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Review

Vancomycin-intermediate resistance in *Staphylococcus aureus*

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ABSTRACT

Vancomycin-intermediate *Staphylococcus aureus* (VISA) and its precursor hetero-VISA (hVISA) were discovered almost 20 years ago and have continued to be a stumbling block in the chemotherapy of methicillin-resistant *S. aureus* (MRSA). Unlike vancomycin resistance mediated by the *van* gene in enterococci and staphylococci, VISA is generated by accumulation of mutations. It displays diverse and intriguing genetic mechanisms underlying its resistance phenotype. Here we make a brief note on our recent understanding of the genetics of hVISA, VISA and the newly discovered phenotype 'slow VISA' (sVISA).

Contents

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1. Introduction

The first clinical vancomycin-intermediate *Staphylococcus aureus* (VISA) strain (Mu50) with a vancomycin minimum inhibitory concentration (MIC) of 8 mg/L and the hetero-VISA (hVISA) strain (Mu3) with an MIC of 2 mg/L were isolated in 1996 [1,2]. hVISA is the precursor of VISA and is composed of cell subpopulations with various degrees of vancomycin resistance [2]. They were initially named vancomycin-resistant *S. aureus* (VRSA) and hetero-VRSA (hVRSA), respectively, because both of them caused infection that was clinically refractory to vancomycin therapy [3]. However, Mu50 and Mu3 were renamed as VISA and hVISA, respectively, according to contemporary vancomycin susceptibility criteria in clinical laboratories, which defined resistance as an MIC $\geq$ 32 mg/L, intermediate resistance as an MIC of 8 mg/L or 16 mg/L and susceptible as an MIC $\leq$ 4 mg/L. Subsequently, the criteria were changed to include an MIC of 4 mg/L in the intermediate group, and 16 mg/L in the resistant group to reflect accumulated clinical experience of frequent vancomycin therapeutic failure against methicillin-resistant *S. aureus* (MRSA) with an MIC of 4 mg/L [4].

The above nomenclature is still incomplete and will remain so as long as it is based on MIC values alone. Some VISA strains recorded MICs of 16 mg/L, and even 32 mg/L in the case of 'slow VISA' (sVISA) (see below). Therefore, it would be better to re-define the terms VISA and VRSA based on their mechanisms of resistance and not on the degree of their resistance, i.e. VRSA for strains whose vancomycin resistance is caused by the horizontally acquired *vanA* gene complex [5], and VISA for strains whose resistance is caused by accumulation of mutations. In the following, we describe recent advances in our understanding of the mechanism of resistance in VISA and hVISA as well as sVISA, a newly identified category of VISA.

2. Elusive nature of vancomycin susceptibility

2.1. Inoculum effect of vancomycin

Vancomycin is unique in its high-inoculum effect against *S. aureus*. The anti-*S. aureus* activity of vancomycin is greatly compromised against a high inoculum of bacteria [6]. This feature of vancomycin is closely associated with the nature of its target of action. Cell wall peptidoglycan (PG) layers contain many free D-alanyl-D-alanine residues in the murein components, to which vancomycin binds with high affinity [7]. These are considered as 'pseudotargets' or 'false targets' of vancomycin, since binding itself does not affect the viability of the cell [3]. Real or vital targets of vancomycin are the lipid–murein monomer precursors on the cytoplasmic membrane that serve as substrates for transglycosylase [3,7]. Transglycosylase does not use the lipid–murein monomer precursors bound by vancomycin as substrates. Thus, vancomycin does not act on the cell-wall synthesis enzyme but on the substrate for the enzyme. This indirect mode of action makes vancomycin an inefficient bactericidal agent [3].

Penicillin-binding proteins (PBPs) function to strengthen the three-dimensional (3D) structure of PG. They cut between the D-alanyl-D-alanine residues of the stem pentapeptide of a nascent PG chain, and cross-bridge the penultimate D-alanine to the tip of the pentaglycine of the neighbouring nascent PG chain [7,8]. In this way, the cell wall PG gains physical strength and the vancomycin binding sites decrease. However, *S. aureus* cells usually have ca. 20% of the PG components uncrosslinked, and there remain ca. 6×10^6 pseudotargets of vancomycin in the PG layers [3]. Then, theoretically, 10^8 *S. aureus* cells can adsorb 1.4 μg of vancomycin without losing their viability. This would lead to a great drawback for vancomycin therapy because this sequestration of vancomycin leads to a significant decrease in the effective vancomycin concentration in the infected tissue of patients. Experimentally, VISA clinical strain Mu50, having two times thicker and less cross-linked PG layers than that of usual *S. aureus* strains, was demonstrated to adsorb as much as 6.3 μg of vancomycin per 10^8 cells [9].

2.2. The pitfall of vancomycin susceptibility tests

Since vancomycin has to pass through the PG layers to reach the cytoplasmic membrane, many molecules are lost before reaching the real targets. This loss of vancomycin by PG layers becomes greater if the number of cells increases. The loss is simply caused by binding of vancomycin to PG, so it does not matter whether the bacteria are dead or alive. If an abscess is formed in the infected tissue, it is expected that live bacteria inside the abscess would never be reached by vancomycin since the antibiotic is consumed by the cells in the outer layers of the abscess.

This nature of vancomycin makes accurate evaluation of vancomycin susceptibility difficult. If a large number of *S. aureus* cells are inoculated on a vancomycin-containing agar plate, a substantial amount of vancomycin is adsorbed by the cells and lost from the surface of the agar. Thus, the effective drug concentration of the agar is drastically decreased because the high molecular weight (1449.27 Da) of vancomycin prevents its quick diffusion across the agar plate. The presence of cell clumps in the dense inoculum also makes it difficult to allow each cell to be exposed to equally selective concentrations of vancomycin. A higher inoculum frequently leads to patchy cell growth even on agar plates containing much greater concentrations of vancomycin than the MIC. Therefore, the level of vancomycin resistance must be carefully evaluated keeping the inoculum size limited per agar plate. In particular, in the analysis of resistant subpopulations [population analysis (PA)] for vancomycin, the inoculum size should not exceed 10^7 CFU for each vancomycin agar plate of 9 cm in diameter [10]. Otherwise, the plate will allow patchy growth of the cells that are susceptible to the nominal concentrations of vancomycin.

2.3. Population analysis and clinical feature of hVISA infection

Fig. 1 shows the PA curves of hVISA strain Mu3 and VISA strain Mu50. Mu3-6R-P is a laboratory sVISA strain derived from Mu3, which will be discussed below. Some subpopulations of Mu3 can grow on agar plates containing $\geq$4 mg/L vancomycin. Thus, the Mu3 cell population is composed of resistant subpopulations with various degrees of vancomycin resistance. This was reflected in the clinical course of pneumonia caused by Mu3. When the patient was treated with vancomycin, the infiltrate of the chest radiograph became faint for the initial 9 days. However, in the next 4 days the infiltrate became dense again despite continued vancomycin treatment with the same regimen [11]. This characteristic clinical picture of the infection (initial improvement and subsequent exacerbation) appears to be a typical pattern of hVISA infection [12]. Most of the cell population of hVISA are depressed of growth by the attainable tissue concentrations of vancomycin, presumably lower than ca. 2–5 mg/L [3], leaving a small number of the VISA subpopulation to survive vancomycin therapy. During continuation of vancomycin therapy, VISA-converted cells multiply, causing recurrence of infection.

2.4. The nature of resistant colonies on the population analysis plates

Although the colonies grown on the PA agar plates can be propagated stably on agar plates containing the same concentrations of vancomycin, their resistance is unstable when the colonies

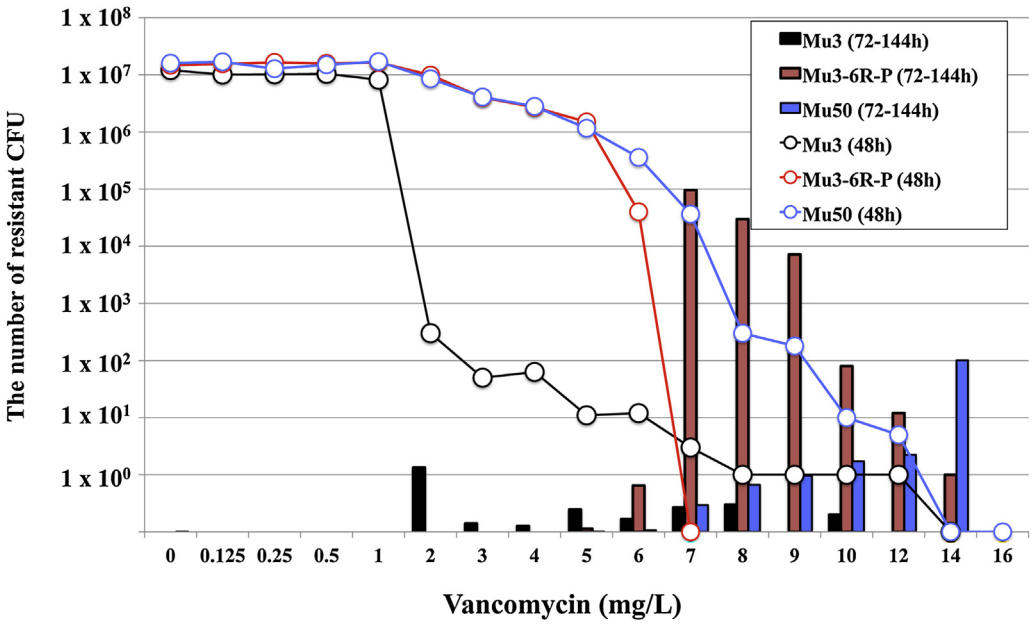

Fig. 1. Vancomycin-resistant subpopulations of vancomycin-intermediate *Staphylococcus aureus* (VISA; Mu50), hetero-VISA (hVISA; Mu3) and 'slow VISA' (sVISA; Mu3-6R-P). Mu3, Mu50 and Mu3-6R-P were compared for their distribution of vancomycin-resistant subpopulations as evaluated after various periods of incubation from 48 h up to 144 h. The population curves were drawn after 48 h of incubation according to the standard protocol [2,10]. Bars are used to show the number of colonies that appeared on each agar plate after 72 h up to 144 h of incubation.

are propagated in drug-free media [2]. The MICs of the strains established from the agar plates tend to be lower than those expected from the nominal vancomycin concentrations of the agar plates on which the colonies were formed. For example, the strain established from the colonies formed on the agar plate containing 4 mg/L may record an MIC of 2–4 mg/L instead of expected MIC of ≥5 mg/L when determined with the MIC value scale with 1 mg/L increment [10]. We consider this decrease in MIC largely due to the inoculum effect as described above, and partially due to instability of certain VISA phenotypes [13]. Therefore, repeated colony purification using agar plates containing the same concentration of vancomycin, or picking the colonies formed on the agar containing higher concentrations of vancomycin, e.g. 6 mg/L instead of 4 mg/L, is necessary to establish VISA strains having a vancomycin MIC of ≥4 mg/L.

3. Unique mechanism of vancomycin resistance in VISA

3.1. Affinity trapping of vancomycin by false targets

Binding of vancomycin to non-vital targets in PG is the essence of vancomycin-intermediate resistance in *S. aureus*. A thick cell wall, as observed by transmission electron microscopy (Fig. 2A), is the cardinal feature of VISA [3,13–16]. In VISA strain Mu50, PG synthesis is accelerated and a greater amount of glucose is incorporated into the PG compared with Mu3 and control vancomycin-susceptible *S. aureus* (VSSA) strains [16]. Cell wall thickness is highly influenced by nutrients in the culture medium. In a medium rich in the structural components of PG such as glucose and glutamine, Mu50 produces an abnormally thickened cell wall (Fig. 2A) [16]. As described below, the extent of thickness of the PG layers directly correlates with the degree of vancomycin resistance. Therefore, nutrient dependence of the cell wall thickness of VISA strains requires special attention in the selection of media for susceptibility tests. Usually, brain–heart infusion supports the expression of vancomycin resistance much better than Mueller–Hinton.

With the activated cell-wall synthesis pathway in Mu50, supply of the precursor metabolites does not appear to catch up with demand. In agreement with this notion is the structural feature of Mu50 PG. High-performance liquid chromatography (HPLC) analysis of the PG structure revealed an increased proportion of glutamine-non-amidated murein monomer versus glutamine-amidated murein monomer (as reflected in the M9/M4 peak ratio) in Mu50 cells grown in a regular medium [15], which is a sign of the

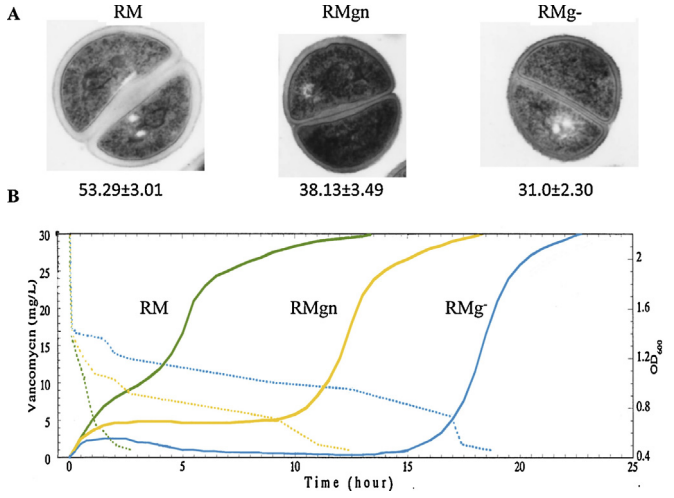

Fig. 2. Nutrient dependence of cell wall thickness and the level of resistance of vancomycin-intermediate *Staphylococcus aureus* (VISA) strain Mu50. (A) Mu50 was grown in brain–heart infusion (BHI) medium, washed twice with resting medium without glucose (RMg^-) and then incubated at 37 °C for 2 h with shaking in either one of the following media: RMg^-; RM (RMg^- plus 30 mM D-glucose); or RMgn [RMg^- plus 30 mM *N*-acetylglucosamine (GlcNAc)]. The values given under each picture are the mean and standard deviation of cell wall thickness (nm). (B) Growth of Mu50 cells with different cell wall thicknesses in BHI medium with 30 mg/L vancomycin was monitored by optical densitometry (right vertical axis). The vancomycin concentration in the medium (left vertical axis) was sequentially monitored by bioassay (dotted line) [9]. Cells with a thicker cell wall started growing earlier than those with a thinner cell wall.

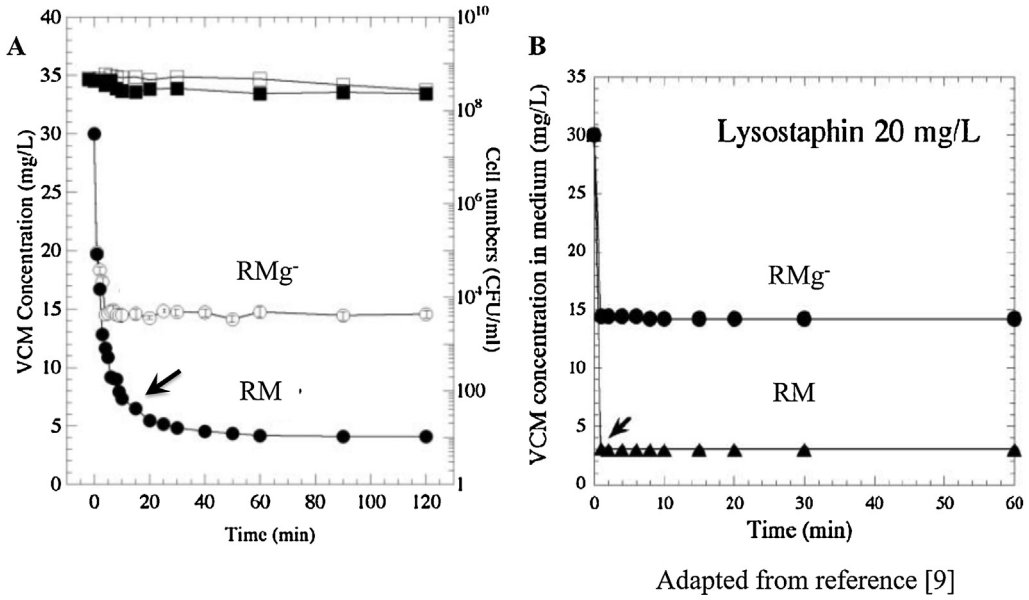

Fig. 3. Prolonged time for vancomycin (VCM) to reach the cytoplasmic membrane through the thickened *Staphylococcus aureus* cell wall. (A) Time course of VCM consumption compared between Mu50 cells with a thin cell wall (RMg^-) and thick cell wall (RM). Mu50 cells were incubated in resting medium without glucose (RMg^-) for thin cell walls and in resting medium containing glucose (RM) for thick cell walls. Cells were then incubated in RMg^- containing 30 mg/L VCM. A portion (0.5 mL) of the cell preparations was taken at selected time intervals to measure VCM consumption and the number of viable cells (see Ref. [9] for details). The arrow indicates slowing of VCM consumption by thick cell wall. Circles, VCM concentration; squares, cell numbers; open symbols, cells with thin cell walls; solid symbols, cells with thick cell walls. (B) The same experiment was done using cells pre-treated with lysostaphin. Lysostaphin is a glycylglycine endopeptidase that specifically cleaves the pentaglycine cross-bridges of peptidoglycan (PG) without cutting off the VCM binding targets D-alanyl-D-alanine residues from the stem pentapeptide. Treated cells consumed amounts of VCM comparable with those consumed by intact PG (A), whereas slowing of VCM consumption observed with intact PG was lost (denoted by an arrow). •, VCM consumption by Mu50 with a thin cell wall; ▲, VCM consumption by Mu50 with a thick cell wall.

deficiency of intracellular glutamine that serves as the donor of the amine group to the murein monomer. Coincidentally, glutamine-non-amidated murein monomer is a poor substrate for PBPs [17]. As a result, a lower rate of PG cross-bridging was observed by HPLC analysis (lowered dimer/monomer ratio) [16]. This further contributed to the increase of free D-alanyl-D-alanine residues in the PG layers and increased consumption of vancomycin. Furthermore, by another curious coincidence, the non-amidated muropeptide happened to have a greater binding affinity to vancomycin than the amidated muropeptide [16]. These observations correlated well with the experimental data that Mu50 consumes ≥2.8 times more vancomycin in its cell wall than VSSA strains [16].

Fig. 2B illustrates that VISA strain Mu50 with a vancomycin MIC of 8 mg/L can grow in medium containing as high as 30 mg/L vancomycin [13]. When the concentration of vancomycin was monitored by bioassay, a significant drop in the concentration was observed from 30 mg/L to ca. 16–17 mg/L within a few minutes of addition of the cells to the medium. This is due to the rapid adsorption of vancomycin to the cell wall PG of Mu50. The vancomycin concentration gradually decreased before the cells started to re-grow. It is noted that prior to the growth initiation of Mu50 cells, the concentration of vancomycin dropped to <10 mg/L. This decline of vancomycin concentration is due to the sequestration of the drug by the false targets in PG of Mu50. The vancomycin MIC of the re-grown Mu50 cells was the same as that of the inoculum. This clearly demonstrates the 'inoculum effect' in *S. aureus* susceptibility to vancomycin [13].

3.2. Clogging of the peptidoglycan mesh and prolonged time for vancomycin to reach the cytoplasmic membrane

When VISA cells are exposed to vancomycin, their PG layers adsorb a huge number of vancomycin molecules as described above. As a result, bound vancomycin molecules obliterate the PG mesh structure and prevent further passage of vancomycin molecules from outside of the cell. This 'clogging effect' is clearly observed in Fig. 3. Fig. 3A shows the time-dependent change in vancomycin concentration of the medium inoculated with ca. 5×10^8 cells with thick and thin PG layers. In less than 5 min, 15 mg/L vancomycin was adsorbed by both cells, after which no more decrease was observed with cells with thin PG layers. Complete saturation of vancomycin binding targets in PG is achieved. In contrast, cells with thick PG continued adsorbing vancomycin from the culture medium, but the rate of decrease in vancomycin concentration was blunted after 5 min and gradually reached saturation (denoted by an arrow in Fig. 3A). The thick cell wall finally adsorbed 25 mg/L vancomycin (Fig. 3A). Slowing of the decrease in vancomycin concentration was lost by brief treatment of the cells with lysostaphin, which breaks the PG mesh and allows vancomycin to penetrate the entire PG layer without hindrance (Fig. 3B). The more critical sequel of clogging is observed in Fig. 3A, in which the timing of saturation of thick PG layers is delayed by 20–40 min, which is almost comparable with the doubling time of *S. aureus*. This signifies that *S. aureus* cells would continue producing PG during the time delay and provide new PG layers from beneath the older PG layers. In this way, with thickened PG layers, vancomycin cannot completely inhibit PG synthesis no matter how high a dose of vancomycin is used. This is another strategy of VISA. PG now works as a shield for vancomycin penetration, like the outer membrane of Gram-negative bacteria.

4. Emergence of hVISA and selective pressure

4.1. 'Regulator mutations' to generate hVISA: vraUTSR, walKR *and* graRS

Evidence is accumulating for the view that vancomycin resistance in VISA is caused by altered cell wall structure and metabolism. Since *S. aureus* cell wall synthesis is regulated by

multiple regulator genes, it is reasonable that hVISA/VISA clinical strains carry various mutations in the regulator genes associated with cell wall biosynthesis [12]. Whole-genome sequencing and microarray analysis of Mu50 identified the *vraSR* two-component regulatory system (TCRS) whose transcriptional upregulation is responsible for the raised vancomycin resistance of Mu50 [18–20]. In Mu50 (and also in Mu3), the sensor kinase gene *vraS* was constitutively activated by the incorporated mutation *vraS*(I5N). The mutated VraS in turn activated the cognate response regulator VraR and raised expression of the genes encoding several key enzymes of cell wall biogenesis such as *murZ*, *pbp2*, *sgtB*, *tarA*, *fmtA* and *lcpC* (SA2103) [20,21]. Experimental introduction of the mutation *vraS*(I5N) as well as of another experimentally obtained mutation *vraS*(S329L) into VSSA strain N315ΔIP conferred an hVISA phenotype on the strain [21]. The mutation *vraS*(I5N) is carried by many VISA strains isolated from various districts of Japan [2,22–24], indicating clonal spread of Mu3 throughout Japan.

Recently, *vraSR* is regarded as part of a four-membered operon, *vraU–vraT* (or *yvqF*)–*vraS–vraR*. *vraT* is reported to be essential for *vraSR* function as the upregulator of cell wall synthesis [25,26]. The *vraT*(Y220C) mutation was shown to activate *vraSR* and raise both methicillin and vancomycin resistance [26]. Another mutation [*vraT*(T125I)] is also shown to raise vancomycin and imipenem MICs [23]. This cross-resistance between vancomycin and β-lactams through activation of the *vraSR* TCRS has a historical implication in the emergence of VISA from MRSA in Japan [22]. Acquisition of homogeneously high β-lactam resistance by hetero-MRSA (see below) appears to have prepared the way for Japanese MRSA to conquer vancomycin as well.

The *vraUTSR* operon is frequently mutated in clinical VISA and hVISA strains (Table 1). Of 33 VISA strains, 14 (42.4%) possessed mutations in either one of the three genes *vraT*, *vraS* and *vraR*. In Japan, however, the *vraTSR* mutation frequency among 86 *S. aureus* clinical strains with reduced teicoplanin susceptibility (teicoplanin MIC ≥ 2 mg/L; equivalent to hVISA or 'pre-hVISA' described below) was as high as 67.4% (58 strains) [23]. We found that the development of hVISA clinical strains among MRSA strains in Japan occurred before the introduction of vancomycin into clinical use [22]. Since injectable vancomycin was not introduced in Japan until 1991, clinical use of potent β-lactam antibiotics such as imipenem, cefmetazole and flomoxef was the national trend to cope with MRSA infection in the 1980s [27]. We also found that hVISA was generated in vitro by selecting a hetero-MRSA strain ΔIP with imipenem [8,21], and that the appearance rate of hVISA from ΔIP was much higher with imipenem than with vancomycin as a selective agent (2.0×10^{-5} vs. 3.6×10^{-7}) [21].

Table 1
rpoB and regulator gene mutations in clinical vancomycin-intermediate *Staphylococcus aureus* (VISA) strains worldwide.

Phenotype	NARSA no.	Strain name	Nationality	DT (min)	*rpoB* sequence	VAN MIC (mg/L)	RIF sensitivity[a]	Sequence of selected genes[b]				
								vraT	*vraS*	*msrR*	*graR*	*walK*
hVISA	NRS2	Mu3	Japan	36.9	wt	2	S	wt	I5N	E146K	wt	wt
sVISA		Mu3-6R-P	In vitro	62.2	R512P	12	S	wt	I5N	E146K	wt	wt
VISA	NRS1	Mu50	Japan	36.5	H481Y	8	R	wt	I5N	E146K	N197S	wt
VISA	NRS29	HIP09735	USA	53.8	Y737F	4	S	wt	wt	wt	wt	A468T
VISA	NRS65	LY-1999-03	Oman	49.3	wt	4	S	wt	wt	K312R	wt	N48K, R222K, A468T
VISA		JCSC7203	Thailand	34.8	H481N, S529L	4	R	nt	wt	K312R	D68Q	R222K, S437F, A468T
VISA	NRS22	HIP07930	USA	47.5	D320N	4	S	wt	G9V	E146D	wt	wt
VISA		JCSC7219	Thailand	28.8	H481N, S529L	4	R	nt	wt	K31R	wt	R222K, A468T
VISA		JCSC7221	Thailand	31.5	wt	4	S	nt	wt	wt	wt	wt
VISA	NRS12	SA MER-S6	France	29.2	wt	8	S	wt	wt	wt	wt	wt
VISA	NRS13	SA MER-S12	France	33.5	wt	4	S	wt	wt	wt	wt	wt
VISA	NRS18	HIP06854	USA	30.2	wt	4	S	wt	wt	wt	wt	T492K
VISA	NRS74	HIP10267	USA	44.8	D471V, A473S, A477S, E478D	4	R	H164R	wt	wt	T11A	wt
VISA	NRS403	HIP13057	USA	36.1	H481Y	4	R	wt	wt	wt	E15K	R282C
VISA	NRS21	HIP07920[c]	USA	39.5	R484H, F1075L	4	R	wt	wt	wt	wt	wt
VISA	NRS23	HIP08926	USA	33.3	wt	4	S	wt	wt	wt	wt	R222I, T492K
VISA	NRS24	HIP09143	USA	33.0	wt	4	S	P126S	wt	wt	wt	wt
VISA	NRS26	HIP09313	USA	41.6	wt	4	S	wt	P327S	wt	wt	L10F, S437T
VISA	NRS27	HIP09433	USA	31.9	D320N	4	S	wt	G9V	E146D	wt	V1454G
VISA	NRS28	HIP09662	USA	41.0	D471N, S486L	4	R	wt	wt	wt	wt	Ins.433N, 434D
VISA	NRS49	AMC11094[d]	Korea	33.8	wt	8	S	wt	wt	wt	wt	wt
VISA	NRS51	HIP09740	USA	27.0	H481D	6	R	wt	wt	wt	wt	V380I
VISA	NRS52	HIP09737	USA	28.9	H481D	4	R	wt	wt	wt	wt	G275V
VISA	NRS63	LY-1999-01	Oman	32.4	R406S	4	S	wt	wt	K312R	wt	N48K. R222K, A468T
VISA	NRS14	SA MER-S20	France	36.1	wt	4	S	wt	wt	wt	wt	wt
VISA	NRS17	HIP06297 (PC)	USA	36.4	Q468L	8	R	W119R	wt	wt	wt	A567D
VISA	NRS73	HIP10540	USA	32.6	V135A, A477V	4	R	L85F	wt	wt	wt	wt
VISA	NRS118	NRS118	USA	29.4	H481N, S529L	8	R	wt	wt	wt	wt	F330S
VISA	NRS76	NRS76	USA	28.8	wt	8	S	wt	A314V	wt	wt	A243T
VISA	NRS79	NRS79	USA	27.4	H481R	8	R	A151T	wt	wt	wt	D496N
VISA	NRS126	NRS126	USA	46.2	H481N	4	R	wt	wt	wt	wt	wt
VISA	NRS272	P1V44[c]	Belgium	46.7	H481N, S529L, E792G, F1075L	16	R	wt	wt	wt	wt	wt
VISA	NRS402	HIP12864[c]	USA	53.1	P519L	4	R	H164R	wt	wt	wt	wt
VISA	NRS404	HIP13036	USA	31.9	wt	8	S	wt	T104A	wt	wt	wt
VISA	NRS3	MI	USA	39.1	R140S	8	S	G32D	wt	wt	wt	V494L

hVISA, hetero-VISA; sVISA, 'slow VISA'; DT, doubling time; VAN, vancomycin; MIC, minimum inhibitory concentration; RIF, rifampicin; wt, wild-type; nt, not tested.
[a] Susceptible (S), MIC ≤ 1 mg/L; resistant (R), MIC > 1 mg/L.
[b] *vraT* data are based on Ref. [23].
[c] sVISA was identified in the stored culture of the strain.
[d] AMC11094 from Korea possesses the *vraR*(A113V) mutation.

Other regulator mutations are found in the *walKR* TCRS, where *walK* (*yycG*/*vicK*) is a sensor histidine kinase and *walR* (*yycF*/*vicR*) is the cognate response regulator. The TCRS is supposed to be the regulator of cell wall metabolism, including expression of autolysins [28–31]. Introduction of a *walK* mutation into VSSA strain N315LR5 raised vancomycin resistance from 1 mg/L to 3 mg/L and significantly depressed Triton X-100-induced autolysis [29]. As many as 18 (54.5%) of 33 tested VISA strains possessed mutations in the *walK* gene (Table 1).

Mutation in the response regulator gene *graR*(N197S) is present in Mu50 [32]. In another clinical MRSA strain, a mutation in the sensor kinase gene *graS*(T136I) was demonstrated to confer an hVISA phenotype on a VSSA strain [33]. In the Mu50 chromosome, *graR*(N197S) is one of the nine non-synonymous mutations compared with the Mu3 chromosome [32]. Introduction of *graR*(N197S) on a multiple-copy plasmid converted Mu3 into VISA with an MIC of 4 mg/L. However, introduction of *graR*(N197S) into Mu3 as a single copy by gene replacement raised the vancomycin MIC slightly from 2 mg/L to 3 mg/L, i.e. to the degree of resistance of hVISA. Conversion to VISA was finally achieved by subsequent introduction of an *rpoB* mutation. The *rpoB* gene encodes the β subunit of RNA polymerase (see below). Introduction of the *graR* mutation enhanced gene expression of the ABC transporter genes *vraDE*-SAS091, *vraFG* and *mprF* [32,34]. *mprF* encodes phosphatidylglycerol lysyltransferase that modifies membrane phosphatidylglycerol with L-lysine [35]. The *graRS* genes together with *vraFG* and the adjacent orf *graX* are proposed to constitute the five-component system *graXRS–vraFG* to sense cationic antimicrobial peptides [36]. Although rarely represented in clinical VISA strains, *graRS* mutation is another regulator mutation that could convert VSSA into hVISA [33].

4.2. rpoB *mutation as a 'regulatory' mutation in hVISA phenotype acquisition*

The most prevalent mutations in VISA clinical strains were those in the *rpoB* gene, which were carried by 21 (63.6%) VISA strains (Table 1). Although *rpoB* is not a regulator gene, its mutation drastically changes the transcription profile of the cell much more than any of the regulator mutations [37]. We therefore regarded *rpoB* mutation as a 'regulatory mutation' [34,38].

As many as 29 (87.9%) of the 33 tested VISA strains possessed mutations in either one of the three genetic loci *rpoB*, *walRK* and *vraUTSR* (Table 1). Considering the low frequency of occurrence of spontaneous back-mutation, the initial chromosome mutation towards a VISA phenotype acquisition would remain in the chromosome of established VISA clinical strains; therefore it would be plausible to consider that the mutations in *rpoB*, *walRK* and *vraUTSR* are the first-step mutations placing a VSSA onto the path towards VISA. In this regard, it is interesting that vancomycin is not the only selective pressure for the generation of hVISA. Use of non-glycopeptide antibiotics such as rifampicin, daptomycin and β-lactams may serve as selective pressures for the emergence of hVISA in the hospital by mutating *rpoB* [39], *walRK* [30,40,41] and *vraUTSR* [20–22], respectively.

As shown in Fig. 4A, PA curves of ΔIP-derived strains introduced with *vraS*(S329L), *walK*(V494L), *graR*(N197S) and *rpoB*(H481Y) as a single mutation acquired small subpopulations of cells capable of growth in the presence of 2–4 mg/L vancomycin. Therefore, all of the tested mutations possessed the potential to raise vancomycin resistance as a single mutation. However, none, even the *vraS* mutation, formed as many colonies as Mu3 on the agar plate with 4 mg/L vancomycin. To distinguish them from hVISA, we designate those strains with reduced susceptibility to vancomycin

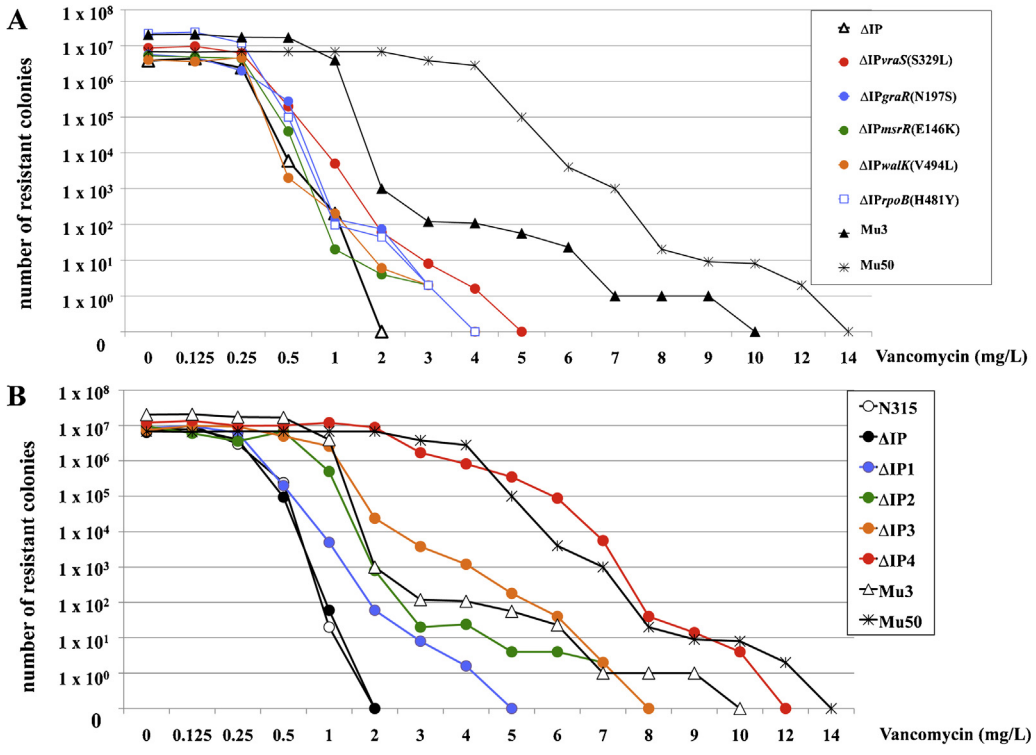

Fig. 4. Effect of individual mutations towards the development of vancomycin-intermediate *Staphylococcus aureus* (VISA). (A) Regulator mutations and an *rpoB* mutation were introduced into vancomycin-susceptible *S. aureus* (VSSA) strain N315ΔIP and their resistant subpopulations were analysed. Note that each mutation developed only small subpopulations with reduced vancomycin susceptibility. Those one-step-mutated strains may well be called 'pre-hVISA'. (B) Sequential introduction of four mutations reconstituted the VISA phenotype of Mu50 in VSSA strain N315ΔIP. ΔIP1, ΔIP-*vraS*(S329L); ΔIP2, ΔIP-*vraS*(S329L)*msrR*(E146K); ΔIP3, ΔIP-*vraS*(S329L)*msrR*(E146K)*graR*(N197S); and ΔIP4, ΔIP-*vraS*(S329L)*msrR*(E146K)*graR*(N197S)*rpoB*(H481Y).

'pre-hVISA', which has a smaller subpopulation ($<1 \times 10^{-6}$) of cells capable of growth on the agar plate containing 4 mg/L vancomycin. The pre-hVISA strains may be correlated with the 'MIC creep' phenomenon observed in hospitals where anti-MRSA chemotherapy is frequently implemented [42].

4.3. Multistep generation of hVISA

Two or more regulator or regulatory mutations may be required for clinical VSSA to become hVISA, which is defined as the ability to generate colonies at a frequency of $\geq 1 \times 10^{-6}$ on the agar containing 4 mg/L vancomycin [2]. In the case of Mu3, we found that another mutation, *msrR*(E146K), was required in addition to *vraS*(I5N) to acquire an hVISA phenotype (Katayama Y, unpublished data). The first-step mutation *vraS*(I5N) or *vraS*(S329L) converted ΔIP (vancomycin MIC = 1 mg/L) into pre-hVISA strain ΔIP1 with an MIC of 2 mg/L. When *msrR*(E146K) was introduced as the second mutation, the strain was converted to hVISA strain ΔIP2 with a raised MIC of 3 mg/L (Fig. 4B). The shape of the population curve of ΔIP2, or ΔIP*vraS*(S329L)*msrR*(E146K), was now equivalent to that of Mu3 (Fig. 4B). The *msrR*(E146K) mutation was previously shown to raise imipenem susceptibility and teicoplanin resistance when overexpressed in VSSA strain N315 [43]. We then noticed that the mutation was shared by Mu3 and Mu50. The *msrR* gene is present on the *S. aureus* chromosome as one of the three paralogues encoding proteins of the LytR–CpsA–Psr (LCP) family [44]. The *msrR* (or *lcpA*) and the other two *lcp* genes *lcpB* and *lcpC* are proposed to function in the last stage of wall teichoic acid (WTA) synthesis, namely attachment of teichoic acid to cell wall PG [44,45]. WTA is proposed to control autolysis of *S. aureus* cells through stabilisation of autolysin [46]. However, it remains to be elucidated how the altered MsrR in Mu3 and Mu50 contributes to raised vancomycin resistance.

5. hVISA to VISA conversion

5.1. Conversion of Mu3 to Mu50

There are two levels in VISA: 'high-level' VISA with vancomycin MICs of ≥8 mg/L; and 'low-level' VISA with MICs of 4 mg/L. Mu50 (NRS1) [1] and MI (NRS3) [47] represent high-level VISA, whereas the majority of clinical VISA strains are low-level VISA (Table 1). As illustrated in Fig. 4B, introduction of *graR*(N197S) raised vancomycin resistance of hVISA strain Mu3 to the level of a low-level VISA, and subsequent introduction of *rpoB*(H481Y) converted it into high-level VISA indistinguishable from Mu50 [34].

Subsequently, the VISA phenotype of Mu50 was successfully reconstituted in VSSA strain N315ΔIP by sequentially introducing four mutations, i.e. *vraS*(S329L), *msrR*(E146K), *graR*(N197S) and *rpoB*(H481Y) (Katayama Y, unpublished data) (Fig. 4B).

However, we were not convinced with the idea that combination of *graR* and *rpoB* mutations was the only way for Mu3 to become high-level VISA. To obtain a more comprehensive view on the genetic events underlying hVISA-to-VISA conversion, we established 45 high-level VISA strains by selecting Mu3 and its related hVISA strains with 6 mg/L vancomycin and determined their whole genome sequences [38].

5.2. Extremely diverse genetic mechanisms for hVISA-to-VISA phenotypic conversion

VISA is generated by spontaneous mutation from hVISA at a frequency of $\geq 10^{-6}$ [3,48,49]. Since the usual appearance rate for a spontaneous mutation is ca. 10^{-8}–10^{-9}, such a high incidence of emergence of VISA from hVISA indicates the presence of great numbers of alternative mutations. The whole-genome sequences of 45 VISA-converted mutant strains revealed a surprising result that each converted strain had one to four mutations, but no two strains shared the same mutation [38]. Table 2 shows the list of non-synonymous single mutation found in 32 of the 45 VISA-converted strains. Each strain carried a unique mutation in 1 of the 20 genes. Therefore, those genes listed in Table 2 were considered to have a direct contribution to the hVISA-to-VISA phenotypic conversion as a single determinant. By far the most frequently affected genes were *rpoB* and *rpoC* in six strains, *cmk* in another six strains, followed by *tarO* in three strains [38]. They affected various cellular processes and metabolic pathways of the cell, and five of them were reported previously in association with raised vancomycin resistance, including SAHV_1209 encoding PP2C phosphatase [50], *pbp4* [51], *rpoB* [34,39,52], *rpoC* [53] and *walK* [28–30,54]. Reduction in PBP4 activity decreases PG cross-linkages and thus increases the number of false targets of vancomycin. PP2C phosphatase and the *walKR* TCRS are considered to be associated with cell wall metabolism and the control of autolysis [50,55]. Accelerated cell wall synthesis and decreased autolysis are two alternative ways to thicken cell wall PG layers. In this regard, it is also noteworthy that the orf SAHV_1760 encoding putative autolysin is among the singly mutated gene list (Table 2).

As shown in Table 1, *rpoB* mutation is frequently found in clinical VISA strains. In a separate experiment using nine Japanese clinical MRSA strains, we established 90 rifampicin-resistant mutant strains. By testing their susceptibility to vancomycin, we found that 86 (95.6%) of 90 rifampicin-selected mutant strains showed decreased vancomycin susceptibilities [39]. Besides *rpoB* encoding the β subunit of the RNA polymerase holoenzyme, genes encoding the other subunits of RNA polymerase holoenzyme, *rpoC*, as a unique mutation, and *rpoD* (*sigA*) and *rpoA*, as one of the double mutations, respectively, were also found in the 45 VISA-converted strains [38]. Therefore, the structural change in RNA polymerase holoenzyme itself appears to raise vancomycin resistance through the alteration of cell physiology and metabolism.

Of the 20 mutations, 15 were newly identified in the above experiment [38]. Among them the most frequently found was *cmk* encoding cytidylate kinase. The enzyme catalyses the formation of cytidine diphosphate (CDP). The mutation decreased the function of *cmk* (Matsuo M, unpublished data). *cmk* dysfunction is considered to result in the depression of not only DNA/RNA synthesis but also WTA synthesis, since supply of CDP–glycerol is required for the synthesis of WTA [56]. Coincidentally, a total of six other strains had mutations in the genes of the WTA biosynthesis pathway (Table 2). Together with *cmk* mutation, as many as 12 (37.5%) of the mutant strains may have depressed WTA synthesis. In Gram-positive bacteria, the structural components of PG and WTA are synthesised on the membrane carrier undecaprenyl phosphate [57]. Since a limited number of lipid carriers are available in the membrane [58], a depressed teichoic acid synthesis pathway may provide an advantage for the synthesis of PG components. A depressed teichoic acid synthesis pathway and *cmk* dysfunction may help raise vancomycin resistance by allowing the PG synthesis enzymes to use more membrane carrier lipids.

It should be noted that the above experiment was done using Mu3 and its derivative strains [38]. Therefore, the cell wall PG synthesis pathways in the recipient strains were already activated by *vraS*(I5N) mutation [20]. This may be the reason why mutations were not observed in the *vraTSR* regulatory system in the experiment. Another mutation in *walKR* TCRS frequently observed in clinical VISA strains was not prevalent either in the experiment; only one strain carried the mutation (Table 2). It is possible that at least part of the effects of *vraSR* mutation is redundant with that of *walKR* mutation in the control of cell wall metabolism.

Table 2
Functional categorisation of genes that are singly mutated in 32 vancomycin-intermediate *Staphylococcus aureus* (VISA)-converted strains.

Mutated genes	Description	The location of Mutation and AA Change[a]	pathways	Number of strains (%)
pbp4	Penicillin binding protein 4	S140N	PG synthesis	1
SAHV_1760	cell wall hydrolase	W200C	PG degradation (Autolysis)	1
tarO (=*llm or tagO*)	Wall teichoic acid biosynthesis protein TarO	P94L 169R F205L	Wall teichoic acid synthesis	6 (19%)
tarA (=*tagA)*	Wall teichoic acid biosynthesis protein TagA	(10bp upstream of the *orf*)	Wall teichoic acid synthesis	
SAHV_0256 (*tarL*)	Wall teichoic acid biosynthesis protein TagL	W13R S224Y	Wall teichoic acid synthesis	
gtaB	UTP-glucose-1-phosphate uridyltransferase	Q220*	Lipoteichoic acid synthesis	1
rpoB	β subunit of RNA polymerase	R406S T480M G540V S746Y A1085V	Transcription	6 (19%)
rpoC	β' subunit of RNA polymerase	P440L	Transcription	
walK or vicK	Involved in the regulation of autolysin genes	Q216E	Transcription regulation	1
cmk	Cytidylate kinase	A20G A24V I128N G129V G201V (13bp upstream of *cmk*)	Pyrimidine metabolism	6 (19%)
pykA	Pyruvate kinase	P12S	Glycolysis glucose → pyruvate Purine nucleotide biosynthesis	1
SAHV_1392	Acylphosphatase	G14R	Pyruvate metabolism (Acetyl-P→Acetate)	1
rpsU	Small subunit ribosomal protein S21	E31*	Protein synthesis	1
trpC	Tryptophan biosynthesis	K31N	Amino acid synthesis	1
ureD	Urease accessory protein	V151I	Urea degradation	1
SAHV_1209 (*pp2c*)	Protein phosphatase 2C	G148(VKLRRKKHLHIHNVILLRR*)[b]	Serine/threonine phosphatase (Autolysis)	1
SAHV_0372	Hypothetical protein	T3I	Unknown function	1
SAHV_0612	Hypothetical protein	V93F	Unknown function	1
SAHV_0741	Hypothetical protein	G93D	Unknown function	1
SAHV_2101	putative *hemK* family modification methylase	G121R	Unknown function	1

PG, peptidoglycan; WTA, wall teichoic acid.
*Denotes a stop codon.
[a]The letters in parenthesis denote the amino acid sequences generated by a frameshift mutation.

Alternatively, the *walKR* system may not be as influential in Mu3 as in other genetic lineages of *S. aureus*.

5.3. *Pleiotropic effects of* rpoB *mutations*

Not all *rpoB* mutations contribute to raised resistance to vancomycin. Such *rpoB* mutations as *rpoB*(S464P), *rpoB*(Q468R) and *rpoB*(Q468K) raised rifampicin resistance of the mutants but did not raise their vancomycin resistance appreciably [34]. Fig. 5 shows the location and amino acid substitution of *rpoB* mutations found in various categories of *S. aureus* strains. The mutations found in clinical strains are described under the scheme of the *rpoB* gene, and those generated in the laboratory are described above the scheme. All of the rifampicin-resistant VISA clinical strains possessed mutations within the rifampicin resistance-determining region (RRDR). Six strains were rifampicin-susceptible VISA strains (MIC < 1 mg/L) whose mutations were found outside of the RRDR of the *rpoB* gene (Table 1 and Fig. 5). Such strains having mutation outside the RRDR can be easily obtained in vitro from hVISA strain Mu3 by selection with vancomycin, but not with rifampicin. Such strains tend to have higher levels of vancomycin resistance than those selected by rifampicin [34]. For example, laboratory-derived mutant strains with *rpoB*(T480M), *rpoB*(R503H) and *rpoB*(S746Y) possessed vancomycin MICs of 7, 9 and 9 mg/L, respectively. These values were significantly higher than 4–5 mg/L of the mutants with *rpoB*(H481Y) and other RRDR mutations [34].*rpoB* mutations affect susceptibility of not only rifampicin and vancomycin but also of other categories of antibiotics. Depending on the location and kinds of amino acid substitution, *rpoB* mutations cause a variety of phenotypic changes. Especially notable are susceptibilities to vancomycin, daptomycin and linezolid (Fig. 5). Daptomycin and vancomycin MICs are positively correlated [32,40,41,52]. In Mu50, *graR*(N197S) mutation appears to have raised daptomycin resistance by increasing the positive charge of the cell surface through enhanced expression of the *mprF* gene [32,35]. *rpoB*-mediated dual resistance to vancomycin and daptomycin was first demonstrated in laboratory strain ΔIP-10*3d1 that acquired reduced susceptibility to both antibiotics after serial daptomycin selection. A single mutation, *rpoB*(A621E), was responsible for the dual resistance phenotype [52]. This indicated that multiple cellular phenotypes separately controlled by independent regulators may be altered by a single *rpoB* mutation. We also noticed a negative correlation between vancomycin MICs and linezolid MICs among clinical VISA strains [60]. Now we found that certain *rpoB* mutations represented by *rpoB*(S746F) increase linezolid susceptibility and decrease vancomycin susceptibility at the same time [34].

Some *rpoB* mutations also have a profound influence on methicillin resistance [61]. Expression of the methicillin resistance gene *mecA* is not enough for *S. aureus* cells to express high-level methicillin resistance (defined as an MIC > 128 mg/L for oxacillin or >4 mg/L for imipenem). Acquisition of certain chromosomal mutations (*chr**) was known to be required to convert the *mecA*-carrying *S. aureus* into highly methicillin-resistant *S. aureus* or homogeneously methicillin-resistant *S. aureus* (homo-MRSA). Without *chr**, *S. aureus* stays as heterogeneously methicillin-resistant *S. aureus* (hetero-MRSA). Curiously, the mutated *vraS* gene of Mu3 causing an hVISA phenotype turned out to be one of the *chr** mutations [19,20]. The mutation raised both methicillin and vancomycin resistance by activating cell wall PG synthesis [20,21]. Such dual activity was also ascribed to several *rpoB* mutations (Fig. 5) in which two mutations, *rpoB*(R512P) and *rpoB*(A621E), were confirmed to have the dual activity by gene replacement experiments. Thus, *rpoB*(A621E) was found to have a triple activity on methicillin, vancomycin and daptomycin susceptibilities. On the other hand, recently identified *rpoB* mutation *rpoB*(I967N) acted as *chr** but did not influence vancomycin susceptibility [61].

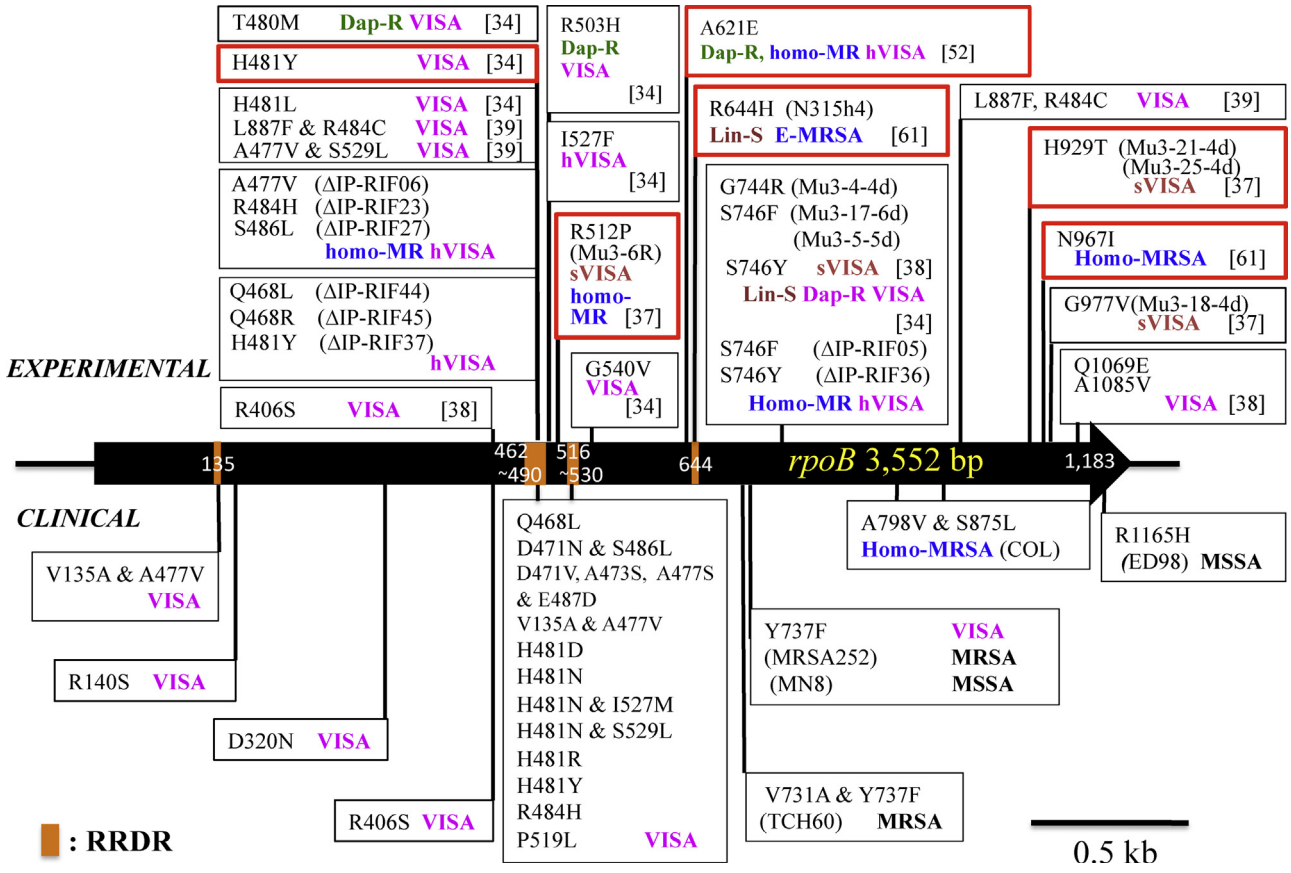

Fig. 5. *rpoB* mutation and antibiotic resistance phenotype. Mutations found in laboratory-derived *Staphylococcus aureus* strains are given above the linear scheme of the *rpoB* gene; those of clinical strains are given under the scheme. Mutations whose direct contribution to the resistance phenotype is genetically proven are in red boxes. Names of the vancomycin-intermediate *S. aureus* (VISA) strains carrying the mutations are either in Table 1 or in the cited references. References for the strains are given in brackets. Otherwise, the names of the strains are given in parentheses. Series of strains ΔIP-RIF are ΔIP-derived in vitro strains selected with 0.125 mg/L rifampicin (Katayama Y, unpublished data). Dap-R, daptomycin-resistant; Lin-S, linezolid-susceptible; homo-MR, homogeneously methicillin-resistant; hVISA, hetero-VISA; sVISA, 'slow VISA'; MSSA, methicillin-susceptible *S. aureus*; RRDR, rifampicin resistance-determining region. E-MRSA (Eagle-type MRSA) is a class of homo-MRSA characteristically resistant to high concentrations of methicillin but susceptible to lower concentrations of methicillin [59,61]. All of the sVISA strains listed are derived from Mu3 by selection with 6 mg/L vancomycin.

6. 'Slow VISA' (sVISA) as a new category of vancomycin resistance phenotype

In PA, hVISA produces VISA, which is observed as a colony formed on the vancomycin-containing agar plates within the incubation time of 48 h [10]. However, we noticed formation of new colonies on the plates left beyond 2 days of incubation at 37 °C (Fig. 1). Almost equal or an even greater number of colonies appeared from the third day (72 h) to the sixth day (144 h) of incubation. On drug-free agar plates they formed pinpoint colonies. However, they rapidly generated small or large colonies during drug-free propagation (Fig. 6). The strains established from pinpoint colonies exhibited degrees of vancomycin resistance equal to or greater than that of extant VISA strains (Table 3). Since the strains grew extremely slowly, we designated them 'slow VISA' (sVISA). The representative strain Mu3-6R-P was further studied and was compared with VISA strain Mu50. Mu3-6R-P had a doubling time (DT) of 62.2 min, which was extremely prolonged compared with 37.1 min for VISA strain Mu50. Otherwise, Mu3-6R-P had the features of a VISA phenotype, i.e. thickened cell wall and reduced autolytic activity (Table 3). The great difference of Mu3-6R-P with extant VISA strains was an extremely prolonged DT and instability of the VISA phenotype and colony morphology. It generated large colonies at a frequency of 3×10^{-7} during overnight drug-free cultivation. The large colonies were found to have returned to hVISA, grew fast, and overgrew the culture by the sixth day of serial daily passage: >99.9% of the cell population formed large colonies. All 28 strains established from the Mu3-derived colonies that appeared after 72 h incubation on agar plates with 6 mg/L vancomycin shared the sVISA features, i.e. prolonged DT, high vancomycin resistance (MIC ≥ 6 mg/L) and unstable expression of the phenotype (Table 3).

6.1. Biological and clinical significance of sVISA

The emergence of sVISA strains appears to have a special biological meaning. Since they can resist greater concentrations of vancomycin than extant VISA, they would serve as temporary shelters for hVISA to survive intensive vancomycin therapy. When vancomycin therapy is over, sVISA can revert to hVISA and may cause recurrence of infection.

The immediate consequence of this phenomenon would be the rare visibility of VISA in the clinical laboratory. Extant VISA with a stable phenotype may be identified in patient's samples after or even before the treatment of infections, but sVISA would not be readily seen on the vancomycin agar plates before 2 days of incubation. However, careful examination of the agar plates for pinpoint colonies after 3 days of incubation would help find sVISA. Even stored culture of VISA strains may contain the original sVISA strain as a minor subpopulation. In fact, we identified a few pinpoint colonies on the drug-free agar plates streaked with the glycerol stocks of 3 of the 33 VISA clinical strains listed in Table 1.

Table 3
Comparison of four categories of methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Category of MRSA	Representative strain	DT (in minutes) (range)[a]	VAN MIC (mg/L)[b] (range[a])	Phenotypic reversion after days of drug-free passage[c] (range[a])	Genetic events underlying the phenotype[d]	Cell wall thickness (nm)	Autolysis (% decrease of OD)[e]
sVISA	Mu3-6R-P	62.2 (43.3–133.3)	12 (8–24)	1 day (1–6)	*vraS*(I5N), *msrR*(E146K), *rpoB*(R512P)	26.1 ± 2.6	11
VISA	Mu50	37.1 (27.0–53.8)	12 (4–16)	30 days (20–84)	*vraS*(I5N), *msrR*(E146K), *graR*(N197S), *rpoB*(H481Y)	32.7 ± 3.0	9
hVISA	Mu3	36.1	3	>70 days	*vraS*(I5N), *msrR*(E146K)	21.5 ± 2.4	68
VSSA	N315ΔIP	26.7	1	N/A	None	16.4 ± 2.9	91

VISA, vancomycin-intermediate *S. aureus*; sVISA, 'slow VISA'; hVISA, hetero-VISA; DT, doubling time; VAN, vancomycin; MIC, minimum inhibitory concentration; N/A, not applicable.
[a] Ranges of 26 sVISA laboratory strains and 16 VISA clinical strains.
[b] E-test using brain–heart infusion agar evaluated after 72 h of incubation.
[c] Different methods were used to evaluate stability of the resistance phenotype. For sVISA, the day of appearance of large colonies among the 10^7 CFU of culture was determined. For VISA strains, the date of passage was determined when the vancomycin MIC came down to ≤2 mg/L. Mu3 stably maintained an MIC of 2 mg/L even after 70 days of propagation.
[d] Mutations of the representative strain are given.
[e] Triton X-100-induced autolysis of the representative strain. Percent decrease in the optical density (OD) at 660 nm after 3 h of incubation.

The slow-growing strains established from these pinpoint colonies expressed high and unstable vancomycin resistance as well as a colony morphology similar to those observed with Mu3-6R-P (Table 3). All of the strains lost the VISA phenotype and returned to the large colony morphology within a week's passage. This indicated that generation of sVISA is not infrequent. They are generated during vancomycin therapy but escaped recognition since they easily reverted to hVISA or became stabilised as VISA during propagation in the clinical laboratory. Generation of sVISA during vancomycin therapy may well explain the discrepancy between the frequency of vancomycin therapeutic failure and the frequency of VISA isolation from patient samples.

6.2. Genetic mechanism of sVISA formation

Whole-genome sequencing of Mu3-6R-P revealed only one single nucleotide polymorphism relative to the Mu3 genome. The mutation was identified in the *rpoB* gene, changing the arginine-512 to proline. Sequence determination of the *rpoB* gene identified non-synonymous *rpoB* mutations in 7 (21%) of 28 sVISA strains; they were *rpoB*(G744R), *rpoB*(S746F) (2 strains), *rpoB*(H929T) (2 strains) and *rpoB*(G977V). Unlike the *rpoB* mutations in extant VISA strains, these mutations were all identified outside of the RRDR and, except for *rpoB*(R512P), were located in the C-terminus half of the RpoB protein (Fig. 5).

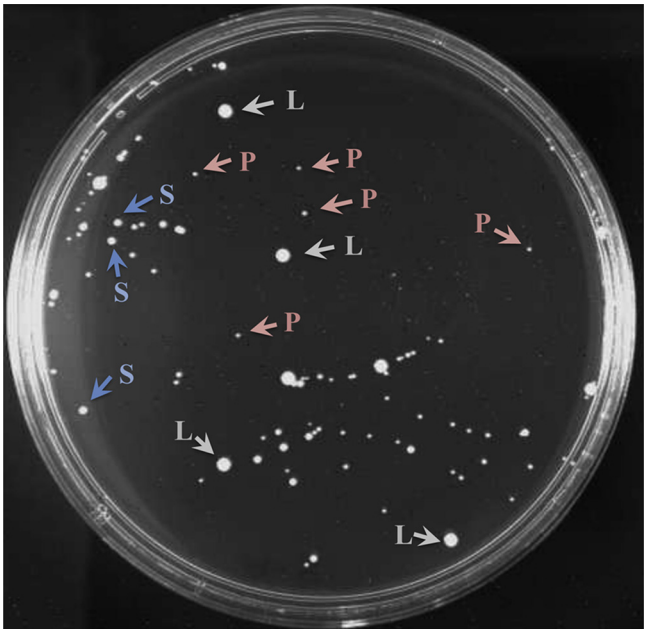

Fig. 6. Instability of 'slow VISA' (sVISA) strain Mu3-6R-P. Heterogeneous colony sizes observed in Mu3-6R-P. The strain with pinpoint colony morphology was propagated for 2 days in drug-free medium. The culture was then spread on a drug-free brain–heart infusion agar plate and incubated for 30 h. P, pinpoint colony; S, small colony; L, large colony.

Three Mu3-derived sVISA strains Mu3-6R-P, 17–6 d and 21–4 d carrying *rpoB*(R512P), *rpoB*(S746F) and *rpoB*(H929T), respectively, were cultivated in drug-free medium, and large-colony derivative strains were established to determine their *rpoB* gene sequences. The *rpoB*(H929T) mutation was back-mutated to wild-type and the *rpoB*(R512P) mutation was replaced by alternate mutations such as *rpoB*(R512L), *rpoB*(R512H) or *rpoB*(R512S). Only the *rpoB*(S746F) mutation was not changed in three independently isolated large-colony strains. All of the large-colony strains reverted to hVISA phenotype with comparable levels of vancomycin resistance and DTs to that of Mu3. Therefore, it is likely that certain *rpoB* mutation does serve as an on-and-off switch for the sVISA phenotype.

A single mutation in the *rpoB* gene can make the cell survive otherwise growth-inhibitory concentration of vancomycin. After the vancomycin selective pressure is lifted, the bacteria can start to diverge themselves beyond the constraints imposed by the *rpoB* regulatory mutation. Some descendants can keep on maintaining the VISA phenotype while slightly improving the severely depressed growth rate by compensatory mutation. Others would compromise a part of or the entire resistance phenotype for a much faster growth rate through back-mutation of the affected nucleotide, alternate mutation within the affected codon, or complementary mutation of the affected gene. In this way, *rpoB* mutation may serve as a remarkable strategy for bacterial evolution to better adapt to a changing environment.

7. Conclusion

In 2002, the first clinical VRSA strain was isolated in Michigan, USA [62]. This news filled people (including the authors [63]) with apprehension that it might rapidly disseminate in hospitals all over the world. However, so far only small numbers of VRSA isolation have been reported. By reviewing the biological properties and resistance mechanism of VISA and sVISA, the authors understand why *S. aureus* does not need to acquire *van* genes from enterococci by horizontal gene transfer. *S. aureus* appears to have already conquered vancomycin as a threat to terminate its existence, just as it dismissed β-lactam antibiotics half a century ago by developing MRSA [64]. By its ingenious genetic strategies,

S. aureus will continue to remain in human flora as our tenacious pathogen.

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Competing interests

None declared.

Ethical approval

Not required.

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