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Review article

Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy

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ABSTRACT

Staphylococcus (S.) aureus silently stays as our natural flora, and yet sometimes threatens our life as a tenacious pathogen. In addition to its ability to outwit our immune system, its multi-drug resistance phenotype makes it one of the most intractable pathogenic bacteria in the history of antibiotic chemotherapy. It conquered practically all the antibiotics that have been developed since 1940s. In 1961, the first MRSA was found among *S. aureus* clinical isolates. Then MRSA prevailed throughout the world as a multi-resistant hospital pathogen. In 1997, MRSA strain Mu50 with reduced susceptibility to vancomycin was isolated. Vancomycin-intermediate *S. aureus* (VISA), so named according to the CLSI criteria, was the product of adaptive mutation of *S. aureus* against vancomycin that had long been the last resort to MRSA infection. Here, we describe the genetic basis for the remarkable ability of *S. aureus* to acquire multi-antibiotic resistance, and propose a novel paradigm for future chemotherapy against the multi-resistant pathogens.

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1. Introduction

Among all the antibiotic resistance achieved by *Staphylococcus aureus*, two most remarkable ones are methicillin and vancomycin resistance. The methicillin resistance was achieved by interspecies transfer of *mecA* gene from an ancestral *Staphylococcus* species to *S. aureus* mediated by a unique staphylococcal mobile genetic element. Vancomycin resistance was achieved by horizontal transfer of a plasmid-born *vanA*-gene transposon from vancomycin-resistant *Enteriococcus* to *S. aureus* across the genus barrier. The other type of vancomycin resistance is expressed by VISA, which is acquired by adaptive mutations incorporated in the genes encoding regulation of bacterial cell physiology. We shall describe below the genetic strategies underlying the organism's admirable adaptability to antimicrobial pressures, and propose the development of 'reverse antibiotics (RA)' as a new paradigm for drug discovery development in the future chemotherapy against the threat of multi-resistant *S. aureus* infection.

2. Great genetic competence of *S. aureus* to acquire antibiotic resistance

2.1. Methicillin resistance in S. aureus

Practically all *S. aureus* isolates were methicillin susceptible until 1961, when Jevons found three MRSA strains among 5440 clinical *S. aureus* strains in England [61]. Then the situation changed as humans started to use methicillin. MRSA became prevalent all over the world, and after five decades, more than half of *S. aureus* clinical strains became methicillin resistant. MRSA is born when methicillin-susceptible *S. aureus* (MSSA) has acquired the methicillin-resistance gene *mecA* by horizontal gene transfer mediated by a mobile genetic element staphylococcal cassette chromosome (SCC) [2]. SCC is a site-specific transposon-like element exclusively used among staphylococcal species [3]. The SCC elements carrying *mecA*, designated SCC*mec*, are integrated in the chromosomes of MRSA strains [2,4]. Fig. 1 illustrates the basic structure of SCC*mec* [5]. The element is composed of *mec*-gene complex encoding methicillin resistance gene *mecA*, and its regulator genes (*mecR1* and *mecI*) and *ccr*-gene complex encoding cassette chromosome recombinase (CCR) that mediates the

Abbreviation: MRSA, methicillin-resistant *Staphylococcus (S.) aureus*; PBP, penicillin-binding protein; VISA, vancomycin-intermediate *S. aureus*; hVISA, heterogeneously vancomycin-intermediate *S. aureus*; sVISA, slow VISA; VRSA, vancomycin-resistant *S. aureus*; SCC*mec*, staphylococcal cassette chromosome *mec*; TCRS, two-component regulatory systems; RA, reverse antibiotic; RNAP, RNA polymerase.

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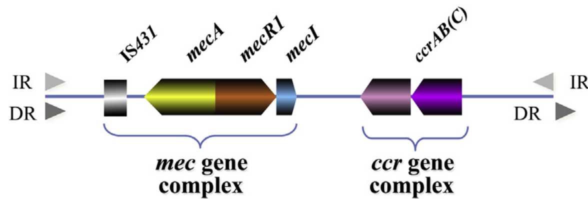

Fig. 1. The structure of SCC*mec*. SCC*mec* is composed of two essential gene complexes. One is *mec*-gene complex, encoding methicillin resistance (*mecA* gene) and its regulators (*mecI* and *mecR1*), and the other is *ccr*-gene complex that encodes the movement, (integration to and precise excision form the chromosome), of the entire SCC element. Abbreviations: IR, inverted repeat; DR, direct repeat.

element's integration into, as well as its precise excision from, the staphylococcal chromosome [3]. There are many structurally distinguishable types and subtypes in SCC*mec*. Detailed description is available elsewhere [5].

1) *oriC* environ as the storage system for useful exogenous genes

SCC is a vehicle for staphylococcal species to exchange genes that are useful for their adaptation to the niches with adverse environmental condition including antibiotic pressure. In the *S. aureus* chromosomal region downstream of the origin of replication (*oriC*), a gene named *orfX* is present. The gene is reported to encode a ribosomal RNA methyltransferase [6]. The *orfX* contains a copy of the direct repeat sequences (DR) that bracket an SCC element (Fig. 1), thus it serves as the unique integration site for SCC elements. Moreover, after the first SCC element is integrated, the second SCC can be integrated at the DR sequence present in the distal side of the first SCC element. In this way, multiple elements can be integrated in tandem forming a cluster of foreign genes downstream of *orfX*. As a result, unique chromosomal region called '*oriC* environ' is formed [5,7].

The *oriC* environ is the most diverged region among *Staphylococcus* chromosomes in terms of its length, GC content, and function of the acquired genes and their integrity. Many transposons and insertion sequences (IS) are found in the *oriC* environ, and they frequently cause deletion, recombination and even a large chromosome inversion across *oriC* [7]. In this way staphylococci can maintain only the genes needed for the survival in the on-going environmental change. Evidently, *mecA* has been the most useful gene ever since the clinical introduction of methicillin in 1960, when a few *S. aureus* strains already seem to have acquired *mecA* [1].

Various functional genes of diverse metabolic pathways are found carried by SCC in the staphylococcal *oriC* environ. Some examples are; *pbp4*, encoding penicillin-binding protein 4 (PBP4) in the cell-wall synthesis pathway [8], arginine catabolic pathway genes (ACME) [9], and *hdc* encoding histidine decarboxylase [10]. However, the genes much more frequently found in the *oriC* environ are drug-resistance genes. Besides *mecA*, such drug-resistance genes against mercury, cadmium, kanamycin, bleomycin, erythromycin, spectinomycin, and fusidic acid have been found in association with SCC elements in *oriC* environ [4,11]. Evidently, the *oriC* environ serves as the storehouse in support for achieving the multi-drug-resistance phenotype. *S. aureus* quickly acquired β-lactamase plasmids soon after the penicillin G was introduced in 1940s, but no plasmid carrying *mecA* has been found. Although the reason is not clear, SCC-mediated acquisition of a single copy of *mecA* gene on the chromosome might have been less effective against penicillin-G as compared to the plasmid-born multiple copies of beta-lactamase encoding *blaZ* genes. On the other hand, *mecA* encodes cell-wall synthesis enzyme PBP2' [12]. PBP2' is a homolog of intrinsic *S. aureus* PBPs and considered to have inefficient transpeptidase activity [13,14]. As such, overproduction of PBP2' may cause turbulence in the cell-wall synthesis and a big fitness cost especially during the growth in the absence of β-lactam antibiotics. Storage of *mecA* as a single gene copy in *oriC* environ and multiple gene doses of *blaI* on the penicillinase plasmid would be the best way to maintain *mecA* in the repressed status in the drug-free growth condition. (Here, note that *blaI* gene is the cognate repressor gene of *blaZ*. The BlaI also cross-represses *mecA* gene because the cognate *mecA*-gene repressor gene *mecI* is usually deleted or inactivated by mutations [15].) Apparently, *oriC* environ is suitable for the storage of foreign genes in single copies that may have a hazardous effect on the cell physiology if overexpressed.

2) The origin of *mecA* gene

We previously identified a *mecA*-gene homolog *mecB* on the plasmids and chromosomes of *Macrococcus caseolyticus* isolates [16,17]. Macrococcal species, disseminated in nature as animal commensals, are immediate antecedents of staphylococcal species (Fig. 2) [17]. The macrococcal *mecB* was distantly related to *mecA* (61.7% nucleotide identity), and was found disseminated among the macrococcal strains as a transposon, designated Tn*6045* [16]. No complete form of SCC*mec* was found in macrococcal strains. However, many *ccr* genes are found on the plasmids and chromosomes of the macrococci, and tandem integration of an SCC element and a *mecB* transposon was observed in the *oriC* environ of a macrococcal strain [16]. Spontaneous excision of an SCC and the *mecB* transposon as a closed circle DNA from the *oriC* environ was observed, suggesting *de novo* synthesis of SCC*mec* is on-going in macrococcal species [16].

Recently, the third *mecA* gene homolog *mecC*, which exhibits 68.7% nucleotide identity with *mecA*, was found in *S. aureus* isolates from cattle and a human by using next generation sequencing technology [18]. The SCCs carrying *mecC* were also found in *Staphylococcus sciuri* [19], and *Staphylococcus xylosus* [20]. Previously, *mecA* was the exclusive genetic marker for MRSA. Now, however, we have to worry about overlooking *mecB* or *mecC*-carrying MRSA in the clinical laboratory. According to recent reports, prevalence of *mecC*-mediated methicillin resistance ranges from 0 to 2.8% among human MRSA isolates [21–25]. There is no report yet of *mecB*-carrying *S. aureus*.

Phylogenetic distribution of the *mecA* homologs illustrated in Fig. 2 suggests that *mecA* had been vertically transmitted as an ortholog for some time during the course of speciation of *sciuri*-group staphylococcal species such as *Staphylococcus fleurettii, Staphylococcus vitulinus, S. sciuri* subspecies *sciuri*, and *Staphylococcus carnaticus*. As the vertically transmitted ortholog, *mecA, mecA1,* and *mecA2* are located at the corresponding loci on the chromosomes of the *sciuri*-group species; *S. fleurettii*, *S. sciuri*, and *S. vitulinus*, respectively. They have 99.8%, 80%, and 91% nucleotide identities, respectively, to the *mecA* gene carried by SCC*mec* on the MRSA chromosome [26]. Thus, apparently, *S. fleurettii mecA* was the original *mecA,* which was adopted as the methicillin-resistance determinant of the SCC*mec* that converted *S. aureus* into MRSA. The comparative structural analysis of the *mecA* loci on the chromosomes of *sciuri*-group species corroborated this historical event [26]. Curiously, however, the *mecA* locus was not preserved intact in certain strains of *sciuri* group. Some of them possessed SCC*mec* elements carrying either *mecA* or *mecC* in the *oriC* environ instead of the functional *mecA* ortholog (Fig. 2) [27]. They seem to have had lost methicillin resistance by either deletion or mutations incorporated in the coding region or promoter sequence of the original *mecA* gene [28].

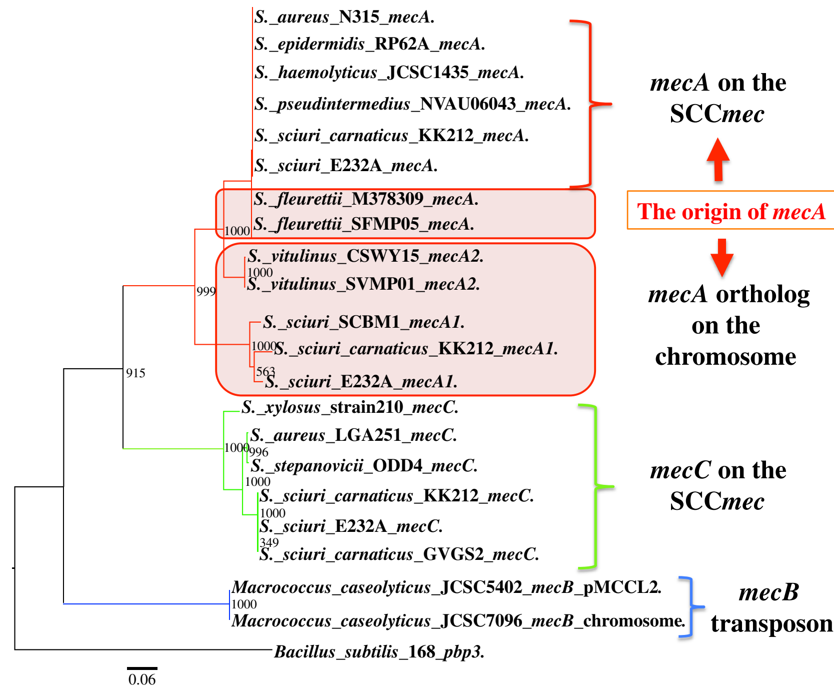

Fig. 2. Phylogenetic tree of *mecA* gene homologs. Nucleotide sequences were obtained from the NCBI database and our ongoing project. The multiple codon alignment was performed using pal2nal v14 from the corresponding aligned protein sequences by MAFFT v7.037. Maximum likelihood tree was built with MEGA5 using a GTR + Gamma model. The numbers at branches are bootstrap values indicating the confidence levels. The scale bar indicates an expected value of base substitution per site.

So far, the original source of methicillin-resistance gene has been identified only for *mecA* gene. In view of the distribution of *mecC* and *mecB* genes (Fig. 2), however, it seems likely that they were derived from the bacteria of the taxonomic positions between contemporary genera *Staphylococcus* and *Macrococcus*, although it is not clear if the bacterial species are still existent or already extinct.

3) Co-evolution of staphylococci and mammals and loss of *mecA*

Some staphylococcal species exhibit evident host-specific colonization. For example, *Staphylococcus epidermidis* is a member of human microflora, and *Staphylococcus pseudintermedius* is isolated specifically from canine hosts [29,30]. In our ongoing ecological study of staphylococci in mammalian species, we found a co-evolutionary relationship between *Staphylococcus delphini*-group (*Staphylococcus hyicus*, *Staphylococcus chromogenes*, *Staphylococcus felis*, *Staphylococcus lutrae*, *Staphylococcus schleiferi*, *S. delphini*, *Staphylococcus intermedius* and *S. pseudintermedius*) and Laurasiatherian hosts after diverging from Chiropter (bats). Based on this observation, the appearance of genus *Staphylococcus* was estimated to be about 250 million years ago by molecular clock method using genome-wide datasets (Fig. 3). Then, the staphylococcal species seem to have started to colonize and co-evolve with mammals that emerged almost simultaneously about 225 million years ago (Fig. 3). It is probable that the antecedents of staphylococci, e.g. macrococcal species and old staphylococcal species of *S. sciuri*-group required the benefit of *mecA or mecC* genes to protect themselves from β-lactam-producing environmental microorganisms before their descendants successfully adapted to mammalian hosts. The descendant staphylococcal species, after successful adaptation as mammalian microbial flora, lost *mecA or mecC* gene, because they became protected from the assault of β-lactam-producing microorganisms thanks to the host's immune system. The situation changed, however, in the 1940s, when humans started to use penicillin G, threatening the colonizing staphylococci. They first acquired penicillinase plasmid. Then, since the introduction of methicillin in 1960, *S. aureus* had to regain *mecA* gene from *S. fleurettii* via the SCC*mec*.

2.2. Vancomycin resistance in S. aureus

1) hVISA, and VISA

Some important antibiotic resistance phenotypes of MRSA are acquired by spontaneous mutations. Rifampin resistance and fluoroquinolone resistance are the most well known examples. Moreover, vancomycin resistance, which has cast a dark shadow on anti-MRSA chemotherapy in the last two decades, is also acquired by mutation. Vancomycin has long been regarded as the last resort for MRSA infection. In 1997, however, the first VISA strain Mu50 was isolated from the surgical wound of a Japanese infant whose infection did not respond favorably to long-term vancomycin therapy [31,32]. The vancomycin MIC of Mu50 was 8 mg/L [31].

Now VISA is defined as *S. aureus* strain having vancomycin MIC of 4 or 8 mg/L. Note that MIC $\leq$ 2 mg/L is defined as susceptible. However, among the susceptible clinical strains, there are precursor strains for VISA. From the precursor strains, one-step selection with vancomycin generates VISA at a frequency of 10^{-6} or above [52]. MIC determination cannot detect such precursor strains. Using 1000 times or more number of cells (or colony forming unit; CFU) of a bacterial strain than used for MIC method (about 10^{4-5} CFU for the test) we can discriminate the precursor strains from really vancomycin-susceptible *S. aureus* (VSSA). This sensitive method is called analysis of resistant subpopulation (population analysis (PA)), and is an essential tool for the study of vancomycin and methicillin resistance [72].

Fig. 4 illustrates the PA patterns of the MRSA strains with different types of vancomycin susceptibility. A fixed number of cells were inoculated on each of the agar plates containing various

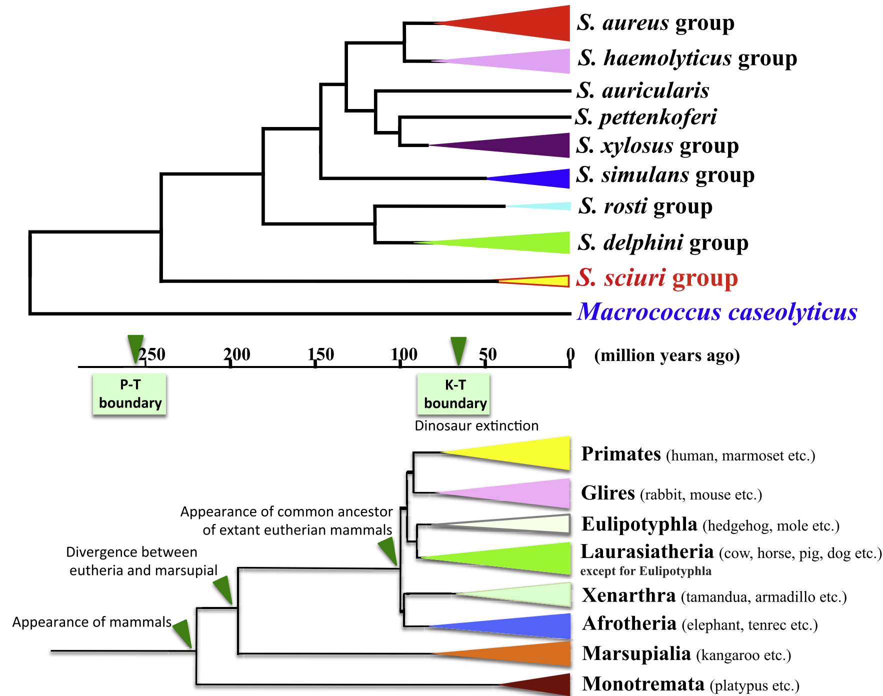

Fig. 3. Comparison of phylogenetic time trees of staphylococci and mammalians. Each cluster was compressed as a colored triangle, whose thickness is proportional to the number of species. Tree topology was constructed by maximum likelihood method using RAxML. Time estimation was performed by bayesian inference using MCMCTREE program included in PAML 4.7 package. Amino acid sequences of 31 staphylococcal core genes were used for the construction of staphylococcal tree. Mammalian time tree was originated from a report by Meredith RW et al. [62].

concentrations of vancomycin (abscissa). The plates were incubated at 37 °C for 48 h. Then the number of grown colonies were counted and plotted on the semi-logarithmic graph. Note that the precursor strain Mu3 with MIC 2 mg/L is distinct from VSSA strain ΔIP (MIC 1 mg/L). Whereas the growth of ΔIP is completely depressed by 2 mg/L of vancomycin, the minor proportions of cells of Mu3 grew up to 12 mg/L of vancomycin though the 99.999% of the entire cell population is depressed with 3 mg/L of vancomycin. This clearly showed that Mu3 is composed of heterogeneous cell subpopulations with different levels of vancomycin resistance. Within the subpopulations grown on the agar plates containing 4 mg/L or greater concentrations of vancomycin, we identified VISA

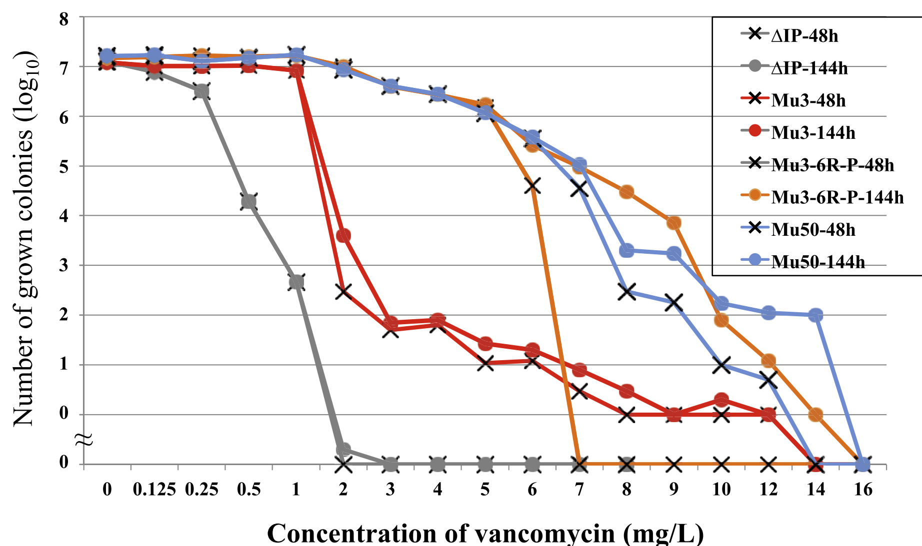

Fig. 4. Population analysis of VSSA, hVISA, VISA and sVISA. The population analysis (PA) was performed with BHI agar plates containing varied concentrations of vancomycin [72] The grown colonies were counted and plotted on the graph after 48 h, (which is the regular practice in PA method), and 144 h of incubation at 37 °C. It is noticed that hVISA strain Mu3 (red lines) generates new colonies on the vancomycin agar plates after 72 h incubation up to 144 h, which was in contrast to the case for VSSA strain N315ΔIP (ΔIP) (dark green line). These late-appearaing colonies of Mu3 contained sVISA strains. Mu3-6R-P (orange lines) was one of them. Its PA curve is drastically changed when analyzed at 48 h and 144 h. In contrast, the two PA curves of extant VISA strain Mu50 (light blue lines) were not that different each other. Symbols: cross, number of colonies determined after 48 h incubation; circle, that determined after 144 h incubation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

converted strains. To distinguish Mu3 from VSSA, we classified it as a heterogeneously vancomycin-resistant *S. aureus* (hVRSA; now is called hVISA) [52].

VISA is generated by accumulation of several spontaneous mutations [33,34]. The VISA phenotype of Mu50, for instance, can be reconstituted in VSSA strain ΔIP by sequentially introducing four mutations in the genes *vraS*, *msrR*, *rpoB* and *graR* (Katayama, Y. in preparation). The first couple of two mutations in *vraS* and *msrR* converted ΔIP into a hVISA strain with a similar PA pattern with that of Mu3, then *rpoB* mutation converted the hVISA into VISA with vancomycin MIC 4 mg/L. Addition of *graR* mutation further increased vancomycin MIC to 8 mg/L.

vraS is the sensor histidine kinase of two-component regulatory systems (TCRS), which is known to up-regulate the genes in the cell-wall synthesis pathway in response to the exposure to cell-wall-acting antibiotics [35,36]. *graR* is a response regulator of another TCRS which is involved in resistance to cationic antimicrobial peptides (CAMP) [37–39]. *msrR* is considered to be involved in the production of wall-teichoic acid (WTA) [40,41]. The RNA polymerase (RNAP) core enzyme is composed of five subunits as represented by $\alpha_2\beta\beta'\omega$. Remarkably, as many as 64% of VISA clinical strains possessed more than one mutation in *rpoB* gene encoding the β subunit of the RNAP core enzyme [42].

When introduced individually into vancomycin-susceptible *S. aureus* strain ΔIP, the above four mutations either increased vancomycin MIC slightly, (i.e, within the susceptible range), or changed the susceptible patterns of PA curves to those of hVISA. Besides the four genes described above, great number of different mutations and their combinations were found to raise vancomycin resistance of *S. aureus*. A single mutation incorporated in any of the 20 genes in diverse metabolic pathways was found to raise vancomycin resistance [33]. They are such genes as *tarO* and *tarA,* in the WTA synthesis pathway; *gtaB* in the lipoteichoic acid biosynthesis pathway; *walk* involved in the regulation of cell-wall synthesis and autolysis; *cmk* responsible for the synthesis of cytidylate diphosphate (CDP); *pykA*, involved in both glycolysis and purine synthesis pathways; *rpsU*, encoding small subunit ribosomal protein S21; *trpC* in Tryptophan biosynthesis pathway, and *ureD* encoding urease accessory protein [33].

Alteration of such a diverse metabolic pathway genes seems to change the flow of nutrients and metabolites towards the enhanced production of cell-wall peptidoglycan (PG) and/or reduction in autolysis [42]. Besides supporting the cell to tolerate the cytokilling activity of vancomycin, reduced autolysis is considered to contribute to the maintenance of thick cell-wall PG layers by decreasing the rate of cell-wall turnover. In fact, considerable number of mutations affecting the above 20 genes are speculated to contribute to the enhanced cell-wall synthesis [33,42]. PG contains many D-alanyl-D-alanine residues to which vancomycin binds. Therefore, thickened PG layers trap more vancomycin molecules than the PG layers of normal thickness [43–45]. Moreover, the PG mesh structure is clogged by the entrapped vancomycin, and serves as an obstacle for further penetration of vancomycin to the cytoplasmic membrane where the real targets of vancomycin exist [46].

2) VRSA: cross-genus transmission of resistance gene.

Vancomycin MIC of VISA is 4–8 mg/L, which 'was not' considered resistant according to the CLSI criteria of the time. Therefore, the word VISA was coined for Mu50 indicating its 'intermediate' level of vancomycin susceptibility. Five years later, in 2002, a VRSA clinical strain with MIC $\geq$ 16 mg/L was isolated [47]. It turned out to have acquired a *vanA*-transposon from vancomycin-resistant *Enterococcus* (VRE). The transposon carried *vanA*-gene complex containing *vanA, vanH, vanX,* and *vanY.* If the four genes function in concert, all the D-Alanyl-D-Alanine residues of the substrate for PG synthesis are replaced by D-Alanyl-D-lactate to which vancomycin cannot bind. This amazing mechanism of resistance is described elsewhere in detail [48]. In spite of the acquisition of this ingenious system, however, so far only a dozen of VRSA clinical strains have been reported in the world after more than a decade of its first isolation. The fitness cost of the carriage of *vanA* plasmid was suspected although growth retardation of the *vanA* plasmid-carrying strain is reported to be minimum [49]. In fact, the *vanA*-mediated vancomycin resistance is an inducible type, and does not cause much fitness cost during the growth in the absence of vancomycin [70]. As an explanation for the unpopularity of the resistance, we initially speculated that the level of methicillin resistance might be much lowered due to the loss of D-Alanyl-D-Alanine residues from the cell wall to which PBP2' is supposed to bind. However, we found that a VRSA clinical strain VRS1 simultaneously expressed high-level resistance to both vancomycin and oxacillin [70]. Therefore, the only remaining barrier for MRSA to acquire *vanA* gene would be the availability of an efficient mobile genetic element carrying it. In this regard, a worrisome report on a transmissible *vanA* plasmid has been published [71]. Future prevalence of VRSA is not an illusion as long as we continue using vancomycin as the first choice for MRSA infection. We have to develop new chemotherapeutic agents against multi-resistant MRSA to prepare for the future.

3) 'sVISA' – an ingenious strategy to survive vancomycin chemotherapy

Vancomycin is still the first-line antibiotic against MRSA infection. However, its clinical effectiveness is compromised even against the strains whose vancomycin MICs are within the CLSI susceptible range ($\leq$2 mg/L) [50,51]. Also, the overall therapeutic failure rates of vancomycin are too high to be explained by the latent infection of VRSA (with vancomycin MIC of $\geq$16 mg/L) or even of VISA (MIC $\geq$ 4 mg/L) [50,67–69]. It seems that many MRSA strains exist whose vancomycin MIC values are in susceptible range ($\leq$2 mg/L), and yet 'resisting' vancomycin killing. hVISA is evidently one of those strains resisting vancomycin by generating VISA at high frequency. However, in this case, hVISA is converted to VISA during the therapy, and the therapeutic failure is ascribed to the VISA strain. In this case, VISA would be detected from clinical specimen after vancomycin therapy. Using hVISA strain Mu3, however, we noticed a transient VISA status designated 'slow VISA (sVISA)' which returns to hVISA quickly once vancomycin is removed from the culture [66]. This implies that hVISA infection may not leave VISA after unsuccessful vancomycin therapy. Only hVISA with susceptible levels of vancomycin MIC values would be present after vancomycin therapy.

Fig. 4 illustrates the PA pattern of hVISA strain Mu3 evaluated after 2 days (Mu3-48 h) and 6 days (Mu3-144 h) of incubation at 37 °C. The usual PA test is evaluated after 2 days. However, when PA was evaluated after 72 h (3 days) to 144 h (6 days) of incubation, additional number of Mu3 colonies appeared on the BHI agar plates containing 4 mg/L or greater concentrations of vancomycin (Fig. 2). In contrast VSSA strain ΔIP did not generate additional colonies after 48 h (Fig. 4). The number of the late-appearing colonies was comparable to the number of the colonies that had appeared within 48 h of incubation. VISA is included within the latter group of colonies, and sVISA was identified within the late-appearing colonies. The first sVISA strain Mu3-6R-P (6R-P) was obtained *in vitro* from hVISA strain Mu3 by the selection with 6 mg/L of vancomycin [52].

6R-P grew extremely slowly, and did not draw our attention until recently. Then its high level of vancomycin resistance was

noticed (MIC = 16 mg/L, with E-test evaluated after 72 h incubation [66].) The strain 6R-P had a VISA phenotype similar to the extant VISA strains; i.e., thickened cell wall and reduced autolytic activity. However, it was distinct from extant VISA strains in its extremely prolonged doubling time (DT) of 62.2 min, forming a pin-point colony (PC) only after two days incubation on a drug-free agar plate. The PA pattern of 6R-P is shown in Fig. 4. In contrast to Mu50, 6R-P does not form colonies on the agar plates containing 7 mg/L or greater concentrations of vancomycin within 48 h incubation, whereas, it does after 72 h–144 h of incubation (Fig. 4).

The most striking feature of 6R-P is the instability of VISA phenotype. When passaged on drug-free agar plates, it generated phenotypic revertants (PR) having larger colony sizes and significantly decreased vancomycin resistance. When 6R-P was passaged in drug-free medium, the culture was quickly overgrown by PR cells within several days. The appearance rate of PRs from 6R-P was around 1×10^{-6} and was comparable to that of the emergence rate of VISA from hVISA [52].

A total of 25 sVISA strains were obtained from Mu3 by selection with 6 mg/L of vancomycin [66]. The colonies that appeared on the vancmycin plates after 72 h (3 days) to 144 h (6 days) incubation at 37 °C were picked, colony-purified, and established as sVISA strains. Their vancomycin MICs increased with time of incubation, while that of clinical VISA strains, represented by Mu50, did not [66]. Some sVISA strains reached to the MIC values of 24 mg/L to 32 mg/L after 48–96 h incubation, whereas Mu50 remained at MIC of 12 mg/L throughout the incubation time up to 144 h [66]. This high MIC values of sVISA strains, however, were very unstable, and PRs with large colony size, and decreased vancomycin resistance appeared quickly in the drug-free culture. Some sVISA strains are much more unstable than 6R-P, and generated large colonies with reduced vancomycin resistance even within 72 h of incubation (Fig. 5).

The biological feature of sVISA is intriguing. The sVISA status is easily acquired by hVISA, and even by VSSA [66]. The sVISA phenotype is a transient phenotype, but it can be maintained stably as long as it is passaged on the vancomycin-containing agar plates. Thus, sVISA phenotype is likely to be maintained as long as vancomycin therapy continues. When vancomycin treatment is lifted, sVISA would quickly revert to hVISA without leaving the evidence of VISA infection. This transient nature of resistance of sVISA may explain at least a part of lower rate of VISA isolation than the occurrence rate of the vancomycin-refractory MRSA infection.

4) RNAP regulatory mutation is a frequent mechanism for VISA phenotype

RNAP mutation has been recognized as one of the major genetic events raising VISA [33]. It was the case for sVISA as well. The whole genome sequence determination of 6R-P revealed a single mutation in *rpoB* gene encoding β subunit of RNAP [66]. The identified mutation *rpoB*(R512P) was introduced into a VSSA laboratory strain ΔIP by an allelic replacement method [66]. The resultant strain ΔIP*rpoB*(R512P) possessed vancomycin MIC of 4 mg/L as compared to 1 mg/L of ΔIP. The slow growth phenotype of sVISA was also transferred to ΔIP, prolonging its DT from 26.7 to 41.2 min [66]. It was remarkable that an *rpoB* mutation as a single agent conferred VISA-level resistance (MIC, 4 mg/L) on even a VSSA strain. The daily passage of 6R-P generated PRs at high frequency, and the culture was 100% replaced by large colony-sized PRs by the 7th day of passages. The four large colonies were picked from independent experiments, and their *rpoB* genes were sequenced for the fate of *rpoB*(R512P) mutation. Three out of the four large-colony variant strains, 6R-P-L1, -L2, and -L3, possessed allelic nucleotide changes in the 512th codon, replacing the Proline of Mu3-6R-P by Leucine,

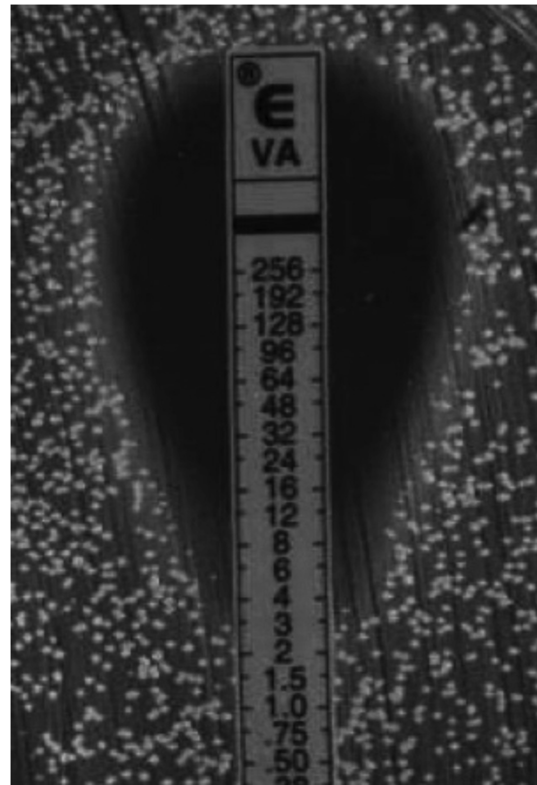

Fig. 5. Vancomycin E-test of a representative sVISA strain. Pin-point colonies of sVISA strain 12-5d were inoculated onto BHI agar plate, and E-test strip of vancomycin was placed followed by incubation for 72 h. Highly frequent appearance of large colonies was observed, which did not grow beyond the MIC level of 4 mg/L. On the other hand, pin-point colonies, whose individual colonies are too small to be discriminated, grew up to the level of MIC 16 mg/L.

Serine and Histidine, respectively. Another sVISA strain 21-4d carrying *rpoB*(H929T) mutation had its *rpoB* mutation back mutated to wild-type in three of the five PR strains tested. The sVISA strain 21-4d produced large-colony PRs at an extremely high frequency of 5.4×10^{-5} after two-days drug-free passages [66]. The mechanism for this high rate of mutations for phenotypic reversion is under investigation.

A total of 25 sVISA strains were tested for their carriage of *rpoB* mutations [66]. Seven (28%) strains possessed *rpoB* mutations. All of them were located out of the rifampin-resistance determining region (RRDR), and did not accompany rifampin resistance. In our current on-going study, some mutations of another RNAP subunit gene *rpoC*; i.e., *rpoC*(L418I) and *rpoC*(N744K) were found to confer sVISA phenotype on hVISA strain Mu3 (Katayama, Y. in preparation). Therefore, sVISA phenotype seems to be expressed via the alteration of the cell physiology brought about by the mutational change in the structure and function of RNAP core enzyme. Besides vancomycin, mutations in RNAP subunits are reported to affect susceptibility of *S. aureus* to such antibiotics as β-lactam [53,54], daptomycin [55–58], and linezolid [55]. Since RNAP is not the direct target of action of any of these antibiotics, RNAP mutation must be preventing the adverse effects of the antibiotics by changing the physiological status of the cell significantly. This should accompany high fitness cost for the cell, and is the cause for the transient nature of the sVISA phenotype.

Finally, there are more number of sVISA strains having no mutation in RNAP [66]. Whole genome sequencing of those sVISA strains are on-going to identify the non-*rpo* gene mutations to

obtain a comprehensive view on the genetic basis for sVISA phenotype.

3. Future chemotherapy for *S. aureus* infection

3.1. Nybomycin as a reverse antibiotic for quinolone resistance

S. aureus is a member of our natural flora. About 20–30% of humans have been reported to possess *S. aureus* in the anterior nares. No trend of decline of *S. aureus* carriage by healthy individuals is noticed after 7 decades of use of man-made antibiotics. This fact shows that *S. aureus* is so well tuned to human body and would never be cleared off from their habitat how energetically we develop new antibiotics with new targets of action. It is evident from the history of chemotherapy in the last century that *S. aureus* can develop resistance to any antibiotic. As seen in the old derivation of *mecA* in the history of life on the earth, antibiotic resistance is the natural consequence of the production of antibiotics. Based on this principle, we should design a new chemotherapeutic strategy. The bacteria of our time is drastically changed as compared to that of the 1940s, when more than half of the hospital-associated *S. aureus* is methicillin-resistant, and more than 80% VISA are quinolone-resistant [59]. Given this, it is much more promising to develop an antibiotic that has stronger activity against the *S. aureus* strains resistant to extant antibiotics rather than against wild-type *S. aureus* strains which are still susceptible to them. If such anti-resistance antibiotics were used in combination with the extant antibiotics, most of the *S. aureus* infections would become treatable.

By screening 1928 culture supernatants of Actinobacteria, we identified a curious substance that possessed a strong bactericidal activity against fluoroquinolone-resistant VISA strain Mu50, whereas only a weak activity against fluoroquinolon-, and methicillin-susceptible VSSA strain FDA209P [59]. The substance was found out to be an old antibiotic Nybomycin (NYB) that had been reported in 1955 [60]. We found that NYB strongly inhibited the function of the mutated DNA gyrase of quinolone-resistant Mu50, but did not inhibit the function of the wild-type DNA gyrase of quinolone-susceptible *S. aureus* [59]. Docking simulation study revealed stable binding of NYB to the quinolone-binding pocket of the GyrA having *gyrA*(S84L) mutation (Fig. 6). On the other hand, fluoroquinolone antibiotics cannot bind to it due to the mutational loss of the Serine residue, which is important to retain hydrogen-bond network for the stabilization of quinolone molecule in the quinolone-binding pocket (Fig. 6).

3.2. Dissolving the vicious cycle between antibiotics and antibiotic resistance

Bacteria always find the way to develop resistance to any antibiotic. As is expected, NYB was not exempt from the emergence of resistance, either. Mu50 did generate NYB-resistant mutants (temporarily defined by MIC $\geq$ 4 mg/L), although at extremely low frequencies: the appearance rates were $0.663–15.3 \times 10^{-11}$ [59]. However, surprisingly, all of the nine independently obtained resistant mutant strains were susceptible to fluoroquinolone antibiotics [59]. Nucleotide sequencing revealed that their *gyrA* genes of the resistant mutants were back mutated to the wild type. Therefore the resistant mutants were genetic revertants [59]. Accordingly, we designated NYB as a ‘Reverse Antibiotic’ (RA) against quinolone-resistant bacteria [59]. Recently, we found that some of the flavones as well are RAs against fluoroquinolone-resistant bacteria (Morimoto, Y. et al. in preparation). Flavones are known as natural antibiotics produced by plants [61]. NYB is also a

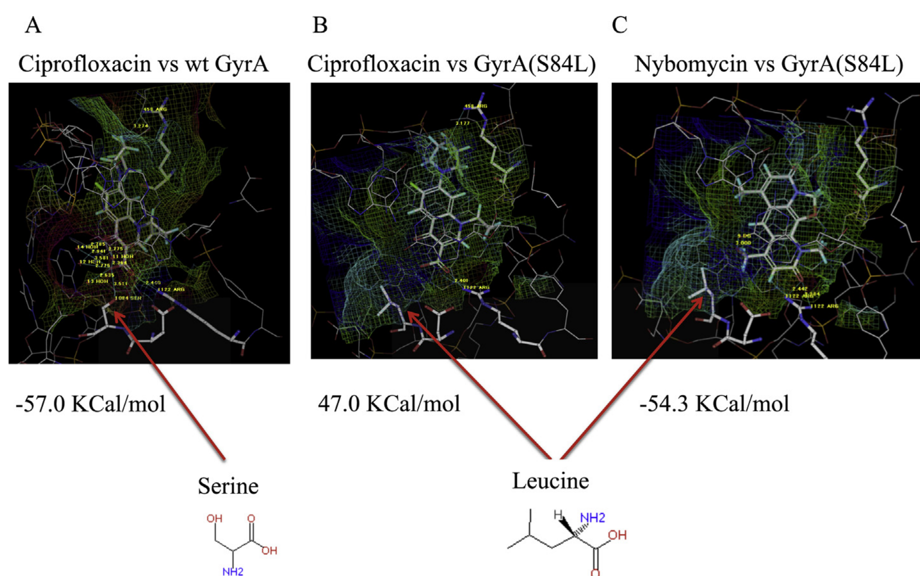

Fig. 6. Nybomycin binding to the Quinolone Pocket of the Gyrase A with its 84th Serine residues replaced by Leucine. The quinolone binding site model was constructed based on the crystal structure of GyrB27-A56 (Tyr123Phe) reported by Bax BD et al. [63]. To reproduce the cleaved state of DNA, the mutated Phenylalanine was corrected to Tyrosine and its hydroxy group was modified to make a phosphoester bond connected to the 5' terminal of DNA. The side chain conformation around the quinolone-binding site was optimized using Bluto [64], with ciprofloxacin kept in the cavity. The docking calculation was performed according to Adam [65], with a docking grid expanded by 1.3 angstrom to accept compounds other than ciprofloxacin. The values under the figures are calculated binding energy. (A) The binding between GyrA and quinolone is stabilized through the octahedral magnesium chelate and the hydrogen bond network surrounding it. The chelate is composed of the oxygen atoms of water, the carbonyl and carboxylic acid groups of the quinolone, and the side chains of Ser84 and Glu88 of DNA Gyrase subunit A. Abbreviation: wt, wild-type. (B) In the interaction between fluoroquinolone and altered GyrA with its 84th Serine replaced by Leucine by a point mutation, the hydrophobic side chain of the Leucine does not support the formation of the hydrogen-bond network. The binding is not stabilized. (C) Nybomycin is stabilized in the mutated quinolone pocket because of its hydrophobic methyl side chain facing the hydrophobic side chain of 84th Leucine. No stable docking mode was found with the combination of Nybomycin and wild-type gyrase.

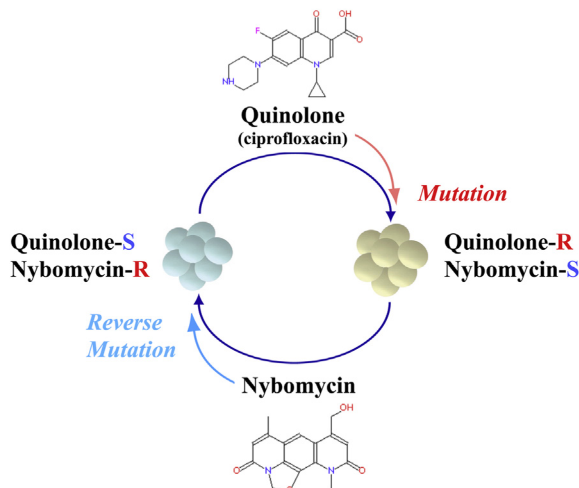

Fig. 7. Resolving the problem of multi-drug resistance by the use of Reverse Antibiotics. The use of quinolone results in the emergence of quinolone-resistant strains with mutated *gyrA*. Nybomycin is effective against the quinolone-resistant mutants. However, the use of Nybomycin generates Nybomycin-resistant mutants with their mutated *gyrA* back (reverse) mutated to wild type. Therefore, quinolones regain activity against the Nybomycin-resistant mutants. In this way, alternate use of Quinolone and Nybomycin always keeps the bacteria susceptible to either one of the two antibiotics, and does not allow development of multi-drug resistance. Abbreviation: *S*, susceptible; *R*, resistant. The structural formula of quinolone is that of Ciprofloxacin.

natural antibiotic. Therefore, it is likely that there are rich repertoires of antibiotics and reverse antibiotics stored in nature. RAs acting on other targets than topoisomerases such as mutated RNAP may be found as well in nature. With reverse antibiotics as a countermeasure for the rise of antibiotic-resistant bacteria, all the living microorganisms co-existed on the earth by maintaining natural homeostasis. Therefore, by developing RAs and using them together with the extant antibiotics developed in the last century, we would be able to control most of the multidrug-resistant bacterial infection without trying in vain to reach the unattainable goal of extinguishing the historically given our natural flora (Fig. 7).

4. Conclusion

The origin of *mecA* gene was traced back to *S. fleurettii* chromosome. Mutation of *rpoB* was found to play a major role in the development of vancomycin resistance in *S. aureus*. Staphylococci never stop evolving: it may acquire a highly efficient plasmid carrying *vanA* gene in near future. We need to be vigilant on the clinical MIC data of *S. aureus*, and have to be prepared for the future by learning from the nature's ecosystem to control them without trying to extinguish them. By using reverse antibiotics, many extant antibiotics will regain their potency, and history of antimicrobial chemotherapy started by the discovery made by Alexander Fleming will finally be completed.

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