Future Chemotherapy Preventing Emergence of Multi-Antibiotic Resistance

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The authors and all of the faculty at the Department of Bacteriology (Dept. of Microbiology from April 2015) have studied methicillin-resistant *Staphylococcus aureus* (MRSA), a representative multi-drug-resistant pathogen prevalent globally. During this study, we have identified the extreme flexibility of this organism to survive antibiotic pressure. *S. aureus* is part of the normal flora of human beings, yet it possesses high pathogenic potential, being aptly called ‘the department of toxins’. We are probably destined to continue our lives in association with this dangerous neighbor. For this reason, in 2008, KH launched a new project searching for novel antibiotics that are effective against MRSA. A newly found antibiotic called nybomycin has a curious property: it is effective against quinolone-resistant *S. aureus* strains (including most MRSA), but not against quinolone-susceptible ones. Moreover, the mutant strains derived from the parent *S. aureus* strain after treatment with nybomycin were cured of their quinolone resistance. Subsequently, we found an antibiotic with the same property in flavones, too. We designated these antibiotics with such unique properties reverse antibiotics (RA). By using RA and extant antibiotics in a well-controlled way, we should be able to establish sophisticated anti-microbial chemotherapy in the future.

**Key words:** reverse antibiotic (RA), MRSA, VISA, quinolone, vancomycin

**Introduction**

After graduation from Tokyo University Medical School in 1975, the author (KH) studied internal medicine (1975–1978). Then he conducted research on cellular immunology (1978–1980), and then retrovirology (Human T-cell Lecukemia Virus I [HTLV–I]) (1984–1988) before he commenced research on methicillin-resistant *Staphylococcus aureus* (MRSA) at Juntendo University. It was a precious experience to have belonged to various fields of medical science and had a chance to extract and learn the essence of science in general. It was also very precious to have learned the cutting-edge of genetic engineering in the S. Tonegawa’s lab at MIT (1981–1983).

Since 1989, KH joined Department of Bacteriology lead by late Professor Takeshi Yokota, and started the study of MRSA. Here briefly described are what he learned through the research and the reason why he commenced a new project of drug discovery and development lately against MRSA and related multi-drug resistant pathogens.

**MRSA is a multi-drug resistant pathogen**

When the author (KH) started the research on MRSA at Juntendo, little was known about the genetic basis for methicillin resistance except for successful cloning of *mecA* gene that encodes the resistance by the M. Matsumashi’s group at Tokyo University1). The author then cloned the same gene from another MRSA strain N315, and found that the *mecA* composes an operon together with *mecR1* and *mecI* regulator, the regulator genes that is involved in the induction of *mecA* gene upon
exposure to β-lactam antibiotics. Meantime, \textit{mecA} was shown to encode a β-lactam non-sensitive cell-wall synthesis enzyme PBP2’ (penicillin–binding protein 2’). It is a transpeptidase used to produce cross–linkage between the peptidoglycan (PG) nascent chains to make a rigid and durable cell–wall of \textit{S. aureus}. As the name suggests the activity of PBP is the target of penicillin and is inhibited by it and other β–lactam antibiotics. Since its first discovery in 1961\textsuperscript{2}, MRSA has turned down all the new antibiotics, the fruits of the anti–bacterial chemotherapy era started in 1940s. By 1970s, MRSA have acquired the resistance to practically all the available antibiotics, and has become the symbol of multi–drug resistant pathogen.

Figure 1  SCC\textit{mec}: Methicillin resistance gene \textit{mecA} is carried by a mobile element Staphylococcal Cassette Chromosome (SCC)

The first step of our study was to reveal that methicillin resistance gene \textit{mecA} and its regulator genes were carried by a novel mobile element staphylococcal cassette chromosome (SCC). SCC is a stretch of DNA demarked by a couple of inverted repeats. Its movement is driven by \textit{ccr}, cassette chromosome recombinases encoded by \textit{ccr} gene complex. We named the SCC containing \textit{mec} gene complex as SCC\textit{mec}.

Figure 2  SCC\textit{mec} is site–specifically integrated in \textit{Staphylococcus aureus} chromosome

The whole genome illustration of three MRSA strains, N315 and Mu50 are Japanese representative Healthcare–associated MRSA strains. MW2 is an USA400 that is a highly virulent representative Community–acquired MRSA.

SCC\textit{mec} in yellow is shown to be site–specifically integrated near the origin of replication. Note that the SCC\textit{mec} in Community–acquired MRSA is much shorter than those in healthcare–associated strains. The chromosomal location of genomic islands encoding pathogenicity factors are illustrated in red or pink: \textit{ν} signifies ‘island’ in Latin, \textit{φ}, stands for integrated bacteriophage. For detail, see references (13, 14, 16).
mecA gene is transferred across staphylococcal species

By chromosome walking of N315 and the other two representative world-wide epidemic MRSA strains, KH and T. Ito discovered the mobile genetic elements, staphylococcal cassette chromosome mec (SCCmec), on which mecA gene is carried (Figure-1). SCCmec is integrated site-specifically near the origin of replication (oriC) of the S. aureus chromosome (Figure-2). SCCmec is an interspecies carrier of mecA gene that confers methicillin resistance on practically any staphylococcal species. By developing the typing method of SCCmec his team proved that new MRSA clones were rapidly emerging from the S. aureus community strains. Type I~III SCCmec are predominant in the healthcare-associated (HA)~MRSA, whereas types IV and V SCCmec were found in newly emerging community-associated (CA)~MRSA (2002~2004). By 1995, elucidation of SCCmec and the sequence around its integration site culminated into the development of the method to discriminate MRSA from methicillin-resistant strains of other staphylococcal species (mec right extremity polymorphism [MREP] typing). With this strategy the PCR-based rapid and specific detection of MRSA became possible, and used all over the world. Until recently, the origin of mecA was not identified, which was finally elucidated in 2010. Practically identical mecA~mecR1~mecI gene complex and surrounding area in SCCmec was found on the chromosome of S. fleurettii, an animal commensal staphylococcus. S. fleurettii is one of the oldest staphylococcal species emerged about 200 million years ago around the historic emergence of mammals. The observation lead us to the hypothesis that mecA gene had existed long before the birth of humans, and then was lost from the genome of the descendant staphylococcal species that successfully established symbiotic relationship with their mammalian hosts (Figure-3).

Discovery of vancomycin resistance in MRSA

In 1997, KH and S. Hori discovered clinical strain
Mu50, the first vancomycin-intermediate *S. aureus* (VISA) strain in the world, and hetero-VISA (hVISA) strain Mu3 as well. In 2001, during the course of genetic investigation on vancomycin resistance of VISA strain Mu50, the first *S. aureus* whole genome sequencing (using strains Mu50 and N315) was achieved in collaboration with several laboratories in Japan and National Institute of Technology and Evaluation (NITE). Then, whole genome sequences of highly virulent CA-MRSA strain MW2 in 2002, and other related species and strains were determined during 2005~2008 at Juntendo.

Based on the continuous research on VISA strains, KH and colleagues proposed thickening of the cell–wall peptidoglycan (PG) layer, and ‘PG clogging’ as the vancomycin resistance mechanism in VISA. PG clogging is a unique mechanism of resistance relevant so far only to vancomycin. The obliteration of PG mesh occurs during the transit of vancomycin molecules from outside the cell to the cytoplasmic membrane where the target of vancomycin, D-Alanyl-D-Alanine residues, are present. Besides these real targets of action, PG layers contain thousands of D-Alanyl–D-Alanine determinants, to which vancomycin binds without causing any disadvantage to the cell. When the cell–wall peptidoglycan thickens, these ‘false targets’ of vancomycin critically serve as the barrier for vancomycin penetration and cause the delay the reach of vancomycin to the real targets on cytoplasmic membrane.

Uncovering genetic changes that cause vancomycin resistance in *S. aureus* has been the major theme of study of our laboratory, and was intensively studied by L. Cui, Y. Katayama, and M. Matsuo. Now, it became clear that vancomycin resistance in VISA are the cumulative outcome of the effects of multiple mutations. Such regulator genes as *vraSR*, *graRS*, and *walK*, *rpoBC* genes encoding RNA polymerase β and β’ subunits, and the genes involved in PG synthesis (*pbp4*) and cell–wall teichoic acid biosynthesis (*tarA, tarO, tarL*) contributed to the VISA phenotype expression. Also mutation of the genes involved in various metabolic pathways such as of pyrimidine, amino acids, and pyruvate were also found to contribute to the rise of vancomycin resistance. Single or combinations of these mutations easily raise vancomycin resistance. Therefore, it is not surprising even if some of the vancomycin–susceptible clinical strains generate VISA strains at a high frequency of around one in 10^6 colony forming units (CFU) when exposed to vancomycin. Such strains are designated heterogeneously vancomycin resistant or hetero-VISA (hVISA).

Figure 4 shows analysis of vancomycin susceptibility of three categories of clinical *S. aureus* strains.
Up to $10^7$ cells of the strains were spread on the agar plates containing varied concentrations of vancomycin. Most of the cell population of Mu3 is susceptible to vancomycin, but it contains highly resistant cells that can grow even in 8 or 9 mg/l of vancomycin (Figure-4). Quite a few fractions of clinical MRSA strains are suspected to be hVISA, but accurate prevalence is difficult to evaluate because of the lack of easy test method.

We also noticed that vancomycin has insufficient cytokilling activity against MRSA. Some MRSA strains are found to be ‘tolerant’ (i.e., they survive the exposure to much higher concentrations of vancomycin than needed to inhibit their growth). ‘Slow VISA’ (sVISA) is a novel phenotype of vancomycin resistance derived from hVISA strains that is characteristic in its extremely prolonged growth rate and resistance as well as tolerance to higher concentrations of vancomycin as compared to VISA\textsuperscript{24}. The remarkable feature of the sVISA phenotype is its instability. The sVISA phenotype of the strains disappears within 7 days’ drug-free passages, and they return to hVISA strains\textsuperscript{24}. It is probable that this phenomenon occur in clinical settings, which causes the therapeutic failure of the infection caused by the MRSA strains having susceptible ranges of vancomycin MICs (i.e. $\leq 2$ mg/l). Vancomycin has long been freed from drug-resistance development from its first discovery in 1956 until recently\textsuperscript{11}. However, it is now clear that vancomycin has gradually had lost reliability in the treatment of MRSA infection, and we have no alternative antibiotic effective against it.

**Discovery of reverse antibiotics (RA)**

Since 2008, KH, and Y. Morimoto, started a project of searching for natural antibiotics effective against multi-drug resistant MRSA. Screening of about 2,000 Actinobacteria cultures hit one queer substance. The substance was identified as a nybomycin, which was first reported in 1955\textsuperscript{25}. Surprisingly, nybomycin was found to be active against quinolone–resistant \textit{S. aureus} but was inactive against quinolone–susceptible \textit{S. aureus}\textsuperscript{26} (Table-1). This curious property of nybomycin was not noticed before, because the year of its discovery was long before the human development of nalidixic acid, the first quinolone antibiotic in 1962. Like quinolone antibiotics, nybomycin also allowed the emergence of resistant mutant strains. However, it turned out that the nybomycin–resistant mutants have lost quinolone resistance and become susceptible to quinolone antibiotics (Figure-5). Quinolone resistance is generated when a type–II topoisomerase gene (\textit{gyrA} or \textit{parC}) is mutated. Topoisomerase inhibition assay revealed that nybomycin binds to the mutated topoisomerases with much greater affinity than to non-mutated wild-type topoisomerases. This discovery prompted KH to look for other substances that bind to bacterial type–II topoisomerases. Literature search hit an old reference that

![Diagram](image_url)

**Figure–5** Nybomycin is a Reverse Antibiotic for Quinolone Resistance
It turned out that nybomycin also produces resistant mutants. But, curiously, they carry back mutated wild-type \textit{GyrA}, to which quinolones are effective.
### Table 1
Alternate susceptibility pattern of Quinolone antibiotics and RA (Nybomycin and Apigenin) against *S. aureus* clinical strains of the world with various genotypes of type-II topoisomerase genes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country</th>
<th>Year of Isolation</th>
<th>AA substitution&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIC (mg/l)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>parC</td>
<td>GyrA</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>UK</td>
<td>1943</td>
<td>MSSA</td>
<td>w</td>
</tr>
<tr>
<td>FDA 209P</td>
<td>UK</td>
<td>1948</td>
<td>MSSA</td>
<td>w</td>
</tr>
<tr>
<td>N315</td>
<td>Japan</td>
<td>1982</td>
<td>MRSA</td>
<td>w</td>
</tr>
<tr>
<td>Mu50</td>
<td>Japan</td>
<td>1996</td>
<td>MRSA</td>
<td>Ser80Phe</td>
</tr>
<tr>
<td>VRS1</td>
<td>USA</td>
<td>2002</td>
<td>VRS A&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Ser80Phe</td>
</tr>
<tr>
<td>HIP07920</td>
<td>USA</td>
<td>1998</td>
<td>MRSA</td>
<td>Ghu84Lys</td>
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<tr>
<td>HIP5836 (NJ)</td>
<td>USA</td>
<td>1997</td>
<td>MRSA</td>
<td>Ser80Tyr</td>
</tr>
<tr>
<td>HIP07930</td>
<td>USA</td>
<td>1999</td>
<td>MRSA</td>
<td>Ile85Met, Ser80Phe</td>
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<tr>
<td>NRS118</td>
<td>USA</td>
<td>2002</td>
<td>MRSA</td>
<td>Ser80Phe, Ser81Pro</td>
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<tr>
<td>VRS3a</td>
<td>USA</td>
<td>2004</td>
<td>VRS A&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Ser80Tyr, Ghu84Lys</td>
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<tr>
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<td>2000</td>
<td>MRSA</td>
<td>Ser80Phe, Ghu84Gly</td>
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<td>USA</td>
<td>2005</td>
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<td>Ser80Tyr, Ghu84Gly</td>
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<td>MRSA</td>
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<tr>
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<td>USA</td>
<td>2005</td>
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<tr>
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<td>KBSA56</td>
<td>Japan</td>
<td>2005</td>
<td>MRSA</td>
<td>Ser80Tyr, Ghu84Val</td>
</tr>
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</table>

MIC, minimum inhibitory concentration; VAN, vancomycin; OXA, oxacillin; NOR, norfloxacin; CIP, ciprofloxacin; OFX, ofloxacin; LVX, levofloxacin; TSX, tosufloxacin; SPX, sparfloxacin; NYB, nybomycin; API, apigenin.

<sup>a</sup> Strains are listed from top to bottom in order of the number of amino acid substitution(s) in the quinolone resistance-determining region (QRDR).

<sup>b</sup> Amino acid substitutions identified in the QRDR of ParC and GyrA: w indicates wild type.

<sup>c</sup> Quinolone and nybomycin MICs ≥8 mg/l are in bold.

<sup>*</sup>VRSA, vancomycin-resistant *Staphylococcus aureus*; defined by VCM MIC of ≥16 mg/l (20).
reported the marginal levels of activity of flavones in the inhibition of *E. coli* DNA gyrase. By testing 104 flavones, we found apigenin and several other flavones shared a property of reverse antibiotic. They inhibited mutated DNA gyrase of *S. aureus*, while they had practically no activity against wild-type DNA gyrase (Table-1).

Nybomycin and apigenin are considered to represent a novel functional class of antibiotics. They are active against quinolone-resistant bacteria but inactive against quinolone-susceptible ones. Thus, they can be used to cope with quinolone-resistant bacteria which are predominant in nosocomial environments. Although at a low frequency of around one in $10^{11}$, resistant mutants against nybomycin do emerge. We found that these resistant mutants harbored the wild-type gyrase gene. Since the quinolone-resistant strain has a mutation in the gyrase gene, the back (or reverse) mutation of which cured the bacteria of quinolone resistance and achieved instead the resistance to nybomycin or flavones. We named these new class of antibiotics as ‘reverse antibiotics (RA)’ for quinolone resistance.

**Future chemotherapy**

Now it is clear that no antibiotic is free from emergence of resistance. That is an invaluable lesson of nature we drew through the experience of anti-microbial chemotherapy since 1940s. Resistance is inevitable and had long existed much before the use of antibiotics by mankind in commercial scale. However, existence of resistant bacterial population is not hazardous to us if we have in our arsenal an antibiotic specifically effective on it. Thanks to the rapid progress in genome sequence technology, many new targets for antibiotic development have been found. However, introduction of any new antibiotic would end up with another cycle of resistance formation. Nybomycin and flavones, on the other hand, share the well known ‘old’ targets of action with quinolone antibiotics. Closer look at the ligand–binding mode by the target molecule revealed an alternate binding of nybomycin and quinolones to the wild-type and mutated DNA gyrase. This provides the basis for the reciprocal nature of antibiotics and RA. RA would not be confined to quinolone targets. Future well-tempered use of RA and antibiotics against various vital targets of action would allow the advent of new era of anti-microbial chemotherapy based on the principles of nature.

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