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Comprehensive Identification of Mutations Responsible for Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* (hVISA)-to-VISA Conversion in Laboratory-Generated VISA Strains Derived from hVISA Clinical Strain Mu3

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Heterogeneous vancomycin-intermediate *Staphylococcus aureus* (hVISA) spontaneously produces VISA cells within its cell population at a frequency of 10^{-6} or greater. We established a total of 45 VISA mutant strains independently obtained from hVISA Mu3 and its related strains by one-step vancomycin selection. We then performed high-throughput whole-genome sequencing of the 45 strains and their parent strains to identify the genes involved in the hVISA-to-VISA phenotypic conversion. A comparative genome study showed that all the VISA strains tested carried a unique set of mutations. All of the 45 VISA strains carried 1 to 4 mutations possibly affecting the expression of a total of 48 genes. Among them, 32 VISA strains carried only one gene affected by a single mutation. As many as 20 genes in more than eight functional categories were affected in the 32 VISA strains, which explained the extremely high rates of the hVISA-to-VISA phenotypic conversion. Five genes, *rpoB*, *rpoC*, *walK*, *pbp4*, and *pp2c*, were previously reported as being involved in vancomycin resistance. Fifteen remaining genes were newly identified as associated with vancomycin resistance in this study. The gene most frequently affected (6 out of 32 strains) was *cmk*, which encodes cytidylate kinase, followed closely by *rpoB* (5 out of 32), encoding the β subunit of RNA polymerase. A mutation prevalence study also revealed a sizable number of *cmk* mutants among clinical VISA strains (7 out of 38 [18%]). Reduced cytidylate kinase activity in *cmk* mutant strains is proposed to contribute to the hVISA-to-VISA phenotype conversion by thickening the cell wall and reducing the cell growth rate.

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains one of the major causes of both health care-associated and community-associated infections. Vancomycin (VAN) has been the first-choice antibiotic for treating serious infections caused by MRSA. However, the emergence of MRSA strains with reduced susceptibility to vancomycin, vancomycin-intermediate *S. aureus* (VISA), and hetero-VISA (hVISA) has become a worldwide problem.

The mechanism of vancomycin resistance in *S. aureus* has been investigated extensively since the first report of VISA strain Mu50 in 1997 (1). Vancomycin resistance is based on an accumulation of spontaneous chromosomal mutations (2, 3). Mutations in the two-component regulatory systems *vraSR* and *graRS* are responsible for the VISA phenotype in Mu50 (4, 5). In VISA clinical strain JKD6008, two mutations in *graS* and the *walK* regulatory gene were involved in the VISA phenotype (6, 7). Moreover, mutation of the *yvqF* gene, a member of the *vraSR* operon, is also associated with vancomycin resistance (8). In addition to the regulator mutations, we recently found that a mutation in the *rpoB* gene, which encodes the RNA polymerase β subunit, also contributes to the VISA phenotype (9, 10). Like mutations in regulators, the mutations of the RNA polymerase subunit can alter the expression of large numbers of genes, enabling the cell to survive in the presence of vancomycin. In that sense, the mutation in *rpoB* may be regarded as a "regulatory mutation" that triggers a great physiological change of the cell as if it were the effect of the global regulator system.

In addition to a rather drastic physiological alteration brought about by regulatory mutations, however, there may be contributions by effector genes that further increase vancomycin resistance. Recently, Passalacqua et al. reported that a mutation of the gene encoding a phosphatase protein was involved in the promotion of vancomycin resistance from hVISA to VISA in a USA300 clinical strain (11).

An exhaustive study of hVISA-to-VISA conversion was planned to obtain a comprehensive picture of genetic events underlying hVISA-to-VISA phenotypic conversion. We raised a total of 38 VISA strains obtained from Mu3 and its related strains Mu3*fdh2** and Mu3p27 (2). Another seven VISA strains previously obtained from Mu3*graR** (10) were added, and a total of 45 *in vitro*-generated VISA strains were subjected to a high-throughput whole-genome sequencing strategy to identify the repertoire of genes that caused the hVISA-to-VISA conversion. The hVISA-to-VISA conversion was caused by mutations affecting dozens of effec-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Description[a]	Source or reference(s)
Strains		
S. aureus		
Mu3	hVISA clinical isolate from JUH in 1996	1
Mu50	VISA clinical isolate from JUH in 1996; Mu3 carrying *graR**, *rpoB**, and *fdh2** mutations	1, 5
Mu3p27	Substrain of Mu3 established from a pinpoint colony generated from overnight culture of Mu3 and carrying 4 additional mutations *tarG**, SAHV_1545*, *alr**, and SAHV_2604*	2; this study
Mu3*graR**	Mu3-derived construct with its *graR* gene replaced by *graR**, carrying *graR**, *tarG***, and *rshB** mutations	10
Mu3*fdh2**	Mu3-derived construct with its *fdh2* gene (SAVH_2293) replaced by *fdh2**, carrying *fdh2**, SAHV_0289*, and SAHV_2293* mutations	This study
Mu3V6-1 to -10	VISA mutant strains obtained by selecting Mu3 with 6 mg/liter of vancomycin	This study
Mu3*graR**V4-1 to -10	VISA mutant strains obtained by selecting Mu3*graR** with 4 mg/liter of vancomycin	10
Mu3*fdh2**V6-1 to -10	VISA mutant strains obtained by selecting Mu3*fdh2** with 6 mg/liter of vancomycin	This study
Mu3p27V6-1 to -19	VISA mutant strains obtained by selecting Mu3p27 with 6 mg/liter of vancomycin	This study
E. coli JM109	General-purpose host strain for molecular cloning	TaKaRa Bio
Plasmids		
pND50	*E. coli-S. aureus* shuttle vector for the complementation of *cmk* locus	12
pN*cmk*	pND50 carrying the 0.9-kb DNA fragment containing the wild-type *cmk*	This study
pKOR1	*E. coli-S. aureus* shuttle vector for the construction of allelic-exchange mutants	13
pKOR*fdh2**	pKOR1 carrying the 1.2-kb DNA fragment containing the *fdh2** sequence	This study
pKOR*cmk*(SD*)	pKOR1 carrying the 0.9-kb DNA fragment containing the mutated SD sequence of the *cmk* gene	This study
pKOR*cmk*(A20G)	pKOR1 carrying the 0.9-kb DNA fragment containing *cmk**	This study

[a] JUH, Juntendo University Hospital; *graR**, *graR*(N197S); *rpoB**, *rpoB*(H481Y); *fdh2**, *fdh2*(A297V) [SAHV_2293(A297V)]; *tarG**, *tarG*(N50Y); SAHV_1545*, SAHV_1545(A303E); *alr**, *alr*(A267P); SAHV_2604*, SAHV_2604(W320stop); *graR**, *graR*(N197S); *tarG***, *tarG*(P115S); *rshB**, SAHV_1000(R116H); SAHV_0289*, SAHV_0289(F85L); SAHV_2293*, SAHV_2293(E8V); *cmk*, SAHV_1466; *cmk**, *cmk*(A20G).

tor genes in diverse functionary categories. We studied in detail one of the affected genes, *cmk*, which revealed an unexpected link between a change in cell metabolism and vancomycin resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Clinically isolated hVISA strain Mu3 and its derivative strains Mu3p27 (2), Mu3*graR** (10), and Mu3*fdh2** were used as parent strains to generate VISA. Details of the bacterial strains and plasmids used in this study are presented in Table 1.

The *Escherichia coli* JM109 strain was used as a host for pND50 (12) derivative and pKOR1 (13) derivative plasmids. The *E. coli* strains transformed with the plasmids were cultivated at 37°C in Luria-Bertani broth containing 100 μg/ml ampicillin for pKOR1 derivative plasmids and 25 μg/ml chloramphenicol for pND50 derivative plasmids. *S. aureus* strains were aerobically cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI, USA) at 37°C, and 10 μg/ml chloramphenicol was added to the medium as necessary.

Recombinant DNA techniques and electroporation. Extraction and purification of plasmid DNA from *E. coli*, DNA isolation from *S. aureus* cells, restriction endonuclease digestion, ligation reactions, and DNA cloning were carried out as described previously (10). *S. aureus* electroporation was performed with a Gene Pulser system (Bio-Rad, Hercules, CA, USA) as described previously (10).

To construct plasmid pN*cmk*, *cmk* (SAHV_1466) of Mu3 chromosomal DNA was amplified using the cmk-CP1 and cmk-CP2 primers (see Table S1 in the supplemental material) and was inserted into the BamHI site in the pND50 plasmid vector (12). The integrity of the cloned *cmk* was ascertained by sequencing the recombinant plasmid using the pND-P1 and pND-P2 primers. The resultant pN*cmk* plasmid was then introduced into Mu3p27V6-10 and Mu3V6-7, giving strains Mu3p27V6-10 (pN*cmk*) and Mu3V6-7(pN*cmk*), respectively.

Construction of gene-replaced derivative strains of Mu3: Mu3*fdh2* (A297V) (= Mu3*fdh2), Mu3*cmk*(A20G), Mu3*fdh2*cmk*(A20G), and Mu3*fdh2*cmk*(SD).** Mu50 has 9 single-nucleotide polymorphisms (SNPs) that are not present in Mu3 (5), and one of them is SAV2309(A297V). The gene encodes a putative formate dehydrogenase (Fdh). To distinguish this gene from another *fdh* gene, SAV0177, we designated SAV2309 *fdh2*. The pKOR1 allele replacement system was used as described previously to replace the *fdh2* ortholog of Mu3 (SAHV_2293) with *fdh2*(A297V) (13). Briefly, a DNA fragment of approximately 1.2 kb encompassing the *fdh2*(A297V) mutation was amplified by PCR using the attB1-fdh2-RP-1 and attB2-fdh2-RP-2 primers, with Mu50 genomic DNA as the template. The primer sequences are listed in Table S1 in the supplemental material. To replace *cmk* (SAHV_1466) of Mu3 with *cmk*(A20G) and the mutated Shine-Dalgarno (SD) sequence, approximately 0.9-kb DNA fragments containing the mutation site were amplified by PCR using the attB1-cmk-RP1 and attB2-cmk-RP2 primers and genomic DNAs of Mu3V6-7 and Mu3p27V6-10, respectively, as the templates. The PCR products with the *attB* site at both ends thus prepared were used for recombination with pKOR1, yielding the pKOR*fdh2**, pKOR*cmk*(A20G), and pKOR*cmk*(SD) plasmids. Sequence integrity of the constructs was confirmed by sequencing using the primers listed in Table S1 in the supplemental material. These plasmids were then used in the allelic replacement procedure (10, 13).

Isolation of VISA strains from vancomycin-resistant subpopulations of hVISA Mu3 and its relative strains. VISA strains were isolated from hVISA Mu3 and three related strains, Mu3p27, Mu3*graR**, and Mu3*fdh2**, by vancomycin selection. The isolation of VISA mutant strains from Mu3*graR** has been described previously (10), and three other strains were also treated in a similar manner. In brief, 10, 10, and 19 independent cultures were prepared by inoculating 10^4 CFU of Mu3, Mu3*fdh2**, and Mu3p27, respectively, into a test tube containing 4 ml BHI. After an overnight culture, 10^6 CFU of each culture was spread on a BHI agar plate containing 6 mg/liter vancomycin. Approximately 20 colonies formed on each agar plate. One colony was arbitrarily picked from each of the BHI agar plates and colony purified on another BHI agar plate containing 6 mg/liter vancomycin. Ten mutually independent mutant strains obtained from each of Mu3, Mu3*fdh2**, and 18 mutually independent mutant strains of Mu3p27 thus established were subjected to whole-

genome sequence analysis together with the previously obtained 7 mutually independent Mu3*graR**-derived VISA strains (10) (Table 1).

Antimicrobial susceptibility testing. Vancomycin MICs were determined with the VISA mutants and parent strains using Etest strips (AB Biodisk, Sweden). A sterile cotton swab was immersed in a bacterial cell suspension adjusted to a 0.5 McFarland standard and was used to streak bacteria on BHI agar plates. When the MICs were measured for the strains harboring pND50 and pN*cmk* plasmids, 10 μg/ml chloramphenicol was added to the BHI agar plates to maintain the plasmids. Plates were incubated at 37°C and read after 24 h and 48 h.

Analysis of vancomycin-resistant subpopulations (population analysis). The cell subpopulations resistant to vancomycin were analyzed as described previously (10). An appropriately diluted overnight culture was spread onto BHI agar plates containing vancomycin ranging in concentration from 0 to 10 mg/liter with 1-mg/liter increments. After 48 h of incubation at 37°C, the colonies were counted and plotted on a graph. VISA strain Mu50 and vancomycin-susceptible *S. aureus* (VSSA) strain FDA209P were included as controls.

Doubling time. Doubling times were determined as described previously (10). When the doubling times for the strains harboring pND50 and pN*cmk* plasmids were determined, 10 μg/ml chloramphenicol was added to the medium to maintain the plasmids.

Whole-genome sequencing and mutation detection. The whole genome sequences of *in vitro*-generated VISA strains were determined using a Solexa/Illumina genome analyzer (Illumina, Inc., San Diego, CA, USA), as described previously (14). The Mu3 chromosomal genome sequence (accession number NC_009782.1) was used as a scaffold to assemble and orient the reads. More than 4,000,000 75-bp-long reads were collected for each VISA and parent hVISA strain, providing total coverage of approximately 100 genome equivalents. Short reads were then aligned to the Mu3 genome using the short-read mapping program in Genome Traveler, version 1.2.5D (In Silico Biology, Inc., Yokohama, Japan). We filtered SNP calls and combined them into a single list using the MGG Assemble program, and the resulting SNPs were verified by manually inspecting multiple alignments of all short reads mapping to each SNP locus. The identified SNPs were then verified by resequencing using an Applied Biosystems 3730 capillary sequencer (Applied Biosystems Ltd., Tokyo, Japan) with forward and reverse primers for each locus.

Transmission electron microscopy. *S. aureus* samples were prepared for transmission electron microscopy as described previously (10). Cell wall thickness was morphometrically evaluated using photographic images taken with an electron microscope (model H-7100; Hitachi, Tokyo, Japan) at a final magnification of 30,000. Cell wall thickness was measured as described previously (10). At least 30 cells of each strain with nearly equatorial cut surfaces were measured to evaluate the cell wall thickness. The results were expressed as means ± standard deviations (in nm).

RESULTS

Isolation and characterization of VISA mutants from hVISA strain Mu3 and its related strains. We used four strains, Mu3, Mu3*graR**, Mu3 *fdh2**, and Mu3p27, as parent strains to generate VISA mutants. Strain Mu3p27 was established from a pinpoint colony of Mu3 which was formed on a drug-free agar plate after a 37°C overnight incubation (2). Mu3*graR** and Mu3*fdh2** were Mu3 derivatives constructed by introducing the *graR*(N197S) and *fdh2*(A297V) mutations, respectively. These two mutations are found in clinical VISA strain Mu50 (5), but the introduction of either mutation did not confer the VISA phenotype on Mu3. As shown in Fig. 1, all the strains showed typical hVISA population curves.

A total of 45 VISA strains were established by vancomycin selection. Table 2 lists all of the strains converted to VISA in comparison with their parent hVISA strains. The VISA strains showed a range of vancomycin MICs, from 6 to 12 mg/liter. Slower growth

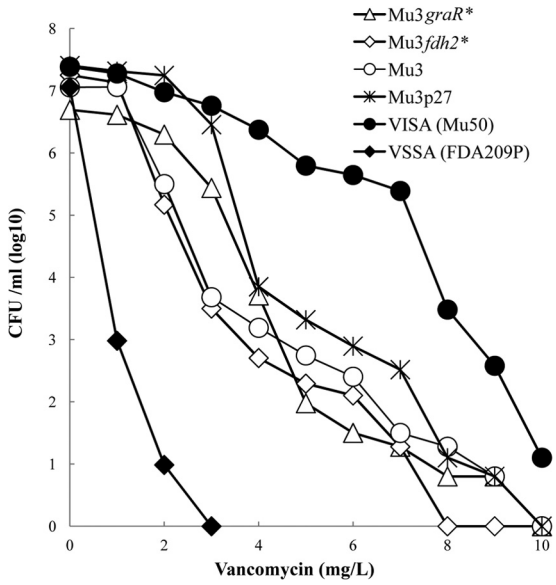

FIG 1 Population analysis of hVISA parent strains. The number of colonies on BHI agar plates containing various concentrations of vancomycin was counted after 48 h of incubation at 37°C.

is a general feature of VISA strains (15). This rule applied to most of the VISA strains obtained in this study. However, a few VISA strains obtained from Mu3p27 had shorter doubling times than the parent strain Mu3p27. This was considered to be due to the exceptionally prolonged doubling time of the parent hVISA strain Mu3p27 with a small-colony variant (SCV) phenotype (46 min). The shortest doubling time of those strains was 41 min, which was still much longer than those of the other three hVISA strains used for VISA selection (30 to 35 min) (Table 2).

Whole-genome sequence comparison between the VISA strains and their parental hVISA strains. A vast number of short-read sequences were obtained using Illumina technology, covering more than 100 times the genome size of each strain. We used the complete genome sequence of hVISA strain Mu3 (accession no. NC_009782.1) as the reference genome. Three parent strains, Mu3p27, Mu3*graR**, and Mu3*fdh2** carried seven, five, and four additional mutations, respectively, compared to the Mu3 chromosome (Table 1; see Table S2 in the supplemental material). It was confirmed that all of the VISA mutant strains obtained in this study retained the mutations of their parents. Because the purpose of this study was to identify the mutations that are correlated with the phenotypic conversion of vancomycin resistance, we searched for the differences between the VISA strains and their parental strains.

The results of genome comparison are listed in Table 2. It was remarkable that each VISA strain carried a unique set of mutations; i.e., every VISA mutant was unique. Among the 45 VISA mutants, 32 strains carried single mutations in their genomes, and the rest (13 strains) carried 2 mutations (10 strains), 3 mutations (2 strains), and 4 mutations (one strain) (see Table S3 in the supplemental material). The 32 singly mutated VISA strains were affected in 20 different genes (see Table 3). Thirty of them had nonsynonymous mutations located within the open reading frame (ORFs), and two mutations of Mu3p27V6-10 and Mu3*fdh2**V6-4 were found in the intergenic region on the chromosome. These two mutations were found in the predicted SD

TABLE 2 Mutations, vancomycin MICs, and doubling times of 45 vancomycin-selected VISA mutant strains

Strain	VAN MIC (mg/liter)[a]	Doubling time (min)	Locus (Mu3 ORF, gene or description)	Amino acid			Nucleotide		
				Position	Mu3	Mutant[b]	Position on Mu3 chromosome	Mu3	Mutant
Mu3*graR**	3	30							
Mu3*graR**V4-1	8	42	SAHV_0540, *rpoB*	480	T	M	606751	C	T
Mu3*graR**V4-2	6	34	SAHV_0012	47	V	F	16232	G	T
			SAHV_2208, *rpoA*	156	T	I	2366727	G	A
			SAHV_2208, *rpoA*	154	N	K	2366732	A	T
			SAHV_2208, *rpoA*	153	Q	K	2366737	G	T
			SAHV_2619, *arcA*	57	A	A (silent)	2792021	C	A
Mu3*graR**V4-3	12	43	SAHV_0540, *rpoB*	503	R	H	606820	G	A
			SAHV_0538, *rplL*	22	L	F	604012	A	T
Mu3*graR**V4-5	6	30	SAHV_0444	41	D	G	491373	A	G
			SAHV_0622	191	S	S (silent)	699386	A	G
			SAHV_1286, *mutL*	648	H	R	1373330	A	G
			SAHV_1898	422	G	S	2059175	C	T
			SAHV_1980	5	K	Q	2122723	T	G
Mu3*graR**V4-6	6	43	SAHV_1562, *rpsU*	31	E	*	1686619	C	A
Mu3*graR**V4-7	12	58	SAHV_0540, *rpoB*	746	S	Y	607549	C	A
			SAHV_2006	337	K	K (silent)	2145135	T	C
Mu3*graR**V4-8	6	34	SAHV_2043, *leuB*	18	L	L (silent)	2182158	G	A
			SAHV_1348, *mprF* (= *fmtC*)	50	R	L	1441489	G	T
			SAHV_2089, *atpA*	218	L	I	2236857	A	T
Mu3p27	3	46							
Mu3p27V6-1	6	46	SAHV_0163, *capP*	126	H	Q	183139	T	A
			SAHV_1422, *ebhA*	3889	V	I	1524217	C	T
Mu3p27V6-2	6	61	SAHV_1358, *trpC*	31	K	N	1453361	G	T
Mu3p27V6-3	6	61	SAHV_2278, *ureD*	151	V	I	2418852	G	A
Mu3p27V6-4	6	56	SAHV_1466, *cmk*	201	G	V	1593457	C	A
Mu3p27V6-5	6	66	SAHV_0256	13	W	R	299992	T	A
Mu3p27V6-6	6	47	SAHV_2484, *gtaB*	220	Q	*	2638030	G	A
Mu3p27V6-8	6	75	SAHV_1683, *pykA*	12	P	S	1813774	G	A
Mu3p27V6-9	8	42	SAHV_0639, *pbp4*	67	I	I (silent)	717477	G	T
			SAHV_0639, *pbp4*	65	Y	*	717483	G	T
			SAHV_1548, *rpoD* (= *sigA*)	266	P	L	1673503	G	A
Mu3p27V6-10	8	56	13 bp upstream of *cmk*				1594071	T	A
Mu3p27V6-11	6	43	SAHV_0639, *pbp4*	140	S	N	717259	C	T
Mu3p27V6-12	7	56	SAHV_1392	14	G	R	1487442	G	C
Mu3p27V6-13	6	41	SAHV_0612	93	V	F	690392	G	T
Mu3p27V6-14	6	50	SAHV_0493, *purR*	244	E	*	551488-9	GA	TG
			125 bp upstream of *fmtB*				2297073	C	T
Mu3p27V6-15	8	58	SAHV_0540, *rpoB*	540	G	V	606931	G	T
Mu3p27V6-16	7	58	SAHV_0540, *rpoB*	1085	A	V	608566	C	T
Mu3p27V6-17	8	43	SAHV_0019, *walK* (= *vicK*)	216	Q	E	26294	C	G
Mu3p27V6-18	6	44	SAHV_0741	93	G	D	823378	C	T
Mu3p27V6-19	8	47	SAHV_1209	148	G	V[c]	1279815	G	Deletion
Mu3*fdh2**	3	35							
Mu3*fdh2**V6-1	8	41	SAHV_0372	3	T	I	418415	G	A
Mu3*fdh2**V6-2	8	43	SAHV_0540, *rpoB*	406	R	S	606528	C	A
Mu3*fdh2**V6-3	8	46	SAHV_0744, *tarO* (= *tagO* or *llm*)	94	P	L	826298	C	T
Mu3*fdh2**V6-4	12	43	10 bp upstream of *tarA* (or *tagA*)				710756	G	A
Mu3*fdh2**V6-5	8	59	SAHV_0541, *rpoC*	440	P	L	610319	C	T
Mu3*fdh2**V6-6	12	50	SAHV_1466, *cmk*	128	I	N	1593676	A	T
Mu3*fdh2**V6-7	8	55	SAHV_0540, *rpoB*	1069	Q	E	608517	C	G
			SAHV_1275, *recA*	45	T	I	1358432	C	T
Mu3*fdh2**V6-8	8	40	SAHV_1466, *cmk*	71	K	N	1593846	T	A
			11 bp upstream of *ansA* (or 66 bp upstream of *cmk*)				1594122	T	A

(Continued on following page)

TABLE 2 (Continued)

Strain	VAN MIC (mg/liter)[a]	Doubling time (min)	Locus (Mu3 ORF, gene or description)	Amino acid			Nucleotide		
				Position	Mu3	Mutant[b]	Position on Mu3 chromosome	Mu3	Mutant
			13 bp upstream of *ansA* (or 64 bp upstream of *cmk*)				1594124	G	A
Mu3*fdh2**V6-9	12	45	SAHV_0744, *tarO* (= *tagO* or *llm*)	205	F	L	826630	T	C
Mu3*fdh2**V6-10	8	43	SAHV_1466, *cmk*	129	G	V	1593673	C	A
Mu3	2	35							
Mu3V6-1	8	43	SAHV_1760	200	W	C	1911964	C	A
Mu3V6-2	12	49	SAHV_2101	121	G	R	2246038	C	G
Mu3V6-3	12	47	SAHV_0933	176	R	Q	981796	C	T
			SAHV_1094, *potD*	19	L	I	1157349	C	A
Mu3V6-4	8	43	SAHV_1031	51	R	C	1089730	C	T
			SAHV_1337, *opuD*	360	A	P	1425502	G	C
			SAHV_2340, *tcaA*	121	E	*	2483747	C	A
Mu3V6-5	8	60	SAHV_0744, *tarO* (= *tagO* or *llm*)	169	G	R	826522	G	A
Mu3V6-6	8	42	SAHV_0197	111	T	K	227487	C	A
			SAHV_1760	251	S	I	1911812	C	A
Mu3V6-7	8	45	SAHV_1466, *cmk*	20	A	G	1594000	G	C
Mu3V6-8	8	46	SAHV_1466, *cmk*	24	A	V	1593988	G	A
Mu3V6-9	12	48	SAHV_0256	224	S	Y	300626	C	A
Mu3V6-10	8	54	SAHV_0506	230	A	T	565169	G	A
			SAHV_1747	57	G	E	1899089	C	T
			SAHV_1906	95	E	*	2070140	C	A

[a] MICs were determined with Etest on BHI agar plates at 37°C after 48 h of incubation.
[b] *, stop codon.
[c] The C-terminal 100 amino acid residues of SAHV_1209 were changed to the 19-amino-acid sequence VKLRRKKHLHIHNVILLRR* because of a deletion mutation.

region of the *cmk* and *tarA* genes, respectively. Among the 30 nonsynonymous mutations, 28 and 2 were missense and nonsense mutations, respectively (Tables 2 and 3). We focused on the 20 genes singly affected in 32 VISA strains, since these were considered to be directly associated with the hVISA-to-VISA phenotypic conversion.

The 20 genes are functionally classified and listed in Table 3. A total of 15 out of 20 genes were newly identified in this study. In addition to the *vraS*(I5N) mutation that is present in the genome of hVISA parent strain Mu3, seven out of 32 VISA mutant strains (22%) acquired the second mutations in either one of the *rpoB*, *rpoC*, and *walK* genes belonging to the regulatory function category (Table 3). Mutations in regulatory systems, such as *vraSR*, *graRS*, and *walKR*, and *rpoB*, encoding the RNA polymerase β subunit, have been shown to be the major contributors in VISA phenotype acquisition (5–10, 16, 17). However, the other 25 mutations (78%) were found to occur in the effector genes encoding functions involved in various metabolic pathways. Nine mutations (28%) affected genes that were in the category "synthesis and modification of cell wall surface structure" (Table 3). They included the *pbp4* and *pp2c* genes, which have previously been reported in association with glycopeptide resistance (11, 18). Quite unexpectedly, however, the most frequently affected effector gene was *cmk* (6 times) of the pyrimidine synthesis pathway, which was almost as frequent as the *rpoB* gene (Table 3).

Impact of *cmk* mutation on vancomycin resistance. Mapping of the *cmk* mutations is illustrated in Fig. 2. Five out of the 6 mutations were found in the *cmk* ORF, leading to amino acid substitutions (A20G, A24V, I128N, G129V, and G201V), and one was found at the 13 bases upstream of the *cmk* gene, which was predicted to be in the SD sequence. To prove that the *cmk* mutations are responsible for the hVISA-to-VISA phenotype conversion, allelic replacement experiments were conducted using the pKOR1 gene replacement system. The effects of mutation *cmk*(A20G), which was found in Mu3V6-7, and another mutation found in the SD region of *cmk* in Mu3p27V6-10 strain were evaluated.

The *cmk*(A20G) mutation of Mu3V6-7 was introduced into hVISA strain Mu3. The resulting strain, Mu3*cmk*(A20G) (= Mu3*cmk**), was tested for vancomycin susceptibility using Etest and population analysis. As shown in Table 4, the gene-replaced strain Mu3*cmk** showed a significantly raised vancomycin MIC of 8 mg/liter, which was identical to that of the mutant strain Mu3V6-7 obtained by vancomycin selection. Population analysis of Mu3*cmk** showed an increase in the proportion of vancomycin-resistant subpopulations compared with that of the parent strain Mu3: the population curve for Mu3*cmk** was almost identical to that for Mu3V6-7 (Fig. 3A). The gene-replaced strain Mu3*fdh2***cmk** was constructed using Mu3*fdh2** instead of Mu3p27 as the parent strain. It was difficult to use Mu3p27 for genetic manipulation, mainly due to its extremely low growth rate. Mu3*fdh2***cmk** also showed reduced vancomycin susceptibility that was comparable to that of Mu3*cmk** (Table 4 and Fig. 3A), indicating that the mutation was commonly effective across the genetic backgrounds of the hVISA strains Mu3 and Mu3*fdh2**.

Slow growth and cell wall thickness have been known as features closely associated with the VISA phenotype (15, 19–21). We compared the doubling time and cell wall thickness of the *cmk*(A20G) mutants with those of the parent strains. As shown in Table 4 and Fig. 4A, both strains carrying the *cmk*(A20G) muta-

TABLE 3 Functional categorization of the genes which are singly mutated in the 32 strains converted to VISA

Category	No. (%) of strains	Subcategory	Locus	Amino acid change(s)[a]	Protein description	Reference(s) for locus previously implicated in vancomycin resistance
Cell envelope synthesis and modification	9 (28)	Peptidoglycan synthesis	*pbp4*	S140N	Penicillin-binding protein 4	18
		Cell wall hydrolase	SAHV_1760	W200C	Autolysis, putative	
		Wall teichoic acid biosynthesis	*tarO* (= *tagO* or *llm*)	P94L, 169R, F205L	Wall teichoic acid biosynthesis protein TarO	
			tarA (= *tagA*)	10 bp upstream of *tarA*	Wall teichoic acid biosynthesis protein TarA	
			SAHV_0256	W13R, S224Y	Wall teichoic acid biosynthesis protein TarL	
		Lipoteichoic acid biosynthesis	*gtaB*	Q220*	UTP-glucose-1-phosphate uridyltransferase	
Transcription	7 (22)	Basic transcription machinery	*rpoB*	R406S, T480M, G540V, S746Y, A1085V	β subunit of RNA polymerase	6, 7
			rpoC	P440L	β′ subunit of RNA polymerase	16
		Regulator	*walK* (= *vicK*)	Q216E	Involved in regulation of autolysin genes	9, 17
Pyrimidine metabolism	6 (19)		*cmk*	A20G, A24V, I128N, G129V, G201V, 13 bp upstream of *cmk*	Cytidylate kinase	
Pyruvate metabolism	2 (6)		*pykA*	P12S	Pyruvate kinase	
			SAHV_1392	G14R	Acylphosphatase, putative	
Ribosomal protein	1 (3)		*rpsU*	E31*	Small subunit ribosomal protein S21	
Amino acid metabolism	1 (3)		*trpC*	K31N	Tryptophan biosynthesis	
Urease formation	1 (3)		*ureD*	V151I	Urease accessory protein	
Serine/threonine phosphatase	1 (3)		SAHV_1209 (*pp2c*)	G148(VKLRRKKHLHIHNVILLRR*)[b]	Protein phosphatase 2C	11
Unknown function	4 (13)		SAHV_0372	T3I	Hypothetical protein	
			SAHV_0612	V93F	Hypothetical protein	
			SAHV_0741	G93D	Hypothetical protein	
			SAHV_2101	G121R	*hemK* family modification methylase, putative	
Total	32 (100)		20			

[a] *, stop codon.

[b] The letters in parentheses denote the amino acid sequences generated by a frameshift mutation.

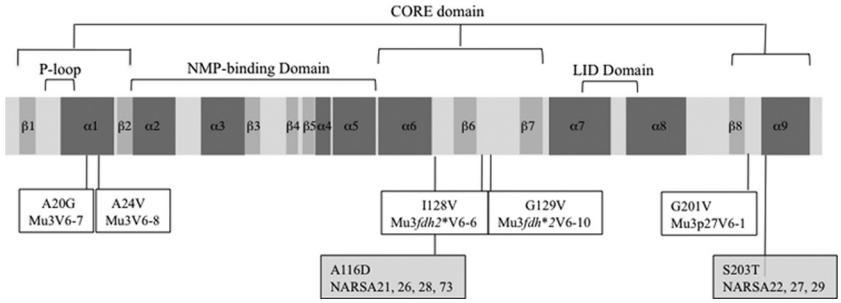

FIG 2 Mapping of the *cmk* mutations of the VISA strains. Substituted amino acid residues of strain Mu3 derivatives and clinical isolates are shown with the strain name in open and gray boxes, respectively. Nine α-helixes and 8 β-sheets assigned previously (22) are shown in gray and dark gray, respectively. The parallel β-sheet consisting of strands β1-β2 and β6-β8 flanked by α-helixes α1, α6, and α9 make up the CORE domain, which contains a highly conserved phosphate-binding loop (22). NMP, nucleoside monophosphate.

tion (Mu3V6-7 and Mu3*cmk**) had prolonged doubling times and thicker cell walls compared to those of their parental strains. These results confirmed that the *cmk*(A20G) mutation was responsible for the hVISA-to-VISA phenotype conversion.

Mu3*fdh2***cmk*(SD*) was also tested for vancomycin susceptibility, doubling time, and cell wall thickness. As shown in Table 4 and Fig. 3B and 4B, Mu3p27V6-10 and Mu3*fdh2***cmk*(SD*) showed reduced vancomycin susceptibilities, prolonged doubling times, and thicker cell walls compared to those of the parent hVISA strains Mu3p27 and Mu3*fdh2**, respectively. These results suggested that the SD mutation of *cmk* was also responsible for the hVISA-to-VISA phenotype conversion.

Introducing the intact *cmk* gene into *cmk*-mutated VISA strains cures them of the VISA phenotype. CMP kinase, encoded by *cmk*, is composed of three domains: the CORE, LID, and nucleoside monophosphate (NMP)-binding domain (22, 23). It catalyzes phosphoryl transfer from ATP to CMP or dCMP. All five *cmk* mutations found in this study were located in the CORE domain (Fig. 2). We performed a complementation experiment to identify the role of the *cmk* mutation in decreased vancomycin susceptibility. We amplified the intact *cmk* gene with its nonmutated SD region and cloned it into the pND50 shuttle vector to obtain pN*cmk*. The effect of the overexpression of the cloned wild-type (wt) gene on vancomycin susceptibility was then evaluated by introducing pN*cmk* into VISA strain Mu3V6-7. As shown in Table 5, introduction of pN*cmk* into Mu3V6-7 significantly decreased the vancomycin MIC from 8 to 2 mg/liter, to a level comparable to that for the parental strain Mu3 (Table 5).

As shown in Table 5, introducing intact *cmk* into Mu3V6-7 shortened the doubling time from 46 to 39 min, which was comparable to that (37 min) of the parental strain with pN*cmk* introduced, Mu3(pN*cmk*), whereas the Mu3V6-7 strain with vector introduced retained the prolonged doubling time (51 min). These results seem to indicate that the *cmk*(A20G) mutation in Mu3V6-7 leads to slower growth in exchange for the acquisition of increased vancomycin resistance through the reduced Cmk activity.

Mutations of SD sequences could either decrease or increase translational efficiency (24). The SD mutation in Mu3V6-7 was also examined by complementation assay to clarify whether the vancomycin resistance caused by the SD mutation was due to reduced translational efficiency. As with Mu3V6-7, introduction of pN*cmk* into Mu3p27V6-10 resulted in a decrease of the vancomycin MIC and a shortened doubling time (Table 5). The results indicated that the SD mutation in Mu3p27V6-10 led to vancomycin resistance through decreased translation of Cmk.

Vancomycin resistance in VISA strains tends to be lost gradually during passage in drug-free medium (15). The transformation process in the gene replacement experiments and drug susceptibility tests contain at least four rounds of drug-free passages. Therefore, we tested the effect of drug-free passage of the *cmk*

TABLE 4 Vancomycin MICs and doubling times of *cmk* mutant strains

Strain	Description	VAN MIC (mg/liter) at 24 h	Doubling time (min)	Nucleotide or amino acid change	
				cmk	*fdh2* (SAHV_2293)
Mu3	Parent strain	2	35	wt	wt
Mu3V6-7	VAN selected	8	49	A20G	wt
Mu3*cmk**	*cmk* replaced	8	52	A20G	wt
Mu3p27	Parent strain	3	46	wt	wt
Mu3p27V6-10	VAN selected	8	56	T(−13)A[a]	wt
Mu3*fdh2**	Parent strain	3	35	wt	A297V
Mu3*fdh2***cmk*(SD*)	*cmk*(SD) replaced	8	46	T(−13)A	A297V
Mu3*fdh2***cmk**	*cmk* replaced	8	49	A20G	A297V
Mu50		8	37	wt	A297V

[a] Nucleotide change 13 bp upstream of the *cmk* gene.

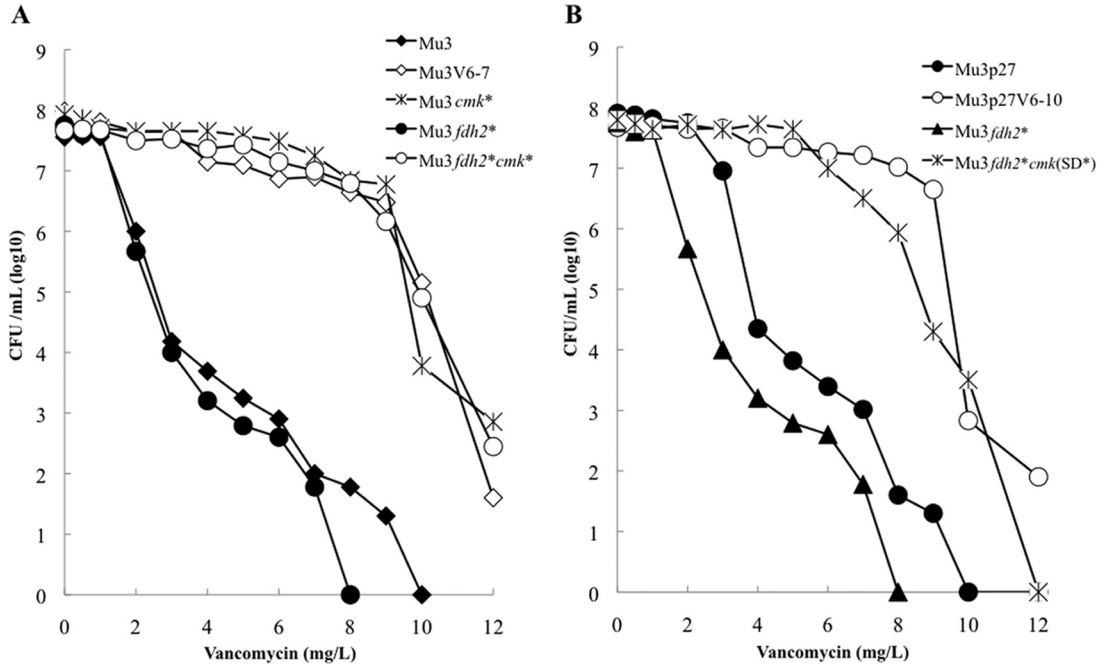

FIG 3 Effects of *cmk* mutations on the vancomycin-resistant subpopulations of Mu3, Mu3p27, and Mu3*fdh2**. (A) Population analysis of the *cmk*(A20G) mutant and the parent strains. Mu3*cmk** and Mu3*fdh2***cmk** are Mu3- and Mu3*fdh2**-derived mutant strains bearing the *cmk*(A20G) mutation introduced by gene replacement methods. Mu3V6-7 is a vancomycin-selected VISA strain from Mu3. (B) Population analysis of the *cmk*(SD*) mutant and parent strains. Mu3 *fdh2***cmk*(SD*) is a Mu3*fdh2**-derived mutant strains with the *cmk*(SD*) mutation introduced by gene replacement methods. Mu3p27V6-10 is a vancomycin-selected VISA strain from Mu3p27.

mutant strains on their vancomycin MICs. Vancomycin MICs for the *cmk* mutant strains, Mu3V6-7 and Mu3p27V6-10, started decreasing gradually after 4 days of passage, suggesting that a slight decrease of the MIC observed with the vector-transformed strains (Table 5) was most likely due to the effect of culture passage (see Table S4 in the supplemental material).

Prevalence of *cmk* mutations among the clinical VISA strains. To know whether the *cmk* mutations occur naturally or not, we conducted a prevalence study of *cmk* mutations among a collection of well-characterized clinical VISA strains isolated from 10 countries (17, 25). Thirty-eight clinical VISA strains isolated from various countries were subjected to the determination of the entire *cmk* gene sequence. The results showed that seven (18%) of the 38 VISA clinical strains carried nonsynonymous mutations in *cmk* (see Table S5 in the supplemental material). Two amino acid substitutions, A116D and S203T, were identified. The A116D mutation was present in four strains: HIP07920, HIP09313, HIP09662, and HIP10540. These four strains had no other mutations in the *cmk* gene or in the up- and downstream regions of the gene. The S203T substitution was found in HIP07930, HIP09433, and HIP09735. The three strains had 7 to 11 synonymous mutations in the ORF of *cmk* and in the immediate upstream and downstream intergenic regions. At least five mutations were shared by the three strains, indicating a possibility that the three strains were phylogenetically related to one another.

DISCUSSION

We isolated 45 independent VISA strains from hVISA strains by one-step vancomycin selection to comprehensively identify the genes involved in hVISA-to-VISA conversion. Comparative genomic analyses of all VISA mutants and the parent strains revealed that there were no VISA strains carrying identical sets of mutations (see Table S3 in the supplemental material). Moreover, the analysis identified a total of 63 mutations in 49 different genes in 45 independently established VISA strains, and 32 single mutations affecting 20 genes were directly associated with the hVISA-to-VISA conversion (Table 3). The result indicates that there are hundreds of alternative sets of mutations underlying the hVISA-to-VISA conversion, assuming that there are hVISA clinical strains with diverse genetic backgrounds in the world besides those with Mu3 genetic lineage studied here.

In this study, we also found that the functional alteration of the genes in multiple metabolic pathways in addition to those with regulatory function (*rpoB*, *rpoC*, *rpoA*, *rpoD*, etc.) can achieve hVISA-to-VISA phenotypic conversion in hVISA strains of the Mu3 lineage (Table 3). Mu3 already carries a regulator mutation, *vraS**(*vraS*(I5N)), that makes itself heterogeneously resistant to vancomycin (5). Without *vraS**, the phenotypic conversion of vancomycin resistance did not occur even in the presence of *graR*(N197S) and *rpoB*(H481Y) mutations in Mu50 (4). The *vraS** mutation augments expression of more than 100 genes, including those encoding the enzymes of the peptidoglycan synthesis pathway, making the cell ready for activated cell wall peptidoglycan synthesis (26). Therefore, it is likely that any additional mutation that directly or indirectly increases the supply of the materials for cell wall synthesis would markedly enhance peptidoglycan synthesis. Thickened peptidoglycan layers will serve as affinity traps for vancomycin molecules and delay their reach to the cytoplasmic membrane through the peptidoglycan layers clogged with bound vancomycin molecules (27). We consider that the regulator mutation can change the physiology of the cell by altering the expression of many genes to prepare for the VISA phenotype expression. The next step, hVISA-to-VISA conversion, can occur with a

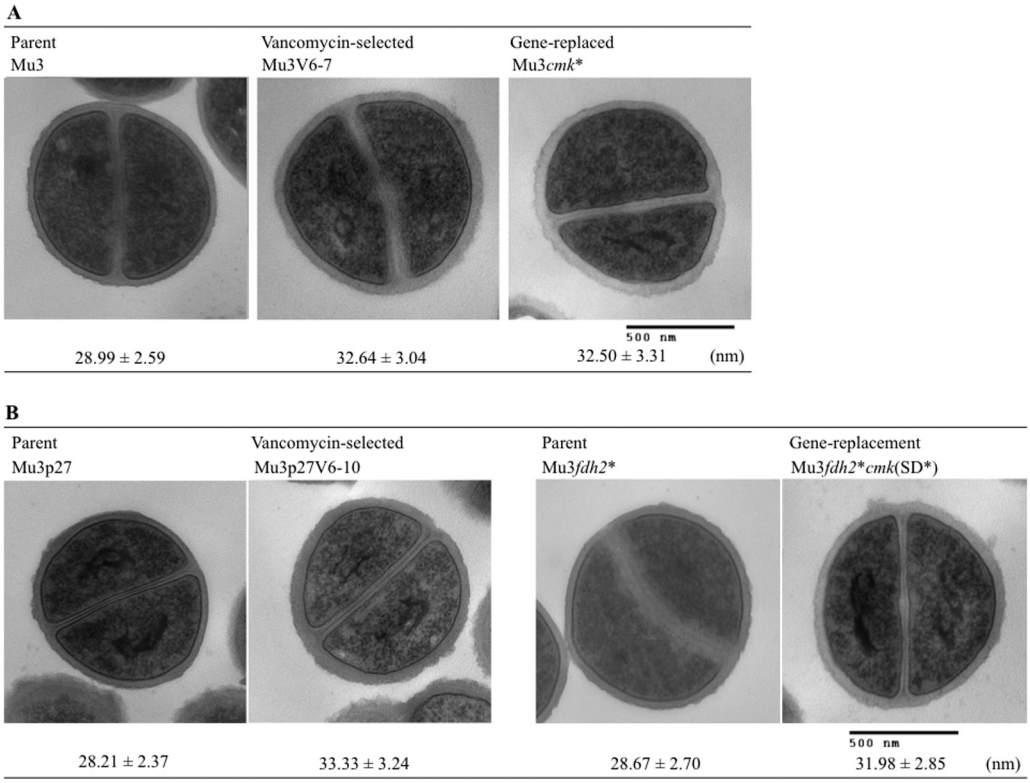

FIG 4 Transmission electron microscopy of the *cmk* mutant strains and their parent strains. (A) *cmk*(A20G) mutants and their parent strain. (B) *cmk*(SD*) mutants and their parent strains. Data are presented as the mean and standard deviation of the cell wall thickness for each strain. Note that all *cmk* mutant cells had thick cell walls compared to those of the parent strain. Magnification, ×30,000.

change in the flow of metabolites triggered by a single mutation occurring in dozens of effector genes in multiple metabolic pathways. This explains the extremely high frequencies (one in 10^{-6} or greater) of hVISA-to-VISA conversion and the characteristic shape of the population curves of hetero-VISA strains (1).

This study presents the identification and preliminary characterization of new mutations that are associated with the conversion of hVISA to VISA. However, we discuss here some plausible speculation with some mutations of interest. The *pykA* mutation may reduce the encoded enzyme activity to convert phosphoenolpyruvate to pyruvate (28). As a result, more phosphoenolpyruvate would be used by *murZ* to the direction of murein monomer production. Enhanced uptake and incorporation of glucose into the cell wall are observed with VISA strain Mu50 (29). Glucose is used to produce both peptidoglycan and teichoic acid (30). If teichoic acid synthesis was reduced by the mutations in the *tarA* or *tarO* gene, more glucose would be utilized to produce peptidoglycan. Mutations in the ribosomal protein genes (*rpsU* and *rplL*) reduce the use of amino acids for the synthesis of protein. Amino acids such as L-alanine, L-glutamate, L-lysine, and glycine are necessary components of peptidoglycan (31). Reduced production of protein will allow more use of amino acids for peptidoglycan synthesis. Many experiments are necessary to test these hypotheses by using the generated mutant strains.

TABLE 5 Effect of introducing the wild-type *cmk* gene into the *cmk* mutant VISA strains on vancomycin susceptibility and doubling time

Strain	Mutation in *cmk*	Plasmid	Vancomycin MIC[b] (mg/liter)	Doubling time[b] (min)
Mu3p27	wt		4	47
Mu3p27(pN)	wt	pND50	3	47
Mu3p27(pN*cmk*)	wt	pN*cmk*	3	49
Mu3p27V6-10	SD[a]		8	59
Mu3p27V6-10 (pN)	SD	pND50	6	65
Mu3p27V6-10 (pN*cmk*)	SD	pN*cmk*	3	49
Mu3	wt		3	36
Mu3(pN)	wt	pND50	2	37
Mu3(pN*cmk*)	wt	pN*cmk*	2	37
Mu3V6-7	A20G		8	46
Mu3V6-7 (pN)	A20G	pND50	6	51
Mu3V6-7 (pN*cmk*)	A20G	pN*cmk*	2	39

[a] SD, mutation at the 13 bp upstream of the *cmk*.
[b] When the MICs and doubling times for the strains carrying the pN and pN*cmk* plasmids were measured, 10 μg/ml chloramphenicol was added to the BHI agar plates and medium to maintain the plasmids.

Cell wall thickening and reduced autolytic activity are consistently observed features of VISA strains (15, 19, 20, 21, 32). Therefore, it appeared reasonable to find the mutations in the genes categorized in the group "Cell envelope synthesis and modification." The genes known to be involved in autolysis or suspected to be (SAHV_1760) are mutated in this study also. Among them, the most frequently affected genes were those in teichoic acid metabolism (Table 3). A link between the D-alanylation of teichoic acids and vancomycin susceptibility has been suggested (33); however, aside from the requirement of glucose in the pathway as discussed above, it is unclear if there is a direct correlation between vanco-

mycin resistance and genes such as *tarO*, *tarA*, and *tarL*, which are responsible for teichoic acid biosynthesis. Atilano et al. have recently reported that teichoic acids regulate peptidoglycan cross-linking through the control of PBP4 activity (34). Thus, another scenario may be present between altered teichoic acid synthesis and reduced peptidoglycan cross-linking that is closely associated with VISA phenotype (27, 32, 35, 36).

cmk and *rpoB* were the most frequently affected genes in this study. We showed that nonsynonymous mutation of *cmk* as well as an SD mutation directly mediated hVISA-to-VISA phenotype conversion of Mu3, which was confirmed by gene replacement experiments (Table 4 and Fig. 3 and 4). In bacteria, the mRNA SD sequence is a ribosomal binding site and the initiator element of translation (37). Mutations in the SD region change the translational efficacy (24, 38). The complementation test with wild-type *cmk* in the Mu3p27V6-10 and Mu3V6-7 strains suggested that a decrease in Cmk activity caused increased vancomycin resistance (Table 5). *E. coli* and *Salmonella enterica* serovar Typhimurium with defective *cmk* are reported to have an increased pool of UTP (39, 40). Therefore, *cmk* mutant strains Mu3p27V6-10 and Mu3V6-7 are expected to have an increased amount of UTP in the cell. On the other hand, UDP-*N*-acetylglucosamine (UDP-GlcNAc), a key intermediate for peptidoglycan biosynthesis, is produced from GlcNAc-1P and UTP (41). Therefore, the increase in the UTP pool may promote cell wall synthesis by facilitating the production of UDP-GlcNAc. In this way, depressed *cmk* activity may lead to thickening of cell wall peptidoglycan layers, increasing vancomycin resistance. This hypothesis needs to be tested by further experiments.

The *cmk* mutations were also found in 7 clinical VISA strains (see Table S5 in the supplemental material). Curiously, all 7 strains were USA isolates, indicating a certain genetic relationship among the strains. Indeed, the four strains sharing the *cmk*(A116D) mutation belonged to ST8, whereas the other three, sharing *cmk*(S203T) (and a number of synonymous mutations as well), belonged to ST45 and ST36 (a genotype closely related to ST45) (42). Since vancomycin-susceptible ST8 strains such as NCTC8325, Newman, FPR3757, and TCH1516 registered at the NCBI site (http://www.ncbi.nlm.nih.gov/genome/) do not carry the *cmk*(A116D) mutation, it is likely that the mutation is associated with the VISA phenotype of the ST8 strains.

The two ST45 strains, HIP07930 and HIP09433, were isolated from New York and Michigan, respectively, and shared the *vraS*(V9V) and *rpoB*(D320N) mutations as well (9, 17), strongly indicating their clonal origin. However, an ST59 community-associated MRSA clinical Taiwanese strain, M013 (43), which shares three *cmk* synonymous and *cmk*(S203T) mutations with the two ST45 strains, is susceptible to vancomycin (MIC, ≤0.5 mg/liter) (T. L. Lauderdale, personal communication). Therefore, it is not clear if *cmk*(S203T) is directly associated with raised vancomycin resistance or not. It could be a part of the polymorphism of *cmk* genes acquired during subspeciation of *S. aureus*. No nonsynonymous *cmk* mutation was found among the 17 strains with the ST5 genotype, which is the most prevalent genotype among VISA clinical strains, including Mu3. This indicates that although it can be obtained under *in vitro* conditions, the *cmk* mutation maybe an effective contributor to the VISA phenotype only for strains of limited genetic backgrounds in clinical settings.

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