Mutation of RNA Polymerase β Subunit (*rpoB*) Promotes hVISA-to-VISA Phenotypic Conversion of Strain Mu3[†]

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The clinical vancomycin-intermediate *Staphylococcus aureus* (VISA) strain Mu50 carries two mutations in the *vraSR* and *graRS* two-component regulatory systems (TCRSs), namely, *vraS*(I5N) and *graR*(N197S) (hereinafter designated *graR**). The clinical heterogeneously vancomycin-intermediate *S. aureus* (hVISA) strain Mu3 shares with Mu50 the mutation in *vraS* that encodes the VraS two-component histidine kinase. Previously, we showed that introduction of the plasmid *pgraR**, carrying the mutated two-component response regulator *graR**, converted the hVISA strain Mu3 into VISA (vancomycin MIC = 4 mg/liter). Subsequently, however, we found that the introduction of a single copy of *graR** into the Mu3 chromosome by a gene replacement method did not confer on Mu3 the VISA phenotype. The gene-replaced strain Mu3*graR** thus obtained remained hVISA (MIC $\leq$ 2 mg/liter), although a small increase in vancomycin MIC was observed compared to that of the parent strain Mu3. Reevaluation of the Mu3 and Mu50 genomes revealed the presence of another mutation responsible for the expression of the VISA phenotype in Mu50. Here, we demonstrate that in addition to the two regulator mutations, a third mutation found in the Mu50 *rpoB* gene, encoding the RNA polymerase β subunit, was required for Mu3 to achieve the level of vancomycin resistance of Mu50. The selection of strain Mu3*graR** with rifampin gave rise to *rpoB* mutants with various levels of increased vancomycin resistance. Furthermore, 3 (33%) of 10 independently isolated VISA strains established from the heterogeneous subpopulations of Mu3*graR** were found to possess *rpoB* mutations with or without an accompanying rifampin-resistance phenotype. The data indicate that a sizable proportion of the resistant hVISA cell subpopulations is composed of spontaneous *rpoB* mutants with various degrees of increased vancomycin resistance.

The first vancomycin-intermediate *Staphylococcus aureus* (VISA) clinical strain, Mu50, was isolated in May 1996 in Juntendo University Hospital (JUH) (20). VISA can be generated *in vitro* from vancomycin-susceptible *S. aureus* (VSSA) by exposing the cells to selective concentrations of vancomycin. At least two steps of selection are required to get VISA when starting from VSSA strains (18). Before the VISA phenotype is attained, mutants in the transitional stage of vancomycin resistance appear. Such mutants are called heterogeneously VISA (hVISA or hetero-VISA). hVISA spontaneously generates VISA cells within its cell population at a high frequency, greater than 1×10^{-6} . The first hVISA clinical strain, Mu3, was isolated in January 1996, 4 months prior to the isolation of Mu50 in JUH (22). hVISA has a characteristic heterogeneous resistance phenotype as observed by analysis of the vancomycin-resistant cell subpopulations (population analysis) (22). While more than 99.99% of the cell populations of hVISA retain susceptible levels of vancomycin MIC ($\leq$ 2 mg/liter), they also contain VISA cells with various resistance levels, capable of growth on the agar plates containing 2 to 10

mg/liter of vancomycin (22). Therefore, the scheme of stepwise acquisition of vancomycin-intermediate resistance is VSSA $\rightarrow$ hVISA $\rightarrow$ VISA (18).

Two mutations were identified in VISA strain Mu50 which were considered to correspond to the two sequential steps in vancomycin-intermediate resistance acquisition; namely, VSSA-to-hVISA and hVISA-to-VISA phenotypic conversion (7). Both mutations are incorporated in the regulator genes of a two-component regulatory system (TCRS), one in *vraSR* (28, 29) and the other in *graRS* (8).

In the case of *vraSR*, the mutation was identified in *vraS*, encoding a sensor kinase or signal transducer. The mutation *vraS*(I5N), which replaces the fifth isoleucine (I) with asparagine (N), was found to be responsible for VSSA-to-hVISA phenotypic conversion (25). *vraSR* is an upregulator of *S. aureus* cell wall synthesis (14, 28). The hVISA strain Mu3 shares this mutation with Mu50 (25). Activation of the regulator induces the transcription of such genes as *murZ*, *pbp2*, and *sgtB*, encoding UDP-*N*-acetylglucosamine enolpyruvyl transferase, penicillin-binding protein 2, and peptidoglycan transglycosylase, respectively (28). Since the accelerated peptidoglycan synthesis and thickening of the cell wall peptidoglycan layers are directly associated with increased vancomycin resistance in VISA strain Mu50 (5, 10, 15), it is reasonable that the first mutation occurred in the *vraSR* TCRS.

In contrast to *vraSR*, the second mutation in Mu50 responsible for hVISA-to-VISA conversion was found in the response regulator gene *graR* of the *graRS* TCRS. *graRS* is known to control more than 200 genes (16) and is involved in the sus-

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TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or bacterial strain(s)	Description ^a	Reference(s) or source
Plasmids		
pYT3	<i>E. coli</i> - <i>S. aureus</i> shuttle-cloning vector	15
pgrar*	pYT3 carrying mutated <i>graR</i> gene with the N197S amino acid substitution (<i>graR</i> *)	34
pKOR1	<i>E. coli</i> - <i>S. aureus</i> shuttle vector for the construction of allelic-exchange mutants	1
pKOR <i>graR</i> *	pKOR1 carrying the 0.9-kb DNA fragment containing the <i>graR</i> * gene	This study
pKOR <i>rpoB</i> (H481Y)	pKOR1 carrying the 3.1-kb DNA fragment containing the <i>rpoB</i> (H481Y) sequence	This study
Strains		
Mu50	VISA clinical isolate from JUH in 1996; carrying <i>vraS</i> (I5N), <i>graR</i> *, and <i>rpoB</i> (H481Y) mutations	20
Mu3	hVISA clinical isolate from JUH in 1996; carrying <i>vraS</i> (I5N) mutation	22
N315	MRSA clinical isolate from NUH in 1982; a representative health care-associated MRSA in Japan	25, 26
Mu3(pYT3)	Mu3 transformed with pYT3	34
Mu3(pgrar*)	Mu3 transformed with pgrar*	34
Mu3 <i>graR</i> *	Mu3 with its <i>graR</i> gene replaced by <i>graR</i> *	This study
Mu3 <i>rpoB</i> (H481Y)	Mu3 introduced with <i>rpoB</i> (H481Y) by gene replacement procedure	This study
Mu3 <i>graR</i> * <i>rpoB</i> (H481Y)	Mu3 <i>graR</i> * with <i>rpoB</i> (H481Y) introduced by gene replacement procedure	This study
Mu3RP1-1 to -3	Mutant strains obtained by selecting Mu3 with 1 mg/liter of rifampin	This study
Mu3 <i>graR</i> *RP1-1, -2, -3, -10, -15, -25, -31, -33, -35	Mutant strains obtained by selecting Mu3 <i>graR</i> * with 1 mg/liter of rifampin	This study
Mu3 <i>graR</i> *V4-1 to -10	Mutant strains obtained by selecting Mu3 <i>graR</i> * with 4 mg/liter of vancomycin	This study
Mu3 <i>graR</i> *V6-25, -36	Mutant strains obtained by selecting Mu3 <i>graR</i> * with 6 mg/liter of vancomycin	This study

^a JUH, Juntendo University Hospital; NUH, Nagasaki University Hospital.

ceptibility of *S. aureus* to lysozyme and cationic antimicrobial peptides (16, 27, 30). The mutation *graR*(N197S), hereinafter designated *graR**, replaced the 197th amino acid, asparagine (N), with serine (S) (34). Introduction of the plasmid carrying *graR** but not introduction of wild-type *graR* increased the vancomycin MIC from 2 to 4 mg/liter (34). The plasmid pgrar* also conferred on Mu3 such phenotypic changes as significant cell wall thickening and greatly reduced growth rate, which are common characteristics of the VISA clinical strains studied so far (34). The introduction of pgrar* did not confer the VISA phenotype on a VSSA strain whose *vraS* was intact (34). Therefore, the VISA phenotype of Mu50 was attained by the combined effects of the two regulator mutations sequentially incorporated in the global regulators *vraSR* and *graRS* (7).

To further confirm the combined effect of the two mutations in an equal gene dose, strain Mu3*graR** was constructed by replacing the *graR* gene of Mu3 with *graR**. Contrary to our expectation, however, the vancomycin MIC of the constructed strain Mu3*graR** did not exceed 4 mg/liter. This study was planned to resolve this question, and we found that, in addition to the two regulator mutations, a third mutation in the RNA polymerase (RNAP) β subunit (*rpoB*) was required for Mu3 to achieve hVISA-to-VISA phenotypic conversion.

MATERIALS AND METHODS

Bacterial strains and growth condition. The bacterial strains and plasmids used in this study are listed in Table 1. LB medium (1% [wt/vol] Bacto tryptone [Becton, Dickinson and Co. {BD}, Franklin Lakes, NJ], 0.5% [wt/vol] yeast extract [BD], and 1% [wt/vol] NaCl [Wako Pure Chemical Industries, Osaka, Japan]) was used to culture *Escherichia coli*, while *S. aureus* was grown in brain heart infusion (BHI) broth (BD) with aeration at 37°C, unless indicated otherwise. In some experiments, antibiotics were added to the media at the following concentrations: ampicillin (Sigma Chemical Co., St. Louis, MO) at 100 mg/liter for *E. coli* and chloramphenicol (Sigma) at 10 mg/liter and tetracycline (Wako) at 150 mg/liter for *S. aureus*.

Genetic manipulation of bacteria. Plasmid DNA was isolated by standard techniques (Promega Co., Madison, WI) and used to transform chemically competent *E. coli* JM109 (Takara Bio, Inc., Shiga, Japan) or electrocompetent *S. aureus* strains as described previously (26). KOD DNA polymerase was purchased from Toyobo Co., Ltd., Osaka, Japan, and was used according to the manufacturer's recommendations.

Isolation of rifampin-resistant mutants from Mu3 and Mu3*graR.** Amounts of 10^7 to 10^8 CFU of overnight cultures of Mu3 or Mu3*graR** were plated onto a BHI agar plate containing 1 mg/liter of rifampin (Sigma). After 24 h of incubation at 37°C, colonies were picked and purified by streaking them on another BHI agar plate containing 1 mg/liter of rifampin before establishing them as rifampin-resistant mutant strains.

Isolation of VISA strains from vancomycin-resistant subpopulations of Mu3*graR.** VISA strains were isolated from Mu3*graR** by vancomycin selection in two separate experiments. In the first experiment, a total of 6.5×10^7 CFU (5×10^6 CFU/plate) of an overnight culture of Mu3*graR** was spread on BHI agar plates containing 6 mg/liter vancomycin. A total of 36 colonies were formed after 48 h of incubation at 37°C. They were tested for rifampin resistance by replica plating onto the BHI agar plates containing 0.5 mg/liter of rifampin. Two colonies were found to grow on the rifampin plates. They were colony purified on drug-free BHI agar plates and established as strains Mu3*graR**V6-25 and Mu3*graR**V6-36 and then were subjected to further analysis (Table 1). In the second experiment, 10 independent cultures were prepared by inoculating 10^4 CFU of Mu3*graR** into each of the 10 test tubes containing 5 ml of tryptic soy broth (TSB; BD). After overnight cultivation, 10^6 CFU of the cell suspension of each tube was spread on a Mueller-Hinton (MH) agar plate containing 4 mg/liter of vancomycin. The numbers of colonies formed on each agar plate were 32, 9, 15, 22, 15, 10, 18, 11, 18, and 20. One colony was arbitrarily picked from each of the 10 MH agar plates and colony purified on another MH agar plate containing 4 mg/liter vancomycin. Ten mutually independent mutant strains (Mu3*graR**V4-1 to -10) of Mu3*graR** were thus established and were subjected to further analysis (Table 1).

Construction of the gene-replaced derivative strains of Mu3: Mu3*graR, Mu3*rpoB*(H481Y), and Mu3*graR***rpoB*(H481Y).** To replace the *graR* of Mu3 with *graR** and the *rpoB* of Mu3 with *rpoB*(H481Y), the pKOR1 allele replacement system was used as described previously (1). Briefly, to replace the wild-type *graR* with *graR**, a DNA fragment of approximately 0.9 kb containing the mutation was amplified by PCR using primers attB1-*graR**-FW and attB2-*graR**-RV and Mu50 genomic DNA as the template. The sequences of the primers used in this study are listed in Table 2. To replace *rpoB* with *rpoB*(H481Y), an approximately 3.1-kb DNA fragment containing the mutation site was amplified by PCR using

TABLE 2. Primers used in this study

Primer	Nucleotide sequence ^a (5'-3')
attB1- <i>graR</i> *-FW.....	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTATTGAAGATTTTCGGCAAAG TAATGGATACATTTGAAAG
attB2- <i>graR</i> *-RV.....	GGGGACCACTTTGTACAAGAAAGCTGGGTATAATCAACTGTATGACGTT
attB1- <i>rpoB</i> -P1.....	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACAAAAATGGTCGTGAA
attB2- <i>rpoB</i> -P2.....	GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTGTTTTGTAAATTGCGTA
<i>rpoB</i> #6746.....	CTGTAATTGGTAATGCTTTCCTGACTC
<i>rpoB</i> #6747.....	CCAGAATCACGTGCTGCAACGTGTTCCA
<i>rpoB</i> -CP1.....	GAACTTTTTGAAATGTAGAAGTGG
<i>rpoB</i> -CP2.....	TTTTGCCTGTTTTGTAAATTGC
<i>rpoB</i> -SP1.....	TTATGCTGCACCTCTTCGTG
<i>rpoB</i> -SP2.....	CGTCGATTTTACGACGATCA
<i>rpoB</i> -SP3.....	TGGAACACGTTGCAGCACGTGATTCTGG
<i>rpoB</i> -SP4.....	GGCGGTATCGTTCTTGATGT
<i>rpoB</i> -SP5.....	CAAGTGCCCATACCTCCATC
<i>rpoB</i> -SP6.....	ATCGGACGTCCATCTGGTAA
<i>rpoB</i> -SP7.....	GATGGAGGTATGGGCACTTG
<i>rpoB</i> -SP8.....	TGATCGTCGTAAAATCGACG
<i>rpoB</i> -SP9.....	GTCCAGGTGAACCACT
<i>rpoB</i> -SP10.....	TGGTCGTTTCATGGATGATG

^a *attB* sites are underlined.

primers attB1-*rpoB*-P1 and attB2-*rpoB*-P2 and Mu50 genomic DNA as the template. The PCR products with the *attB* site at both ends thus prepared were used for recombination with pKOR1, obtaining the plasmids pKOR-*graR** and pKOR-*rpoB*(H481Y), which were then used for the allelic replacement procedure (1, 7).

DNA sequencing. The location of the *rpoB* mutation and the amino acid substitution it causes in the rifampin-resistant (MIC $\geq$ 1 mg/liter) mutant strains were determined by DNA sequencing as described previously (29). Briefly, the rifampin resistance-determining region (RRDR) of the *rpoB* gene (3, 47), with an approximate size of 1 kb, was amplified using primers *rpoB*#6746 and *rpoB*#6747 (Table 2), and the resulting DNA fragments were sequenced in reciprocal directions from *rpoB*#6746 and *rpoB*#6747 using an Applied Biosystems 377 DNA sequencer. For rifampin-susceptible (MIC < 1 mg/liter) mutants, the entire *rpoB* gene was amplified by using primers *rpoB*-CP1 and *rpoB*-CP2 (Table 2). With the amplified DNA as a template, the entire *rpoB* gene sequence was determined by using all the primers listed in Table 2.

Antibiotic susceptibility tests. MIC determination by the agar dilution method was performed according to the recommendation of CLSI (4), with some modifications as follows. BHI agar was used instead of MH agar, because BHI agar is more supportive of the expression of the VISA phenotype than any other tested agar (17). In addition to the orthodox 2-fold antibiotic dilution, linear sets of vancomycin concentrations with 1-mg/liter increments were used for the range of 1 to 10 mg/liter. Therefore, the drug concentrations for vancomycin were 0.5 mg/liter, 1 to 10 at 1-mg/liter increments and 16, 32, 64, and 128 mg/liter. For the evaluation of small changes in susceptibility to daptomycin and linezolid, the Etest method (AB Biodisk, Solna, Sweden) using BHI agar plates was adopted. MICs were determined in at least three independent experiments to confirm reproducibility. Representative data are presented.

Analysis of resistant cell subpopulations (population analysis). Analysis of the cell subpopulations resistant to vancomycin was performed as described previously (22). An appropriately diluted overnight culture was spread onto BHI agar plates containing vancomycin at various concentrations. After 48 h of incubation at 37°C, the colonies were counted and plotted on a graph.

Doubling time. Determination of doubling time was performed as described previously (34). Overnight cultures of the test strains were diluted 1,000 times into 10 ml fresh BHI and were grown at 37°C with shaking at 25 rpm in an automatic photorecording incubator (TN-2612, Advantec, Tokyo, Japan). The optical density at 600 nm (OD₆₀₀) was automatically monitored and recorded every 2 min. For doubling-time determinations, the OD versus time was plotted for each strain in the exponential growth phase. The doubling times were then calculated as follows: $[(t_2 - t_1) \times \log 2] / (\log OD_{600} \text{ at } t_2 - \log OD_{600} \text{ at } t_1)$. Doubling time was measured in at least three independent experiments.

Transmission electron microscopy. *S. aureus* sample preparation for transmission electron microscopy was performed as described previously (10). Morphometric evaluation of cell wall thickness was performed by using photographic images taken by an electron microscope (model H-7100, Hitachi, Tokyo, Japan) at a final magnification of $\times 30,000$, and the cell wall thickness was measured as

previously described (10). At least 30 cells of each strain with nearly equatorial cut surfaces were measured for the evaluation of cell wall thickness; the results are expressed as the mean values $\pm$ standard deviations, and the significance of the differences in the mean values were evaluated by using Student's *t* test.

RESULTS

A single dose of the mutated *graR* gene (*graR) did not confer on Mu3 the VISA phenotype of Mu50.** As shown in Fig. 1, the population analysis of the *graR* gene-replaced strain Mu3*graR** showed increased vancomycin resistance compared to that of its parent Mu3. However, it did not satisfy the criterion of VISA, which is defined as having a vancomycin MIC of 4 or 8 mg/liter. We have previously reported that Mu3(*pgraR**), a transformant of Mu3 with the plasmid *pgraR**, expressed a vancomycin MIC of 4 mg/liter (34). Figure 1 shows that Mu3(*pgraR**) does exceed Mu3*graR** in the level of van-

FIG. 1. Effect of *graR*(N197S) mutation (*graR**) on the vancomycin-resistant subpopulations of Mu3. The numbers of colonies on BHI agar plates containing various concentrations of vancomycin were counted after 48 h of incubation at 37°C. Mu3*graR** is a Mu3-derived mutant strain with the *graR** mutation introduced by a gene replacement method. Mu3(*pgraR**) is a transformant of Mu3 with the plasmid *pgraR**. L, liter.

TABLE 3. The antibiograms, doubling times, and predicted RpoB amino acid substitutions of the Mu3-derived strains

Strains ^a	Amino acid substitution in RpoB	MIC (mg/liter) ^c					Doubling time ^c (min)
		Rifampin	Teicoplanin	Vancomycin	Daptomycin	Linezolid	
Mu50	H481Y	>128	16	6	4	0.5	35.7
N315		0.031	2	1	0.5	1	25.7
Mu3		0.031	16	2	2	1	28.9
Mu3graR*		0.031	16	3	3	0.75	29.8
Rifampin-selected or rpoB-replaced strains							
Mu3RP1-1	Q468L	>128	32	3	2	0.5	42.8
Mu3RP1-2	D471Y	16	16	3	2	0.75	32.0
Mu3RP1-3	H481Y	>128	16	4	1.5	0.5	31.3
Mu3rpoB(H481Y)^b	H481Y	>128	16	4	1.5	0.5	31.0
Mu3graR*RP1-35	Q468K	>128	16	3	4	0.38	31.0
Mu3graR*RP1-10	S464P	8	16	3	3	0.5	32.6
Mu3graR*RP1-25	Q468R	>128	16	3	4	0.25	38.5
Mu3graR*RP1-2	I527F	8	16	4	3	0.5	32.4
Mu3graR*RP1-3	A477D	>128	16	4	3	0.5	34.2
Mu3graR*RP1-31	S486L	>128	16	4	4	0.38	39.1
Mu3graR*RP1-15	Q468L	>128	32	5	3	0.38	33.1
Mu3graR*RP1-1	H481L	128	16	5	3	0.5	31.9
Mu3graR*RP1-33	H481Y	>128	16	6	3	0.38	32.1
Mu3graR*rpoB(H481Y)^b	H481Y	>128	16	6	3	0.38	32.5
Vancomycin-selected strains							
Experiment 1							
Mu3graR*V6-25	A477V	1	32	8	4	0.5	36.0
Mu3graR*V6-36	H481Y	>128	16	6	3	0.5	31.3
Experiment 2							
Mu3graR*V4-1	T480M	0.016	32	7	6	0.5	42.2
Mu3graR*V4-3	R503H	0.031	32	9	6	0.38	42.9
Mu3graR*V4-7	S746Y	0.25	32	9	4	0.25	57.5
Mu3graR*V4-2		0.063	32	5	3	0.75	33.8
Mu3graR*V4-4		0.031	32	6	3	0.75	30.5
Mu3graR*V4-5		0.031	32	6	3	0.75	30.2
Mu3graR*V4-6		0.063	16	7	4	0.75	42.5
Mu3graR*V4-8		0.031	16	5	8	0.75	34.0
Mu3graR*V4-9		0.063	16	6	4	0.5	31.6
Mu3graR*V4-10		0.031	16	6	3	0.75	35.2

^a Strains carrying the *rpoB*(H481Y) mutation are denoted in boldface.

^b *rpoB* gene-replaced strains.

^c The MIC determination and doubling-time measurement were repeated at least three times to confirm reproducibility.

comycin resistance. However, Mu3(*pgraR**) also was still short of Mu50 in the level of vancomycin resistance. Mu50 has a MIC of 8 mg/liter, and its population curve is evidently shifted more to the right than that of Mu3(*pgraR**). Since both Mu50 and Mu3graR* have a single copy of *graR** in the chromosome, we considered that some thus-far-unnoticed additional genetic alteration that promotes vancomycin resistance was present in Mu50.

We again reviewed the whole-genome sequence data of Mu50 and Mu3 and noticed that Mu50 harbored a mutation in the *rpoB* gene which was absent in Mu3 (34). The gene encodes RpoB, the β subunit of RNA polymerase (RNAP). In Mu50, the 481st amino acid, histidine (H), of RpoB was replaced by tyrosine (Y). Consistent with the presence of the *rpoB* mutation was the fact that Mu50 was resistant to rifampin and Mu3 was susceptible to it (Table 3). To test whether the mutated *rpoB*, *rpoB*(H481Y), contributed to the expression of the VISA phenotype in Mu50, we obtained *rpoB* mutant strains from Mu3 and Mu3graR* by selecting them with 1 mg/liter of rifampin. The *rpoB* genes of the established mutant strains were sequenced, and their vancomycin MICs determined (Table 3).

The *rpoB*(H481Y) mutants of Mu3graR* but not those of Mu3 expressed VISA phenotypes comparable to that of Mu50.

Totals of 8 and 34 rifampin-resistant mutant strains were obtained from Mu3 and Mu3graR*, respectively, by selection with 1 mg/liter of rifampin. All the rifampin-resistant mutants harbored *rpoB* mutations. The kinds of RpoB amino acid substitutions and the numbers of Mu3 mutants harboring them were H481Y, 5 strains; D471Y, 2 strains; and Q486L, one strain. The amino acid substitutions and numbers of the Mu3graR*-derived rifampin-selected mutants were H481L, 9 strains; A477D, 9 strains; H481Y, 5 strains; Q468L, 3 strains; S486L, 3 strains; S464P, 2 strains; Q468K, 1 strain; Q468R, 1 strain; and I527F, 1 strain. Rifampin-resistant strains carrying the same *rpoB* mutation as Mu50, *rpoB*(H481Y), were frequently obtained from both strain Mu3 and Mu3graR*.

Table 3 shows the vancomycin and teicoplanin MICs for the rifampin-resistant mutant strains representing each of the RpoB amino acid substitutions. Mu3graR*RP1-33, representing the five *rpoB*(H481Y) mutants obtained from Mu3graR*, had the highest vancomycin MIC, which was comparable to that of VISA strain Mu50 (Table 3). The *rpoB* gene-replaced

strain Mu3*graR***rpoB*(H481Y) had a vancomycin MIC of 6 mg/liter, identical with that of Mu3*graR**RP1-33, supporting the idea that the increase in the vancomycin MIC was due to the *rpoB* mutation (Table 3). The rifampin-selected mutant strain Mu3RP1-3 harboring the *rpoB*(H481Y) mutation and the gene-replaced strain Mu3*rpoB*(H481Y) had higher vancomycin MICs than Mu3. However, the level of resistance of these strains did not reach that of VISA strain Mu50 (Table 3). Therefore, both the *graR** and the *rpoB*(H481Y) mutation were needed for Mu3 to achieve the VISA phenotype of Mu50.

Three mutations, causing RpoB amino acid substitutions H481Y, H481L, and Q468L, were associated with clear increases in vancomycin resistance (the MIC increased by at least 2 digits, from 3 to 5 or 6 mg/liter). RpoB amino acid substitutions I527F, A477D, and S486L were associated with marginal increases in vancomycin MICs, from 3 to 4 mg/liter. The vancomycin MIC was not influenced appreciably by the S464P, Q468K, or Q468R amino acid substitution (Table 3). No *rpoB* mutation, however, was negatively associated with the vancomycin MIC change before or after its introduction (Table 3).

The MIC for the other glycopeptide antibiotic, teicoplanin, was not much influenced by *rpoB* mutations (Table 3). hVISA strain Mu3 was already fully resistant to teicoplanin (MIC of 16 mg/liter versus 2 mg/liter for a control VSSA strain, N315) (Table 3). Teicoplanin resistance has been associated with the *vraS*(I5N) mutation in the *vraSR* TCRS of Mu3 (28). No more significant increases or decreases in teicoplanin MICs were observed with the addition of *rpoB* mutations, except for *rpoB*(Q468L), which slightly increased the teicoplanin MIC of both Mu3 and Mu3*graR**.

More detailed evaluation of changes in vancomycin susceptibility due to the *rpoB*(H481Y) mutation was done by using population analysis (Fig. 2). Figure 2A illustrates the analysis of vancomycin-resistant subpopulations of Mu3 and its derivative strains. Mu3RP1-3, the rifampin-selected *rpoB*(H481Y) mutant strain of Mu3, had moderately increased vancomycin resistance compared to that of Mu3. However, Mu3RP1-3 and Mu3*graR** contained only small subpopulations of cells that could grow on the plates containing more than 4 mg/liter of vancomycin (Fig. 2A). This was in contrast to Mu3*graR**RP1-33, having both the *graR** and *rpoB*(H481Y) mutations, which showed a population curve almost identical to that of Mu50. All five Mu3*graR**RP1 mutants having the *rpoB*(H481Y) mutation exhibited population curves practically identical to that of Mu50 (data not shown).

Figure 2B shows the population curves of the strains Mu3*graR***rpoB*(H481Y) and Mu3*rpoB*(H481Y). They were the derivative strains of Mu3*graR** and Mu3, respectively, constructed by replacing their *rpoB* genes with *rpoB*(H481Y) using the single-gene replacement procedure (1). Both strains exhibited practically the same population curves as Mu3*graR**RP1-33 and Mu3RP1-3, respectively. The data strongly suggested that the *rpoB* mutation itself and not any other incidental genetic alteration(s) caused by rifampin selection was responsible for the increased vancomycin resistance. Although not so significantly as in Mu3*graR**, *rpoB*(H481Y) did increase vancomycin resistance when introduced in Mu3 as well (Fig. 2).

The resistant subpopulations of hVISA strain Mu3*graR contain *rpoB* mutants at high frequency.** To further ascertain

FIG. 2. Effect of *rpoB*(H481Y) mutation on the vancomycin-resistant subpopulations of Mu3 and Mu3*graR**. (A) Mu3RP1-3 is a rifampin-resistant mutant of Mu3 having the *rpoB*(H481Y) mutation. Mu3*graR**RP1-33 is a rifampin-resistant mutant of Mu3*graR** having the *rpoB*(H481Y) mutation. (B) Mu3*rpoB*(H481Y) and Mu3*graR***rpoB*(H481Y) are derivative strains of Mu3 and Mu3*graR**, respectively, with the *rpoB*(H481Y) mutation introduced by a gene replacement method.

the importance of *rpoB* mutations in the hVISA-to-VISA phenotypic conversion, we established VISA strains from the vancomycin-resistant cell subpopulations of hVISA strain Mu3*graR** by vancomycin selection. They were selected by their capability to grow in 4 or 6 mg/liter of vancomycin. In the first experiment (experiment 1), a total of 6.5×10^7 CFU of a single overnight culture of Mu3*graR** was spread on BHI agar plates containing 6 mg/liter of vancomycin. After 48 h of incubation, 36 discrete colonies were observed. The rifampin susceptibilities of the colonies were tested by replicating them on BHI agar plates containing 0.5 mg/liter of rifampin. Two colonies, Mu3*graR**V6-25 and Mu3*graR**V6-36, were found to grow on the rifampin plates (Table 3).

The next experiment (experiment 2) was performed to roughly evaluate the frequency of *rpoB* mutations among the VISA subpopulations of Mu3*graR**. We obtained 10 independent Mu3*graR** mutant strains capable of growth on MH agar plates containing 4 mg/liter of vancomycin (Materials and Methods and Table 3). All of the strains had vancomycin MIC values of 4 to 6 mg/liter on MH agar, satisfying the VISA definition. Their *rpoB* sequencing results and their susceptibilities to anti-methicillin-resistant *S. aureus* (MRSA) antibiotics

are shown in Table 3. It was remarkable that three *rpoB* mutant strains were identified among 10 independently obtained VISA strains from Mu3*graR**. This strongly indicated that *rpoB* mutations are one of the major contributors to the hVISA-to-VISA phenotypic conversion of Mu3*graR**. Also remarkable was that all three of the VISA strains with *rpoB* mutations were either susceptible to rifampin (Table 3) or only marginally resistant to it.

The *rpoB* mutants selected with vancomycin had higher vancomycin MIC values than the ones selected with rifampin. The most notable were *rpoB*(A477V), *rpoB*(R503H), and *rpoB*(S746Y), which increased vancomycin resistance beyond that of Mu50 (MICs of 8 to 9 versus 6 for Mu50) (Table 3). However, the *rpoB*(H481Y) mutants found in both groups (denoted by boldface in Table 3) had equal vancomycin MICs whether they were selected with vancomycin or rifampin (Table 3). This indicated that the type of *rpoB* mutation, namely, the position and the kind of amino acid change, but not the selection method determined the level of vancomycin resistance.

There were seven vancomycin-selected VISA mutants without *rpoB* mutations. Therefore, it is evident that there are other genetic mechanisms than *rpoB* mutation that can increase vancomycin resistance. It was curious, however, that within the vancomycin-selected mutants, the level of resistance associated with *rpoB* mutation was higher than that caused by non-*rpoB* mutations (MICs of 6 to 9 versus 5 to 7) (Table 3). Together with rifampin-selected mutants, a total of 13 different types of *rpoB* mutants were obtained from Mu3*graR** and 3 from Mu3. Although certain *rpoB* mutations, such as *rpoB*(Q468K), *rpoB*(S464P), and *rpoB*(Q468R), were not associated with increased vancomycin resistance, all the other *rpoB* mutants analyzed here demonstrated higher MIC values than their parents (Table 3). Therefore, we concluded that, in addition to *rpoB*(H481Y), some other types of *rpoB* mutations also promote hVISA-to-VISA conversion.

Influence of *rpoB* mutation on daptomycin and linezolid susceptibilities. The VISA phenotype has been associated with decreased daptomycin susceptibility (6, 33) and increased linezolid susceptibility (45). We tested whether *rpoB* mutation was responsible for the curious correlations or not. The introduction of *graR** into Mu3 was accompanied by a small increase in the daptomycin MIC, from 2 to 3 mg/liter (Table 3). The subsequent introduction of *rpoB* mutations causing Q468K, Q468R, S486L, A477V, and S746Y amino acid substitutions further increased the daptomycin MICs of the Mu3*graR** mutants to the level of Mu50 (up to 4 mg/liter) (Table 3). Two *rpoB* mutations resulting in T480M and R503H amino acid substitutions increased the daptomycin MICs even beyond the level of Mu50 (up to 6 mg/liter). Therefore, increases in the daptomycin resistance of Mu3*graR** were dependent on the type of *rpoB* mutation. Curiously, *rpoB*(H481Y), which significantly promoted vancomycin resistance, did not add much to the daptomycin resistance of Mu3*graR** (MIC = 3 mg/liter) (Table 3).

With regard to linezolid susceptibility, all the *rpoB* mutants of Mu3*graR** selected with rifampin or constructed by gene replacement were associated with significant decreases in linezolid MICs (Table 3). Moreover, all five *rpoB* mutants selected with vancomycin also had decreased linezolid MICs,

whereas six of the seven vancomycin-selected non-*rpoB* mutants retained the same linezolid MIC as Mu3*graR** (Table 3). Therefore, all 19 *rpoB* mutant strains obtained from Mu3*graR** and Mu3 demonstrated decreased linezolid MICs, whereas only 1 of the 7 non-*rpoB* mutant strains had a decreased linezolid MIC (Table 3). The data clearly showed that *rpoB* mutation itself and not vancomycin resistance was directly associated with the decreases in linezolid susceptibility in at least the Mu3 and Mu3*graR** genetic backgrounds.

rpoB* mutation prolongs the doubling times of Mu3 and Mu3*graR**.** The introduction of the *graR** mutation into Mu3 did not affect the doubling time appreciably (Table 3). This posed a great contrast to the previously observed extremely prolonged doubling time of Mu3(*pgraR**) (41.5 min) (34). On the other hand, the subsequent introduction of *rpoB*(H481Y) into Mu3*graR** either by selection with rifampin (Mu3*graR** RP1-3) or vancomycin (Mu3*graR** V6-36) or by gene replacement [Mu3*graRrpoB*(H481Y)] prolonged the doubling time of Mu3*graR** to almost the same degree (Table 3). The doubling-time-prolonging effect of the *rpoB*(H481Y) mutation was also observed with Mu3-derived mutant strains Mu3RP1-3 and Mu3*rpoB*(H481Y) (Table 3).

Table 3 shows that, actually, all the *rpoB* mutant strains used in this study had prolonged doubling times compared with those of their parent strains. However, the degree of prolongation was not associated with the degree of vancomycin resistance. For example, although *rpoB*(Q468R) caused an extremely prolonged doubling time (38.5 min versus 29.8 min for Mu3*graR**), it did not increase vancomycin resistance appreciably (Table 3). This indicates that the *rpoB* mutation itself has a growth-delaying effect on the cell that is independent from its effect on vancomycin susceptibility. On the other hand, among the seven VISA strains without *rpoB* mutations, two strains had only marginal increases in doubling time (Mu3*graR** V4-4 and V4-5) (Table 3), whereas they expressed high vancomycin resistance comparable to that of Mu50 (vancomycin MIC = 6 mg/liter). Therefore, *rpoB* mutation seemed to be directly associated with delayed cell growth. However, the prolonged doubling time itself was not sufficient for the expression of the VISA phenotype. It may not be a prerequisite condition for VISA phenotype expression, either.

The *rpoB*(H481Y) mutation thickens the cell wall of Mu3 and Mu3*graR.** Besides the prolonged doubling time, cell wall thickening has been considered a cardinal feature of the VISA phenotype (10, 11, 18, 31, 37). The cell wall thicknesses of Mu3 and its derivative strains were evaluated by using transmission electron microscopy. The mean cell wall thicknesses and standard deviations were as follows: Mu3, 27.40 ± 2.41 nm; Mu3*graR**, 27.37 ± 3.09 nm; and Mu3*graR**RP1-33, 30.67 ± 3.27 nm. As reported previously, introduction of the plasmid carrying the *graR** gene into Mu3 brought about significant thickening of the cell wall (37.88 ± 11.31 nm) (34). In contrast, there was no increase in cell wall thickness caused by the introduction of a single copy of *graR** in this study. Subsequent introduction of the *rpoB*(H481Y) mutation, however, significantly thickened the cell wall (from 27.37 to 30.67 nm). Although statistically nonsignificant, a slight increase in the cell wall thickness was also observed with Mu3RP1-3 (28.67 ± 2.53 nm) in comparison to that of Mu3. A small increase in vancomycin resistance observed with Mu3RP1-3 and Mu3*rpoB*(H481Y)

may be correlated with the cell wall thickening effect of the *rpoB*(H481Y) mutation (Table 3).

DISCUSSION

VISA emerges from cell populations of hVISA, which, represented by Mu3 or Mu3*graR**, has a characteristic vancomycin-resistant subpopulation profile, as illustrated in Fig. 1 (18, 19). hVISA is composed of the cell subpopulations with various degrees of vancomycin resistance (22). The high frequency ($>10^{-6}$) of emergence of VISA from within hVISA cell populations has long been an enigma. The genetic basis for the VISA phenotype has been sought, and many candidate genes have been proposed, such as *agr* (40, 41), *ccpA* (43), *graRS* (23, 32), *mgrA/sarA* (44), *mprF* (35, 39), *pbp4* (13), the σ^B gene (2, 42), *trfAB* (38), and *walRK* (24). It is noted that the genes, except for *mprF* (36) and *pbp4*, are reported to have regulatory functions. Thus, regulator mutation seems to be a feature of genetic alteration underlying the VISA phenotype (21). In addition to the list of regulator genes, we recently reported that the *rpoB*(A621E) mutation, which replaces the 621st amino acid, alanine (A), with glutamic acid (E) in the RNAP β subunit, conferred dual heteroresistance to daptomycin and vancomycin on a VSSA strain (9). In this study, we further demonstrated that 3 (30%) of 10 VISA strains independently established from the heterogeneous cell population of hVISA strain Mu3*graR** possess *rpoB* mutations that do not accompany rifampin resistance. We also observed that the selection of hVISA with rifampin established rifampin-resistant *rpoB* mutant strains whose levels of vancomycin resistance were increased in various degrees, depending on the location of the mutations and the kinds of amino acid substitutions (Table 3). Based on these data, it is likely that the spontaneous occurrence of *rpoB* mutations with different abilities to increase vancomycin resistance constitutes the characteristic heterogeneous population curve of hVISA.

In our previous study, we observed that Mu3(*pgraR**), the transformant of Mu3 with the plasmid *pgraR**, expressed the VISA phenotype with a thickened cell wall and extremely prolonged growth rate (34). The reason why introduction of the *graR** plasmid alone conferred the VISA phenotype on Mu3 in our previous study is not evident at the moment. However, our preliminary microarray analysis of Mu3(*pgraR**) showed down-regulated expression of ribosomal protein genes and increased expression of biosynthesis pathways for some amino acids (T. Hishinuma, unpublished observations). These data indicated that the strain was in a stringent-response-like status (12). The extremely slow growth rate observed for Mu3(*pgraR**) is compatible with this hypothesis, since slow growth is one of the cardinal features of the stringent response. In fact, in *Escherichia coli*, *rpoB* mutations are reported to mimic the stringent response (48). Therefore, it is possible that the *rpoB*(H481Y) mutation helped the effect of the *graR** mutation by triggering the stringent response.

This study demonstrated the promotional effect of *rpoB* mutation in the development of the VISA phenotype in the hVISA strain Mu3. This leads to the prediction that VISA clinical isolates, irrespective of their rifampin susceptibilities, would frequently carry *rpoB* mutations. We tested our collection of clinical VISA strains from various countries and found

that this was the case: more than 70% of the VISA strains carried *rpoB* mutations (46). This study also showed that rifampin selection of hVISA strains frequently converts them to VISA. This implies that the combined use of rifampin and vancomycin in the treatment of MRSA may have to be reevaluated in view of the risk of facilitating MRSA to acquire vancomycin resistance.

VISA clinical strains tend to have reduced susceptibility to daptomycin (6, 33) and increased linezolid susceptibility (45). The results of this study indicate that *rpoB* mutation is directly involved in the linezolid susceptibility change in the VISA phenotype. With regard to daptomycin, different *rpoB* mutations had different degrees of effect. While *rpoB*(H481Y) did not increase the daptomycin MIC of Mu3*graR** appreciably, *rpoB*(T480M) increased the daptomycin MIC significantly.

The *rpoB* mutation was also found to be involved in other features of the VISA phenotype, such as delayed growth and cell wall thickening. Although we do not know the exact mechanism of the effect of the *rpoB* mutation, it would be reasonable to assume that an *rpoB* mutation would change the transcriptional profile of the cell by producing an RNAP β subunit with altered preference for the genes to be transcribed. A different transcriptional profile would produce a different status of cell physiology with altered susceptibilities to various antibiotics. Extensive research would be needed to understand the effect of the *rpoB* mutation, an ultimate "regulatory mutation."

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