS*, and *walkR* (4) as well as *trfA* (teicoplanin resistance factor A), a gene that was shown to be linked to decreased susceptibility to vancomycin (20). This gene is highly expressed in glycopeptide-resistant strains, compared with their susceptible counterparts (20). *trfA* gene deletion leads to a significant loss of oxacillin and glycopeptide resistance. The TrfA protein also demonstrates inducible expression following brief exposure to several cell wall-active antibiotics, including oxacillin (33). Our results showed that *trfA* is required for h-VISA induction upon beta-lactam pressure. Interestingly, *trfA* gene expression is controlled by the oxidative stress response regulator Spx (33). Cell wall antibiotic exposure leads to stabilization of the Spx protein. It can be speculated that antibiotic control of Spx protein stabilization may be part of the genetic explanation for the reversible phenotype observed in our strains. Antibiotic exposure stabilizes Spx and leads to increased expression of *trfA*, whereas antibiotic clearance decreases Spx protein amounts, with concomitant decreases in TrfA levels. Study of the molecular function of the *trfA* gene is

crucial for understanding how it modulates glycopeptide resistance. However, based on homology with the *Bacillus subtilis mecA* gene, we can speculate that *trfA* facilitates the assembly of functional ClpC and thus influences ClpC molecular processes, including cell wall metabolism (34).

Certain studies have already suggested that h-VISA and VISA strains are linked to a decrease in daptomycin susceptibility (35). The induction of heterogeneous daptomycin resistance has been observed after vancomycin exposure (36). Our results showed a slight decrease in daptomycin susceptibility after beta-lactam exposure, but without reaching the resistance level. However, this finding suggests that the use of beta-lactams can also impair daptomycin efficacy. The potential mechanism for the decrease in daptomycin susceptibility in VISA isolates is unclear. This decrease may be linked to changes in cell wall thickness that impair the diffusion of daptomycin through the cell wall (37). Additional theories, such as alterations in cell wall surface charge or alterations in drug binding, have also been suggested (35).

Clinical extrapolations of our *in vitro* results must be made with caution. Our results show that beta-lactams can induce h-VISA from the 3 clinical VSSA strains tested. We placed our strains under conditions of subinhibitory concentrations to maximize the risk of resistance emergence. These extreme conditions may be found *in vivo* because MRSA strains have very high beta-lactam MICs and because antibiotic diffusion may be compromised in certain clinical situations such as cystic fibrosis, due to an abundance of mucus and *Pseudomonas aeruginosa*-dependent biofilm (38). Cystic fibrosis patients are thus a population at risk of h-VISA development due to a combination of many factors, including *S. aureus* carriage (with variable proportions of MRSA) and repeated beta-lactam treatments (notably targeting Gram-negative bacteria such as *P. aeruginosa*), potentially at subinhibitory concentrations, against *S. aureus*.

Our *in vitro* results suggest that previous exposure to beta-lactam antibiotics at subinhibitory concentration can decrease the efficacy of vancomycin when it is needed to treat MRSA infections. This induction of h-VISA resistance by beta-lactam antibiotics requires an intact *trfA* locus, thus confirming the role of this gene in glycopeptide resistance pathways. These observations support the difficulty of predicting the global impact of antibiotic therapy on patients' resistomes. Clinical studies need to be conducted to evaluate the relevance of such unpredicted observations in patients undergoing beta-lactam antibiotic therapy.

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