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Selection of Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* by Imipenem▽

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Vancomycin (VAN)-intermediate *Staphylococcus aureus* (VISA) and heterogeneous VISA (hVISA) isolates are considered to have emerged from VAN-susceptible *S. aureus* (VSSA) by spontaneous mutation during VAN exposure. We previously reported that laboratory mutant H14, obtained from VSSA strain ΔIP by exposure to imipenem (IPM), showed overexpression of the *vraSR* two-component system and a typical hVISA phenotype. In the present study, to elucidate the mechanism of VSSA conversion to hVISA, we further characterized strain H14 by determining its whole-genome sequence, morphology, cell wall synthetic activity, and gene expression. Genome sequencing revealed that H14 harbored a mutated *vraS* (designated $vraS_{H14}$) that caused an amino acid substitution (S_{329}→L). This mutation is different from the VraS mutation (N_5→I) identified in representative clinical hVISA strain Mu3. However, H14 exhibited a phenotype similar to that of Mu3, including heterogeneous resistance to VAN, enhanced cell wall synthetic activity, and *vraSR* overexpression. Replacement of the *vraS* gene of ΔIP with the mutated $vraS_{H14}$ gene confirmed that the S_{329}→L substitution was responsible for both the upregulation of *vraSR* and conversion to the hVISA phenotype. This conversion was also achieved by using the *vraS* gene of Mu3, which carries a mutation (N_5→I), but not with the native *vraS* gene of strain N315. Finally, we carried out a study to analyze the appearance of hVISA from VSSA by exposure of ΔIP to selective concentrations of VAN and beta-lactam antibiotics. A total of 8 and 5 hVISA isolates were detected among 50 isolates selected with VAN and IPM, respectively. Among the 13 hVISA mutants, mutation in *vraSR* was detected only in mutant strain H14, suggesting that additional mutational mechanisms can be responsible for evolution to the hVISA phenotype. We conclude that exposure not only to VAN but also to beta-lactams may select for reduced glycopeptide susceptibility in *S. aureus*.

Methicillin (meticillin)-resistant *Staphylococcus aureus* (MRSA) is a major cause of serious nosocomial infections, and the emergence of virulent MRSA strains in the community is of particular concern (6). Vancomycin (VAN) still serves as the main therapeutic agent for infections caused by multiresistant MRSA strains (17). However, MRSA strains with various degrees of reduced susceptibility to glycopeptides, vancomycin-intermediate *S. aureus* (VISA) and heterogeneous VISA (hVISA) strains, have emerged among multidrug-resistant MRSA clinical strains (9, 16).

Recently, we identified several genes that are overexpressed in VISA strain Mu50 and hVISA Mu3 compared to their levels of expression in their isogenic VAN-susceptible *S. aureus* (VSSA) strain, strain Mu50Ω (13); and among these, we found the overexpression of the *vraSR* two-component system (TCS), an upregulator of the *S. aureus* cell wall biosynthesis pathway (12, 13). We also demonstrated that the *vraS* gene is overexpressed in ΔIP-H14 (H14), a laboratory-derived hVISA strain obtained by selecting VSSA strain N315ΔIP (ΔIP) with 8 mg/liter of imipenem (IPM) (12) and showed that a single amino acid substitution in VraS was present in H14, Mu3, and Mu50 (10a).

In the study described here, we further characterized hVISA strain H14, investigated the role of the *vraS* mutation on the phenotype of H14, and evaluated the rates of selection of hVISA from VSSA ΔIP following exposure to VAN and beta-lactam antibiotics.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth condition. The *Staphylococcus* strains and plasmids used in the present study are listed in Table 1. The cloning and transformation of *Escherichia coli* JM109 were carried out by standard techniques (http://catalog.takara-bio.co.jp/en/PDFFiles/9052_e.pdf; Takara-Bio Co., Ltd., Shiga, Japan). All *S. aureus* strains were cultivated in brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, MI) with aeration at 37°C, unless indicated otherwise. The antibiotics tetracycline and chloramphenicol (Sigma Chemical Co., St. Louis, MO) were used for the selection of the *S. aureus* transformants. VAN (Sigma), teicoplanin (Astellas Pharma Inc., Osaka, Japan), ceftriaxone (CRO; Sigma), IPM (provided by Banyu Pharmaceutical Co., Tokyo, Japan), ampicillin-sulbactam (SAM; provided by Pfizer Pharmaceuticals Inc., Tokyo, Japan), and rifampin (RIF; rifampicin; Sigma) were used for antibiotic susceptibility tests. RIF-resistant (Rif^r) strain ΔIP-rifR was selected by culturing ΔIP on BHI agar containing 1 mg/liter of RIF and was obtained at a frequency of 2.8×10^{-7}. The isolates were evaluated for the presence of the His481→Tyr mutation in the *rpoB* gene of ΔIP-rifR, which confers RIF resistance, by DNA sequencing, as reported previously (26).

DNA methods. DNA manipulations were performed by standard methods (11). Restriction enzymes were used as recommended by the manufacturer (Takara). Routine PCR amplification was performed with an Expand High Fidelity system (Roche, Mannheim, Germany).

Transmission electron microscopy. The preparation for and the examination of *S. aureus* cells by transmission electron microscopy were performed as described previously (4).

Incorporation of ^{14}C-labeled D-glucose or ^{14}C-labeled *N*-acetyl-D-glucosamine into cells. The preparation of the cells and the purification of peptidoglycan were performed as described previously (8), and the rate of incorporation was determined as described previously (8). These assays were performed in triplicate.

Whole-genome sequencing of H14 and identification of SNPs compared to the sequence of parent strain ΔIP. Sequencing of the strain H14 genome was performed with a Genome Sequencer 20 system, a recently introduced highly par-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
N315	Pre-MRSA strain carrying a functional *mecI* encoding *mecA* gene repressor, hetero-Met^r, Nagasaki, Japan	10
N315P	Penicillinase plasmid-free strain derived from N315	10
ΔIP	*mecI* null mutant derived from N315P (*mecI*::*tetL*), hetero-Met^r Tet^r	15
H14(or IPM8–14)	Mutant derived from ΔIP by selection with 8 mg/liter of IPM, VraS (S_{329}→L) homo-Met^r Tet^r	12
ΔIP-KVR	*vraSR* null mutant from ΔIP (*vraSR*::*cat*), Met^s Tet^r Chl^r	12
H14-KVR	*vraSR* null mutant from H14 (*vraSR*::*cat*), Met^s Tet^r Chl^r	12
ΔIP-rifR	Mutant derived from ΔIP by selection with 1 mg/liter of RIF, RpoB (H_{481}→Y) hetero-Met^r Tet^r Rif^r	This study
ΔIP::*vraS*$_{H14}$	ΔIP in which *vraS* is replaced by *vraS*$_{H14}$ (S_{329}→L), homo-Met^r Tet^r	This study
ΔIP-rifR:: *vraS*$_{H14}$	ΔIP-rifR in which *vraS* is replaced by *vraS*$_{H14}$, homo-Met^r Tet^r Rif^r	This study
Mu3	hVISA clinical strain, Tokyo, Japan	9
Mu50	VISA clinical strain, Tokyo, Japan	9
NJ	VISA clinical strain, New Jersey	23
Plasmids		
pKOR1	*S. aureus* allele replacement vector	2
pKO- *vraS*$_{H14}$	pKOR1 harboring 1.0-kb PCR product of *vraS*$_{H14}$	This study

[a] Abbreviations: Met^s, methicillin susceptible; Met^r, methicillin resistant; Tet^r, tetracycline resistant; Chl^r, chloramphenicol resistant; Amp^r, ampicillin resistant; Rif^r, RIF resistant. Strains obtained by antibiotic selection are denoted "mutant."

allel genome sequencer from 454 Life Sciences (Branford, CT). In a duplicate run with this instrument, 757,227 short reads, each of which had an average length of 108 bp, were collected. The short-read genome assembly generated by using the Genome Sequencer 20 system Assembler software contained 616 sequence contigs, including 95 contigs of >500 bp, for a total of 2,735,083 bp (average contig size, 28,790 bp). The chromosomal genome sequence of strain N315 (GenBank accession number BA000018) was used as a scaffold to assemble and orient the H14 contigs, and gap closing was carried out by long-range PCR primer walking. Sequencing of the PCR products was performed with a model 3730 capillary sequencer (Applied Biosystems Japan Ltd.). The resulting sequence of the H14 genome was then compared to that of N315, and the discovery of genome-wide single nucleotide polymorphisms (SNPs) was achieved by whole-genome alignments with the Mummer (version 3.20) software package (http://mummer.sourceforge.net/). The discrepancies identified between the two genomes were then verified by resequencing of the corresponding loci of N315 and H14 genomic DNAs that were as long as the corresponding loci of ΔIP, the parent strain of H14, with forward and reverse primers for each locus.

Construction of ΔIP::*vraS*$_{H14}$ strain. For the replacement of the serine amino acid residue with the leucine at position 329 in *vraS* of ΔIP, we used the pKOR1 allele replacement system, as described previously (2). In brief, a 1.0-kb *vraS* insert DNA encompassing 1-kb flanking sequences of phage attachment sites was generated by PCR from the chromosomal DNA of strain H14 by using the following primers: attB1-vraS (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTACTTACGTGAATGAAAAATCATATAAAGTTGAAAATAACAAT-3′) and attB2-vraS (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCATGATCATCCACAAACAATACTTTAATCGTCATACGAATCCTC-3′). The PCR product was used for recombination with pKOR1, and then recombination products were transformed to *E. coli* DH5α, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). The resulting plasmid, pKO-*vraS*$_{H14}$ (where *vraS*$_{H14}$ indicates the mutated *vraS* gene harbored by strain H14) was introduced into *S. aureus* ΔIP by electroporation, generating transformant ΔIP(pKO-*vraS*$_{H14}$). Replacement of the *vraS*$_{H14}$ gene allele with *vraS* in ΔIP was carried out by a two-step procedure, as described previously (1, 2). In this study, ΔIP-rifR was also used for the construction of a *vraS* mutant. Because it became apparent by whole-genome sequence determination that the nucleotide sequences of ΔIP and H14 did not differ except for the *vraS* mutation described above, it was necessary to introduce an artificial marker to exclude the unnoticed contamination of H14 in the culture of ΔIP::*vraS*$_{H14}$. We utilized the amino acid substitution His481→Asn on *rpoB*, which confers RIF resistance, as a marker for mutant strain ΔIP-rifR::*vraS*$_{H14}$ in which the *vraS* gene was replaced by *vraS*$_{H14}$. In the final step of the procedure for mutant construction, 40 ΔIP-rifR(pKO-*vraS*$_{H14}$) transformants were picked from tryptic soy agar plates containing 1 ng/liter of anhydrotetracycline, as described previously (1, 2), from which we identified the desired strains in which the *vraS* gene was replaced by *vraS*$_{H14}$ by determination of the nucleotide sequence.

Northern blot analysis. Preparation of total RNAs and Northern blot analysis for *vraS* and *vraR* were performed as described previously (12) (20). The *vraSR* probe was amplified by PCR with primers SR1 (5′-TCGATGAACCACTACATTAGAACA-3′) and SR2 (5′-CTGCGAATCTTGAACCATTTTCTCT-3′) (see Fig. 3A).

Antibiotic susceptibility tests. Antibiotic susceptibility was examined by Etest (AB Biodisk, Solna, Sweden) and population analysis, as described previously (9). To detect hVISA strains among the 50 antibiotic-selected isolates, we determined the plating efficiency of each isolate on 4 mg/liter of VAN by population analysis. A VAN MIC of 4 mg/liter is the breakpoint for VISA (according to the criteria of the Clinical and Laboratory Standards Institute). hVISA status was defined for isolates that grew on VAN at 4 mg/liter at a frequency of greater than 10^{-6} when a total of 10^8 cells was inoculated.

Statistical analysis. Two-by-two contingency tables were evaluated by Fisher's exact test. The statistical significance of the data was evaluated by Student's *t* test.

RESULTS AND DISCUSSION

Characterization of laboratory-derived hVISA mutant H14, which originated from VSSA strain ΔIP. To investigate the phenotypes associated with reduced susceptibility to VAN, we analyzed the cell wall thickness and cell wall biosynthetic activity of H14, along with those of *vraSR* null mutant strain H14-KVR and parent strain ΔIP, which were used as controls. Electron microscopy analysis showed that H14 had a significantly thicker cell wall and a rougher cell surface than ΔIP, while H14-KVR had a cell wall much thinner than that of the parent strain (Fig. 1A). Student's *t* test demonstrated that the increase in cell wall thickness was statistically significant in all cases ($P < 0.0001$).

To test for cell wall biosynthetic activity, the rates of incorporation of ^{14}C-labeled D-glucose and *N*-acetyl-D-glucosamine into the cell wall peptidoglycan fraction were evaluated by using a fixed number of nondividing cells. As shown in Fig. 1B, the incorporation of *N*-acetyl-D-glucosamine and D-glucose by H14 was significantly enhanced compared to that by ΔIP and H14-KVR. These data indicate that the *vraS* mutation could cause enhanced cell wall biosynthesis activity.

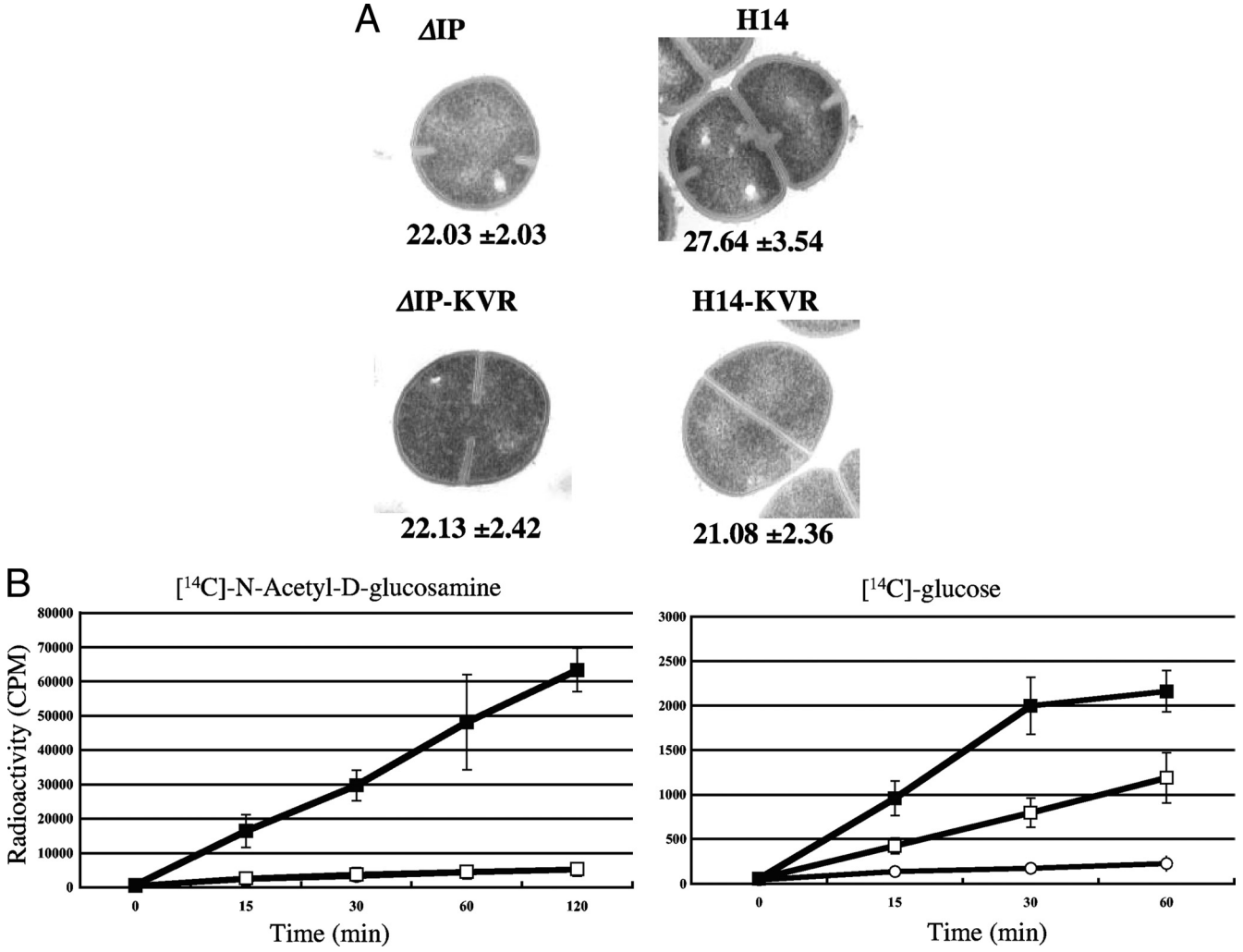

FIG. 1. (A) Transmission electron micrographs of ΔIP derivative strains. The values given under each panel are the means and standard deviations of the cell wall thicknesses of the cells (in nanometers). Magnifications, ×30,000. (B) Incorporation of [^{14}C]*N*-acetyl-D-glucosamine or [^{14}C]D-glucose into the cell wall of the N315 derivative strains. Open squares, parent recipient ΔIP; open circles, H14-KVR; closed squares, H14. The counts per minute were measured at the indicated time points. The experiment was performed in triplicate on three independent occasions, and the results are shown as the mean values ± the standard deviations.

The *vraS* mutation is the only genetic event responsible for hVISA phenotype acquisition in H14. To clarify the genetic mechanism of hVISA phenotype acquisition, genome sequencing of H14 was carried out. The 2,795,992-bp-long whole-genome sequence of H14 was determined. Since H14 is an in vitro derivative of N315, the sequence was practically identical to that of N315 (14), with the exceptions being 42 SNPs and 5 insertions or deletions between the two chromosomes (data not shown). PCR amplification and sequencing of ΔIP chromosomal DNA for the 45 differences found no additional mutations except for the previously reported *vraS* mutation, which causes the replacement of a single amino acid, Ser_{329} with Leu, in H14. This result confirms that the *vraS* mutation causing the replacement of Ser_{329} with Leu was the only genetic event responsible for the acquisition of the hVISA phenotype in H14.

Phenotypic change conferred by *vraS* gene replacement. To see if the single amino acid substitution in the sequence encoding $VraS_{H14}$ is responsible for both the phenotypic expression of hVISA and the overexpression of *vraSR*, we constructed two *vraS* mutants from ΔIP and its RIF-resistant strain, ΔIP-rifR. The native *vraS* gene of the parent strains was replaced by $vraS_{H14}$ to obtain ΔIP::$vraS_{H14}$ and ΔIP-rifR::$vraS_{H14}$. Of 40 transformants analyzed, 3 (7.5%) were found to carry $vraS_{H14}$.

To evaluate the $vraS_{H14}$-mediated phenotypic conversion, we determined the VAN susceptibilities of ΔIP::$vraS_{H14}$ and ΔIP-rifR::$vraS_{H14}$ ($vraS_{H14}$ mutant strains) by the Etest method and population analysis. As shown in Table 2, the MICs of glycopeptides for the $vraS_{H14}$ mutant were higher than those for the parent strain. The population analysis of ΔIP::$vraS_{H14}$ and ΔIP-rifR::$vraS_{H14}$ showed an increase in the proportion of VAN-resistant subpopulations compared with that for parent strain ΔIP (Fig. 2). The patterns of the population curves for H14 and ΔIP::$vraS_{H14}$ were almost superimposable on each other. The same result was obtained by using ΔIP with the RIF resistance marker (the *rpoB* mutation), ex-

TABLE 2. Antibiotic susceptibility profile of ΔIP and its derivative strains determined by Etest

Strain	MIC (mg/liter)								
	RIF	Oxacillin	IPM	VAN	Teicoplanin	Fosfomycin	Bacitracin	Daptomycin	Gentamicin
ΔIP	0.004	6	0.75	1	1	1	32	0.75	0.75
H14	0.004	>256	>32	2	8	96	96	1.75	0.75
ΔIP-KVR	0.004	1.5	0.19	0.75	0.25	0.38	4	0.5	0.75
H14-KVR	0.004	1.5	0.19	0.75	0.25	0.38	4	0.5	0.75
ΔIP-rifR	>32	5	1	1	1	1.5	24	0.5	1
ΔIP::$vraS_{H14}$	0.004	>256	>32	2	8	96	96	1.75	0.75
ΔIP-rifR::$vraS_{H14}$	>32	>256	>32	3	12	96	96	2	1
Mu3	0.006	>256	>32	3	24	>1,024	256	2	384

cept that the population curve for ΔIP-rifR::$vraS_{H14}$ was slightly deflected with 4 mg/liter of VAN. These data clearly demonstrate that the *vraS* mutation alone can confer the phenotypic expression of heterogeneous VAN resistance.

The MICs were then determined, and the MICs of IPM, fosfomycin, bacitracin, and daptomycin for ΔIP::$vraS_{H14}$ and ΔIP-rifR::$vraS_{H14}$ were found to be increased compared with the MICs for ΔIP for which the MICs were similar to those for H14 (Table 2). Recently, Muthaiyan et al. reported that the inactivation of *vraSR* significantly increased susceptibility to daptomycin (18). It was concluded that the *vraS* mutation (Ser329→Leu) alone was responsible for the altered antibiogram of H14 compared to that of its parent, strain ΔIP.

Assessment of *vraS* activation among VISA strains carrying a mutation in the *vraSR* operon. By Northern blot analysis, we examined the transcriptional level of *vraSR* in the hVISA and VISA strains in which a single amino acid substitution in the *vraSR* operon has already been identified (12). The positions of mutations in the *vraSR* operons of different strains are shown in Fig. 3A. Northern blot analysis demonstrated the overexpression of *vraS* in ΔIP-rifR::$vraS_{H14}$, which was indistinguishable from the level of expression by original mutant strain H14 (Fig. 3B). The fact that the whole genome of H14 has only a single mutation, in the *vraS* gene, compared to the sequence of ΔIP suggests that the $VraS_{H14}$ S329→L substitution (on the ATP-binding domain [amino acids 248 to 340]) affects the activation of the VraS sensor histidine kinase by modulating its autophosphorylation (3). The overexpression of *vraS* was also observed in VISA strains NJ and Mu50 and hVISA strain Mu3 (Fig. 3B). The VraS A260→V substitution of NJ may also be associated with the activation of histidine kinase activity. These data are consistent with the findings of this study that implicate the increased expression of the *vraSR* system in the conversion of VSSA to hVISA. It has not previously been reported that amino acid mutations in a global regulator such as the *vraSR* TCS are involved in heterogeneous VAN resistance in *S. aureus*. Further investigation is needed to clarify the relationship between the function of TCS and the role of a mutation in *vraS*.

Recently, evidence of the evolution from hVISA to VISA in vivo during infection has been reported (5, 7, 21, 22, 24, 25). However, the present study described for the first time that a mutation in *vraS* is responsible for the increased level of transcription of *vraS* and the phenotypic expression of hVISA by the gene replacement experiment. It was also confirmed that this mutation could cause cell wall thickening and increase the

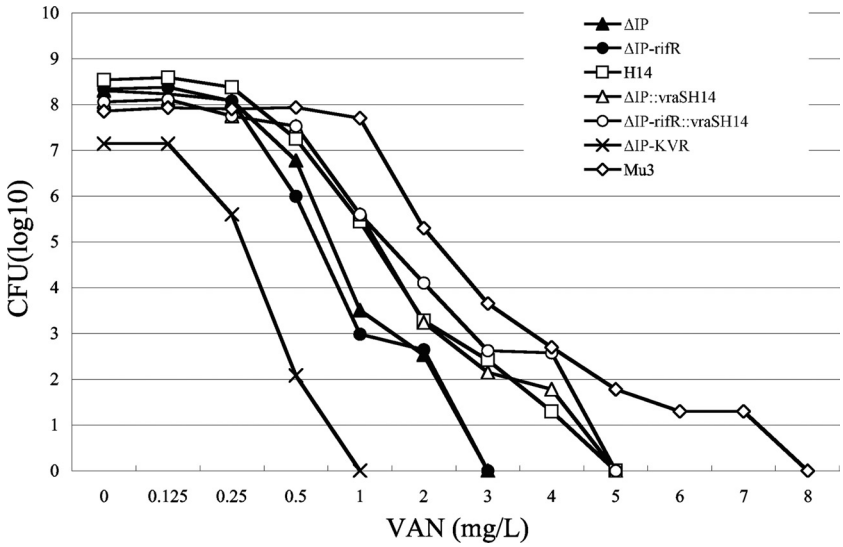

FIG. 2. Resistant subpopulation profiles of ΔIP, its derivative *vraSR* mutants, and hVISA Mu3. The numbers of cells (log_{10} CFU per milliliter) growing on BHI agar containing VAN are shown on the *y* axis; the VAN concentrations are shown on the *x* axis. The number of colonies that grew was counted after incubation at 37°C for 48 h.

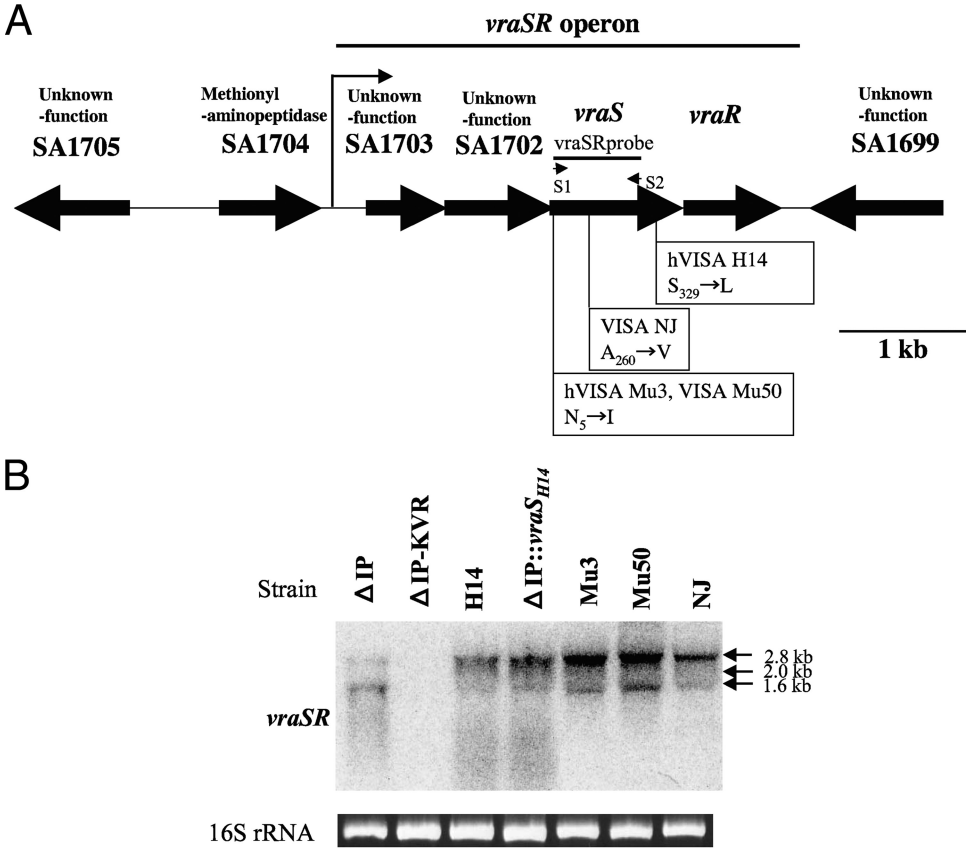

FIG. 3. (A) Structural map of the *vraSR* operon in strains ΔIP, H14, Mu3, NJ, and Mu50 and the positions of the missense mutation in four strains. The *vraSR* probes used for Northern blotting analysis are illustrated by lines. The probe was amplified by PCR with the primers (short arrows) described in Materials and Methods. The arrows below the genes indicate the direction of transcription. The VraS histidine kinase has two amino-terminal transmembrane domains, located between residues 7 and 25 and between residues 49 and 67, as determined by the use of von Heijne's algorithm. The VraS kinase orthologs are similar in domain organization and contain a PAS (*p*eriod clock protein, *a*ryl hydrocarbon receptor nuclear translocator, and *s*ingle-minded protein) family domain which has signaling modules essentially found in proteins involved with signal transduction. A major transcription start codon located in the 133 bp upstream of SA1703 is indicated by a thin arrow above SA1703 (27). The position of a single amino acid substitution in VraS in each VISA strain is shown on the map (10a, 19). (B) Northern blot analysis of *vraSR* in ΔIP, its derivative strains, hVISA strains, and VISA strains with a *vraSR*-specific DNA probe. Ethidium bromide staining of total RNA was used to demonstrate the equivalent loading of RNA. All strains were grown under noninducing conditions.

rate of incorporation of *N*-acetyl-D-glucosamine and D-glucose into cells (Fig. 1B). Although the location of the VraS mutation identified in H14 is different from that identified in clinical hVISA strain Mu3, both mutations had the same effect on conferring VAN resistance in gene replacement experiments (data not shown).

Beta-lactam antibiotics can select hVISA mutants from VSSA strain ΔIP. We exposed VSSA strain ΔIP to selective concentrations of three beta-lactams, IPM, SAM, and CRO, to determine the ability to select mutants with reduced VAN susceptibility. ΔIP is a derivative of N315 in which the *mecI* repressor was inactivated and from which the β-lactamase-carrying plasmid was cured (Table 1). The genotype of inactivated *mecI* and the absence of the β-lactamase-carrying plasmid are characteristic of hVISA strain Mu3 and VISA strain Mu50, as reported previously (9). Therefore, for this study we used ΔIP as a representative Japanese health care-associated MRSA strain from which VISA has emerged. We spread 10^8 CFU from an overnight culture on BHI agar plates containing several concentrations of antibiotics. As a control for this assay, we also spread them onto the agar plate containing VAN. We then picked 50 putative mutant colonies from each plate containing a selective concentration of antibiotic (VAN, 2 mg/liter; IPM, 8 mg/liter; SAM, 8 mg/liter; or CRO, 128 mg/liter) and established them as mutant strains by a colony purification procedure. A selective concentration was defined as the concentration which resulted in a reduction of the initial bacterial population by approximately 6 log units (with VAN) or 4 log units (with IPM, CRO, and SAM) (Fig. 4).

To detect the presence of hVISA isolates among the selected isolates, all isolates were investigated for their VAN susceptibilities by population analysis. Eight and 5 hVISA isolatess were obtained from among the 50 isolates selected with VAN and IPM, respectively. No hVISA isolate was detected among the isolates selected with CRO or SAM. The rates of appearance of hVISA isolates among the 50 isolates selected with VAN and IPM isolates were 3.6×10^{-7} and 2.0×10^{-5}, respectively.

We determined the nucleotide sequence of *vraSR* from 13 hVISA isolates, and a mutation was only in mutant strain H14. This suggests that different genetic mechanisms, other than

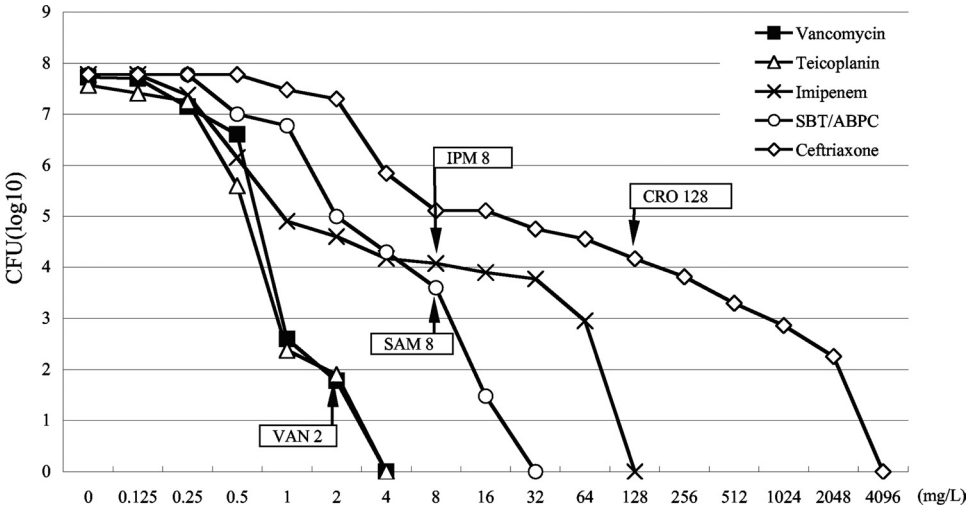

FIG. 4. Profiles of antibiotic-resistant subpopulations. The numbers of cells ($\log_{10}$ CFU per milliliter) growing on antibiotic-containing BHI agar are shown on the *y* axis. The number of colonies of VSSA strain ΔIP growing was counted after incubation at 37° C for 72 h. The concentrations of VAN, IPM, CRO, and SAM are shown on the *x* axis. The numbers after the antibiotic designations indicate selective antibiotic concentrations (in mg/liter). SBT-ABPC, sulbactam-ampicillin.

vraS mutations, are used to acquire hVISA phenotypic expression. From these results, we also confirmed that hVISA can be selected not only by VAN but also by IPM. Since IPM was frequently used for the treatment of MRSA infections before the clinical introduction of VAN in 1991 in Japan, we suspect a possible role of IPM in the early emergence of hVISA in Japan, ahead of its emergence in other countries in 1996.

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