Multiple pathways of cross-resistance to glycopeptides and daptomycin in persistent MRSA bacteraemia

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Background: The development of non-susceptibility to glycopeptides and daptomycin in MRSA during persistent bacteraemia has become a significant therapeutic challenge. However, the in vivo evolution and mechanism of the dual resistance have remained incompletely understood.

Methods: A series of MRSA blood isolates with incremental non-susceptibility to glycopeptides and daptomycin were consecutively recovered from a bacteraemic patient who was failing chemotherapy. The evolutionary pathways during conversion from a glycopeptide- and daptomycin-susceptible phenotype into a vancomycin-intermediate Staphylococcus aureus (VISA) and a daptomycin-resistant S. aureus (DRSA) phenotype were then traced by WGS of the isogenic strains.

Results: A total of six non-synonymous mutations and three evolutionary pathways were identified during the development of the VISA/DRSA phenotype. The first pathway involved two steps of evolution, with an initial 1 bp insertion into yycH and a subsequent gain-in-function point mutation in mprF (S295L). The two mutations were correlated with heteroresistance to daptomycin/vancomycin and full development of the VISA/DRSA phenotype. The second pathway involved an 11 bp deletion mutation in yycH and point mutations at two genes, correlating with the development of the VISA phenotype and heteroresistance to daptomycin. Mutation in mprF (S295L) and a 5 bp deletion mutation in yycH were identified in the third pathway and corresponded to conversion into the full VISA/DRSA phenotype. The mutations in yycH resulted in premature terminations of YycH with variable lengths.

Conclusions: Multiple evolutionary pathways involving yycH and mprF can proceed simultaneously and may mediate cross-resistance to glycopeptides and daptomycin during persistent MRSA bacteraemia under antibiotic selective pressure.

Introduction

Persistent bacteraemia caused by MRSA is a serious disease with high morbidity and mortality.1,2 Glycopeptides have long been considered as the therapeutic drugs of choice and they remain the mainstay of treatment for this type of infection. However, delayed clearance of MRSA from the bloodstream and an increased incidence of treatment failure have been documented in patients receiving vancomycin treatment.3 - 5 High-dose daptomycin (10–12 mg/kg) with potent bactericidal activity has been frequently used as salvage therapy following glycopeptide treatment failure.6,7 Nevertheless, the clinical outcome and microbiological success rate have remained unsatisfactory.5 However, a greater challenge is encountered when treatment failure is associated with the emergence of non-susceptibility to both glycopeptides and daptomycin.8 - 10

An understanding of the in vivo evolutionary pathways of MRSA strains exhibiting the vancomycin-intermediate Staphylococcus aureus (VISA) phenotype and the daptomycin-resistant S. aureus (DRSA) phenotype may assist in the development of effective therapeutic strategies against these difficult-to-treat infections. Mutations in the two-component systems of S. aureus, including walRK, vraSR and graSR, have been among the most commonly identified VISA- or heterogeneous VISA (hVISA)-associated genetic alterations.11 - 15 Point mutations in the mprF gene, resulting in a gain-in-function phenotype and enhanced dlt operon function, are the most common bacterial alterations shown to mediate daptomycin resistance in S. aureus, affecting the cell surface charge.16 - 18 However, how the cross-resistance to glycopeptides and daptomycin developed and how it is related to the above-mentioned genetic alterations remain incompletely understood.

In the present study, we characterized the evolution of the dual resistance in a series of isogenic MRSA blood isolates. The isolates belonged to a pandemic clone, MLST ST239, and were recovered from a patient with suspected vascular implant infection who...
failed initial teicoplanin and subsequent daptomycin treatment. By comparing the genetic compositions at the whole-genome level, we were able to comprehensively track all in vivo evolutionary pathways, with incremental vancomycin and daptomycin non-susceptibility, for this clone. We also directly observed a correlation between the resistance-associated genetic alterations and the use of antimicrobial agents in distinct evolutionary pathways.

Materials and methods

Strains and growth conditions

The MRSA strains were consecutively isolated from the bloodstream of a patient with Down’s syndrome and tetralogy of Fallot. A Blalock–Taussig shunt with a Gore-Tex graft was created in this patient to treat his progressive cyanotic heart disease. One and a half months later, he developed an episode of MRSA bacteraemia. The bacteraemia could not be eradicated by teicoplanin followed by daptomycin treatment. A total of seven successive MRSA strains (CH1, CH2, CH3, CH4, CH5, CH6 and CH7) were grown from blood cultures obtained during the bacteraemic episode over the course of 40 days. Although a microbiological cure was finally achieved, the patient died of respiratory and heart failure. The successive MRSA strains (CH1, CH2, CH3, CH4, CH5, CH6 and CH7) were consecutively isolated from the bloodstream of a patient with persistent bacteraemia who failed teicoplanin and subsequent daptomycin treatment.

Doubling time measurement

The measurement of the growth rate was modified from a procedure described elsewhere. Briefly, overnight cultures of the strains were diluted to an OD of 0.05 in 60 mL of brain heart infusion broth and grown at 37°C with shaking at 150 rpm. The OD was monitored every 30 min for 8 h. Doubling times were calculated as follows: \( t_2 - t_1 \times \log 2 \) / (\( \log \text{OD}_{t_2} - \log \text{OD}_{t_1} \)), where \( t_1 \) is the first sampling time and \( t_2 \) is the second sampling time. The measurements were replicated three times for each strain.

Next-generation sequencing and mapping of the short reads to a reference genome of the ST239 lineage using SHRiMP

WGS of two VISA/DRSA strains (CH5 and CH7), the VISA/daptomycin-susceptible S. aureus (DSSA) strain CH4 and the isogenic vancomycin-susceptible S. aureus (VSSA/DSSA) strain CH1 was performed using the Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) (Table 1). A range of 1.6 million to 1.8 million reads was generated for each of the four strains, with similar average read lengths (\( \sim 379 \) bp) for strains CH1, CH4 and CH5 and a slightly shorter average length (363 bp) for strain CH7. The software SHRiMP 2.2.2 was used to map the reads to the reference genome of T0131 (GenBank accession number CP002643), which is an MRSA strain with the same genetic lineage (ST239) as the sequenced strains.

Identifying genetic alternations using Nesoni

Genetic alternations between the sequenced strains and the reference strain, including SNPs and insertions and deletions (INDELs), were identified using Nesoni 0.58 (http://vicbioinformatics.com/nesoni.shtml) as described elsewhere. Nesoni is a tool for high-throughput sequencing data analysis. By tallying the raw counts at each position in the reference strain, the raw counts of bases at each position can be compared using Fisher’s exact test. The VISA phenotype-associated SNPs and INDELs identified by WGS comparison were subsequently verified by PCR sequencing. The oligonucleotide sequences and conditions of each PCR are displayed in Tables S1 and S2 (available as Supplementary data at JAC Online).

Susceptibility tests

The MICs of teicoplanin, vancomycin, daptomycin, linezolid and rifampicin were determined by Etest (bioMérieux) according to the manufacturer’s instructions. Briefly, S. aureus isolates were grown in TSB at 37°C to a cell density equivalent to that of a 0.5 McFarland standard. The bacteria were then streaked onto Mueller–Hinton agar plates and the plates were incubated at 37°C for 24 h before reading.

Modified population analysis profile–area under the curve (PAP-AUC) analysis

A modified PAP-AUC analysis of the vancomycin and daptomycin susceptibilities was performed according to a procedure described previously.

Table 1. Phenotypic features of seven isogenic MRSA blood isolates from a patient with persistent bacteraemia who failed teicoplanin and subsequent daptomycin treatment

<table>
<thead>
<tr>
<th>Strain</th>
<th>Date of isolation (dd/mm/yyyy)</th>
<th>TEC MIC (mg/L)</th>
<th>MIC (mg/L)</th>
<th>PAP-AUC PAP-AUC* (versus Mu3)</th>
<th>MIC (mg/L)</th>
<th>PAP-AUC</th>
<th>LZD MIC (mg/L)</th>
<th>RIF MIC (mg/L)</th>
<th>Mean doubling time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1</td>
<td>16/10/2011</td>
<td>2</td>
<td>2.0</td>
<td>0.89 VSSA</td>
<td>0.25</td>
<td>7.28</td>
<td>1.0</td>
<td>16</td>
<td>59.8</td>
</tr>
<tr>
<td>CH2</td>
<td>20/10/2011</td>
<td>2</td>
<td>2.0</td>
<td>0.83 VSSA</td>
<td>0.38</td>
<td>7.04</td>
<td>1.0</td>
<td>8</td>
<td>57.9</td>
</tr>
<tr>
<td>CH3</td>
<td>25/10/2011</td>
<td>2</td>
<td>2.0</td>
<td>0.96 HIVSA</td>
<td>0.75</td>
<td>29.95</td>
<td>1.0</td>
<td>6</td>
<td>62.6</td>
</tr>
<tr>
<td>CH4</td>
<td>29/10/2011</td>
<td>2</td>
<td>3.0</td>
<td>1.06 VISA</td>
<td>0.38</td>
<td>30.35</td>
<td>1.0</td>
<td>32</td>
<td>59.9</td>
</tr>
<tr>
<td>CH5</td>
<td>07/11/2011</td>
<td>8</td>
<td>3.0</td>
<td>1.17 VISA</td>
<td>2</td>
<td>63.91</td>
<td>0.75</td>
<td>6</td>
<td>71.1</td>
</tr>
<tr>
<td>CH6</td>
<td>21/11/2011</td>
<td>8</td>
<td>3.0</td>
<td>1.20 VISA</td>
<td>2</td>
<td>62.20</td>
<td>0.75</td>
<td>8</td>
<td>68.5</td>
</tr>
<tr>
<td>CH7</td>
<td>24/11/2011</td>
<td>8</td>
<td>4.0</td>
<td>1.22 VISA</td>
<td>3</td>
<td>65.02</td>
<td>0.75</td>
<td>6</td>
<td>69.7</td>
</tr>
</tbody>
</table>

LZD, linezolid; RIF, rifampicin; TEC, teicoplanin.

*The phenotypes of VSSA, HVISA and VISA were defined by PAP-AUC results irrespective of MIC.
Briefly, bacteria were incubated in 5 mL of TSB at 37°C overnight with shaking at 150 rpm. The bacterial solution was then diluted with sterile saline to 10⁻¹ or 10⁻⁶. Aliquots of 100 mL of the diluted bacterial solutions were serially plated onto brain heart infusion agar plates containing vancomycin or daptomycin at concentrations of 0, 0.5, 1, 2, 2.5 or 4 mg/mL. The medium was also supplemented with 50 mg/L Ca²⁺ for the measurement of the daptomycin PAP-AUC. The plates were then incubated at 37°C for 48 h before counting the colony numbers. The PAP-AUC was calculated using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). A vancomycin PAP-AUC ratio of ≥0.9 between the tested strain and Mu3 indicated the presence of the hVISA phenotype.

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis

S. aureus strains were inoculated onto tryptic soy agar with 5% sheep blood from stock and incubated at 37°C for 16–20 h. A single colony was selected for inoculation into 5 mL of TSB, followed by incubation at 37°C for 16 h. A liquid culture volume of 50 mL was then inoculated into 5 mL of fresh TSB to obtain a starter culture. The liquid cultures were grown to mid-log phase (3 h) and early stationary phase (8 h) at 37°C with aeration (150 rpm), followed by centrifugation to obtain the bacterial cells. The cell pellets were resuspended in TE buffer and lysed with lysostaphin (final concentration: 0.5 μg/μL) at 37°C for 15 min. The lysed cell suspensions were used for RNA isolation using TRIzol (Invitrogen) according to the manufacturer’s instructions. For quantification of gene expression, 1 μg of total RNA was reverse transcribed using random hexamers and SuperScript® III Reverse Transcriptase (Invitrogen). Next, qRT-PCR was performed using KAPA SYBR® FAST qPCR Kit Master Mix (Kapa Biosystems, USA) according to the manufacturer’s instructions, together with the primers listed in Table S1. Real-time PCR was conducted using an IQ5™ Real-Time PCR Detection System (Bio-Rad). Reactions were run in duplicate in two independent experiments.

Results

Genotypes of and phenotypic changes in successive MRSA strains

All of the seven MRSA strains were of ST239 and carried a type III SCCmec element, lacked Panton–Valentine leucocidin genes and had indistinguishable band patterns in PFGE (Figure S1), indicating that these were isogenic strains of the isolates. The strains were consistently susceptible to linezolid, tigecycline and fusidic acid, but resistant to rifampicin, erythromycin, clindamycin and trimethoprim/sulfamethoxazole. The successive strains also exhibited incremental non-susceptibility to glycopeptides, which involved two steps of phenotypic conversion: from VSSA (strains CH1 and CH2) into hVISA (strain CH3) and from hVISA (strain CH3) into VISA (strains CH4, CH5, CH6 and CH7), as defined by the measurements of vancomycin MICs and PAP-AUC ratios (Figure 1 and Table 1). A similar two-step evolution was observed for the phenotypic conversion into daptomycin non-susceptibility. The first step specifically involved conversion from DSSA (strains CH1 and CH2) into heteroresistance (strains CH3 and CH4) as demonstrated by the increase in PAP-AUC values from ~7.0 to ~30.0 (Table 1). The second step involved conversion from heteroresistance into the full DRSA phenotype (strains CH5, CH6 and CH7), with an MIC of ≥2 mg/L. It appeared that the phenotypic conversion from VSSA into VISA was correlated with the DSSA-into-DRSA conversion. This observation suggested coevolution of resistance to both glycopeptides and daptomycin in the ST239 strains. To gain further insight into the genetic evolution during the development of dual resistance, a comparative genomics analysis of the seven isogenic MRSA strains was performed.

Comparative genomics of the isogenic ST239 strains (Table 2)

Using next-generation sequencing technology, the whole-genome sequences of the first strain (CH1), with susceptibility to both vancomycin and daptomycin, and of three successive strains (CH4, CH5 and CH7) with significant elevation of the MICs of the two antibiotics were determined (Table 1). Nesoni analysis identified five mutations among the genomes of the three successive strains, including an 11 bp deletion and a 1 bp insertion in gene yycH and a single nucleotide substitution in each of three genes: gene mprF; the gene encoding carbamoyl phosphate synthase L chain ATP-binding protein (SAT0131_01702); and the gene encoding phage HNH endonuclease (SAT0131_02103) (Table 2). The five predicted mutations were further confirmed by PCR sequencing. To more comprehensively trace the genetic evolution accounting...
Table 2. Types and locations of nucleotide alterations and the effect of mutations in isogenic MRSA strains with incremental non-susceptibility to vancomycin and daptomycin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation profile</th>
<th>SAT0131_00022/yycH</th>
<th>SAT0131_01432/mprF</th>
<th>SAT0131_01702/—</th>
<th>SAT0131_02103/—</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH1</td>
<td>A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH2</td>
<td>A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH3</td>
<td>B</td>
<td>INS (T) 28501</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH4</td>
<td>C</td>
<td>DEL (AATGTAATAA) 27599–27609</td>
<td>—</td>
<td>SNP (G/A) 1686208</td>
<td>SNP (C/T) 2089673</td>
</tr>
<tr>
<td>CH5</td>
<td>D</td>
<td>INS (T) 28501</td>
<td>SNP (C/T) 1414097</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH6</td>
<td>E</td>
<td>DEL (GATAT) 27953–27957</td>
<td>SNP (C/T) 1414097</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CH7</td>
<td>D</td>
<td>INS (T) 28501</td>
<td>SNP (C/T) 1414097</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Effect of mutation</td>
<td>frameshift, premature termination</td>
<td>S295L</td>
<td>Q97stop</td>
<td>M2I</td>
<td></td>
</tr>
</tbody>
</table>

DEL, deletion; INS, insertion.

*TO131 is a whole-genome-sequenced strain of the ST239-MRSA-SCCmec type III clone isolated in China. The locations of nucleotide alterations indicated in the table are in accordance with the nucleotide numbers in the genome of TO131.

The three frameshift mutations resulted in truncated YycH proteins with respective lengths of 89 amino acids in strain CH4, 207 amino acids in strain CH6 and 389 amino acids in strains CH3, CH5 and CH7.

for the dual resistance, the four alleles containing mutations were amplified and sequenced in the other three strains (CH2, CH3 and CH6). The sequencing of strain CH6 identified another mutation, consisting of a 5 bp deletion in gene yycH. Taking these results together with the results of the Nesoni analysis, a total of six mutations were identified in four alleles in the ST239 strains. The detailed distributions of the nucleotide alterations and the effects of the mutations in the seven strains are displayed in Table 2.

**Genetic evolution during the development of dual resistance (Figure 2)**

The six mutations in four alleles constituted a total of five mutation profiles (profiles A–E) in the seven isogenic strains (Table 2). The first two isolates (strains CH1 and CH2) harboured the WT alleles (profile A) and exhibited similar antibiograms and doubling times, indicating the same strain in the two isolates. The first mutation was identified in strain CH3 (profile B), which was a one-nucleotide (thymidine) insertion into yycH, resulting in a truncated YycH protein of 389 amino acids (WT allele, 444 amino acids; Figure S2). The insertion in strain CH3 was associated with an elevated PAP-AUC ratio that fell within the range defining DRSA strain CH5 at day 12 of daptomycin use.

Strain CH4 harboured three genetic alterations, including an 11 bp deletion mutation in yycH and two SNPs (mutation profile C, Table 2), which were completely different from those identified in the previous strain (CH3). This observation suggested derivation from the parental strain CH2 (mutation profile A), and not from strain CH3, via another evolutionary pathway. The evolution from mutation profile A to profile C occurred during the treatment with teicoplanin (6 days) followed by daptomycin (3 days) and was accompanied by an elevation of the vancomycin MIC from 2 to 3 mg/L and of the teicoplanin MIC from 2 to 8 mg/L (Table 1). The vancomycin PAP-AUC ratio also increased from 0.96 to 1.17 between strains CH3 and CH5. These observations suggested that the mechanism of daptomycin resistance might be shared by S. aureus to promote non-susceptibility to glycopeptides. Strain CH7 was isolated 17 days apart from strain CH5. Without undergoing major genetic evolution, the MICs of vancomycin and daptomycin further advanced for strain CH7 (Table 1), suggesting that the increased glycopeptide and daptomycin non-susceptibility between strains CH5 and CH7 was achieved by a regulatory mechanism.

Strain CH6 exhibited the VISA/DRSA phenotype and harboured mutations in yycH and mprF, as in strain CH5. However, strain CH6 harboured a 5 bp deletion mutation in yycH, which was distinct from the 1 bp insertion in strain CH5, suggesting that strain CH6 was derived via its own evolutionary pathway, from mutation profile A to profile E. The evolution was associated with an increased vancomycin MIC, from 2 to 3 mg/L, and an increased daptomycin MIC, from 0.38 to 2 mg/L. The strains CH6 and CH7, with dual resistance to vancomycin and daptomycin, were finally eradicated or inhibited by linezolid plus rifampicin treatment.

Detection of the same insertion in yycH in two other strains (CH5 and CH7) with mutation profile D suggested that the two strains were descendants of strain CH3. Strain CH5 was isolated on day 13 of daptomycin treatment and harboured an SNP mutation in mprF. The mutation resulted in an amino acid substitution in MprF (S295L) and was associated with phenotypic conversions from DSSA (MIC of 0.75 mg/L for strain CH3) into DRSA (MIC of 2 mg/L for strain CH5) and from hVISA into VISA. It is intriguing to note that without glycopeptide treatment, strain CH5 continued to exhibit incremental glycopeptide non-susceptibility, as demonstrated by the elevation of the vancomycin MIC from 2 to 3 mg/L and of the teicoplanin MIC from 2 to 8 mg/L (Table 1). The vancomycin PAP-AUC ratio also increased from 0.96 to 1.17 between strains CH3 and CH5. These observations suggested that the mechanism of daptomycin resistance might be shared by S. aureus to promote non-susceptibility to glycopeptides. Strain CH7 was isolated 17 days apart from strain CH5. Without undergoing major genetic evolution, the MICs of vancomycin and daptomycin further advanced for strain CH7 (Table 1), suggesting that the increased glycopeptide and daptomycin non-susceptibility between strains CH5 and CH7 was achieved by a regulatory mechanism.

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Strain CH6 exhibited the VISA/DRSA phenotype and harboured mutations in yycH and mprF, as in strain CH5. However, strain CH6 harboured a 5 bp deletion mutation in yycH, which was distinct from the 1 bp insertion in strain CH5, suggesting that strain CH6 was derived via its own evolutionary pathway, from mutation profile A to profile E. The evolution was associated with an increased vancomycin MIC, from 2 to 3 mg/L, and an increased daptomycin MIC, from 0.38 to 2 mg/L. The strains CH6 and CH7, with dual resistance to vancomycin and daptomycin, were finally eradicated or inhibited by linezolid plus rifampicin treatment.
The expression of RNAIII and selected genes within the regulatory network of *yycH*, including *walRK*, *sdrD*, *ssaA*, *lytM* and *isaA*, and genes related to the resistance mechanism of daptomycin, including *mprF* and *dltA*, were determined (Figure 3a–d). An increasing trend in *mprF* and *dltA* expression and a declining trend in RNAIII expression were observed in the seven successive strains with incremental non-susceptibility to daptomycin and glycopeptides in either the log (3 h) or the stationary (8 h) phase of growth. In contrast, there was no obvious trend in the expression of *walRK*, *sdrD*, *ssaA*, *lytM* or *isaA*, in correlation with the increased non-susceptibility to glycopeptides and daptomycin in the series of MRSA strains.

**Expression of selected resistance-associated genes (Figure 3)**

The expression of RNAIII and selected genes within the regulatory network of *yycH*, including *walRK*, *sdrD*, *ssaA*, *lytM* and *isaA*, and genes related to the resistance mechanism of daptomycin, including *mprF* and *dltA*, were determined (Figure 3a–d). An increasing trend in *mprF* and *dltA* expression and a declining trend in RNAIII expression were observed in the seven successive strains with incremental non-susceptibility to daptomycin and glycopeptides in either the log (3 h) or the stationary (8 h) phase of growth. In contrast, there was no obvious trend in the expression of *walRK*, *sdrD*, *ssaA*, *lytM* or *isaA*, in correlation with the increased non-susceptibility to glycopeptides and daptomycin in the series of MRSA strains.

**Discussion**

A growing body of work on the genetic evolution of the VISA phenotype has shown that VISA-associated genetic mutations usually occur sequentially and accumulate during the development of incremental vancomycin non-susceptibility. Accordingly, the data from the present study further demonstrated that multiple evolutionary pathways might proceed simultaneously during the development of resistance to vancomycin and daptomycin. Indeed, by tracking the genetic changes in the isogenic isolates at the whole-genome level, we captured three distinct evolutionary pathways during the phenotypic conversion from VSSA and DSSA into VISA and DRSA, respectively, in the ST239 strains. By integrating the information regarding antimicrobial therapy, the susceptibility data and the mutation profiles of the strains, we also directly observed how the genetic evolution of drug resistance was driven by the use of antimicrobial agents.

The initial use of teicoplanin effectively inhibited the VSSA strains, but rapidly selected an hVISA strain (CH3, profile B) and a VISA strain (CH4, profile C) through two distinct pathways (Figure 2). However, the shift from teicoplanin to daptomycin use eliminated VISA strain CH4. Daptomycin treatment promoted further evolution of hVISA strain CH3 and selected a strain with the VISA/DRSA phenotype (strain CH5, profile D). The pathway...
from profile A to B to D was finally terminated when strain CH7 of profile D was eradicated after a 10 day course of linezolid treatment. The strain of profile E, representing the third evolutionary pathway, was identified at a very late stage of the treatment course. With no other strains available in between, we were unable to determine the sequence of acquiring the mutations in yycH and mprF in the pathway from profile A to E. The strain diversification should have occurred in an early stage of antibiotic treatment. It is possible that the strains of the pathway from profile A to E represented a minority in the bacterial population and were not chosen when picking colonies for strain preservation. This speculation was supported by the relatively slow growth rate of strain CH6 among the seven isogenic strains (Table 1).

MprF is the synthase for positively charged lysylphosphatidylglycerol (L-PG), which is considered to be among the major contributors to the positive charge on the S. aureus cell surface. Deletion of mprF reduces the cell surface positive charge in S. aureus and increases the susceptibility of S. aureus to daptomycin and host defence peptides. It has also been frequently reported that the DRSA phenotype in S. aureus is associated with gain-in-function SNP mutations in the mprF gene. A recent report further demonstrated that two SNPs in mprF, resulting in amino acid alterations in S295L and T345A, were causally related to daptomycin resistance by enhancing the synthesis of L-PG and reducing daptomycin binding to S. aureus. Consistent with this observation, the phenotypic conversion into full DRSA in the ST239 strains in the current study was associated with the occurrence of the SNP mutation MprFS295L in the pathways from profile B to D and profile A to E.

In addition to mprF mutations, it appeared that another mechanism might be implicated in daptomycin non-susceptibility. Indeed, without involvement of mprF mutation and without daptomycin selective pressure, elevation of the daptomycin PAP-AUC from 7.28 to 29.95 (in the susceptible range) and of the daptomycin MIC from 2 to 3 mg/L (in the resistant range) was observed for strain pairs CH1/CH3 and CH5/CH7, respectively (Table 1 and Figure 1). The elevation of daptomycin PAP-AUC values and MICs for the two strain pairs was accompanied by the increased expression of mprF and dltA and correlated with reduced susceptibility to vancomycin, indicating linked non-susceptibility to daptomycin and vancomycin via a common pathway. The linked non-susceptibility was also evidenced by the observation that without ongoing exposure to glycopeptides, MprFS295L was accompanied by elevation of the vancomycin MIC from 2 to

![Figure 3. Expression of selected drug resistance-associated alleles in successive MRSA strains with incremental non-susceptibility to vancomycin and daptomycin. Reactions were run in duplicate in two independent experiments.](image-url)
3 mg/L for strain pair CH3/CH5 (Table 1 and Figure 1). The reduced susceptibility to vancomycin may have been caused by a mechanism other than MprF activity, given that the MprF S295L mutation has been shown to have no impact on the vancomycin MIC. Furthermore, with linezolid monotherapy for 10 days, a concomitant elevation of the vancomycin and daptomycin MICs was observed for strain CH7 compared with its parental strain (CH5). Taken together, the data strongly suggest that anti-MRSA agents are capable of providing selective pressure, prompting coevolution of resistance to both glycopeptides and daptomycin via a general, but not yet identified, mechanism.

**yycH**, **yycI** and **yycJ** are the downstream genes of **walR** and **walK**, which together form an essential two-component signal transduction system. This system, known as the **walRK** (synonyms, **vicRK** and **yycFG**) regulon, is present in a variety of Gram-positive bacteria, including *S. aureus*. The **walRK** system plays a crucial regulatory role in cell wall metabolism and mutations with SNPs occurring in either the **walR** or the **walK** gene have been demonstrated to confer co-resistance to vancomycin and daptomycin on *S. aureus*. In the current study, we did not identify **VISA**- or **DRSA**-associated mutations in **walR** or **walK**, but rather in their downstream gene, **yycH**. In *Bacillus subtilis*, it has been shown that YycH and YcI are transmembrane proteins that act with the WalK (YycG) histidine kinase as a complex and are able to modulate the kinase activity of WalK. Moreover, WalK kinase activity may be maintained when the YycH protein is truncated to as few as 60 N-terminal amino acids (composed of the transmembrane helix) in *B. subtilis*. This observation indicated that the transmembrane helix is the major functional domain of YycH and that the C-terminal extracytoplasmic domain is dispensable for WalK activity modulation. Intriguingly, data from the current study demonstrated that three **VISA**- or **DRSA**-associated frameshift mutations in **yycH** resulted in premature termination of the YycH protein at 82 to 383 WT N-terminal amino acids (Figure S2). Furthermore, the yycH mutations were involved in three distinct evolutionary pathways during the development of resistance to glycopeptides and daptomycin. This observation raised the possibility that distinct from the condition in *B. subtilis*, the C-terminal extracytoplasmic domain of YycH may play an important role in the modulation of WalK kinase activity in *S. aureus*. However, the expression of WalK and other alleles under the **walRK** regulon, including **sdhD**, **ssaA**, **lytM** and **isaA**, did not change consistently in the strains with incremental non-susceptibility to glycopeptides or daptomycin (Figure 3c and d). Pathways other than regulation of WalK activity may thus account for the association of mutations in **yycH** with the **VISA** or **DRSA** phenotype. A follow-up study is currently under way to address the role of **yycH** mutations in vancomycin and daptomycin non-susceptibility in *S. aureus*.

It appeared that the persistent MRSA bacteraemia was finally eradicated by simultaneous administration of linezolid and rifampicin. However, the *in vivo* effect of rifampicin on inhibiting the MRSA strains was questionable, given that the seven successive MRSA strains carried an **rpoB** mutation (H481N, data not shown) and were highly resistant to rifampicin, with an MIC of 6 – 32 mg/L (Table 1). We have previously shown that the use of non-susceptible agents *in vitro*, such as rifampicin, to treat VISA infections not only was unable to eradicate the strains, but also may have increased the non-susceptibility to glycopeptides. Microbiological cure in this case most likely would have been achievable by salvage use of linezolid. Two Korean groups have demonstrated that the success rate of salvage treatment for persistent MRSA bacteraemia was higher for linezolid-based combination therapy than for glycopeptide-based combination therapy. The data suggested a promising role for linezolid in the treatment of Gram-positive bacteraemia, including MRSA infection. The observation that the MIC of linezolid declined for the MRSA strains with increasing resistance to daptomycin (Table 1) further highlighted the potential benefit of linezolid in bacteraemic patients who fail daptomycin and glycopeptide therapy.

This study has several limitations. First, not all of the seven successive strains were sequenced. Mutations in alleles other than the four screened genes might have occurred in strains CH2, CH3 and CH6. The number of evolutionary pathways could therefore be underestimated. Second, the WGS was not complete and severe unaligned gaps remain. Mutations occurring in the unaligned sequence gaps were not identified in this study. Third, the mechanism of the multiple evolutionary pathways was based on the identified isolates, which suffered from potential bias because isolates of minor clones may not be readily identified by the traditional culture method.

In conclusion, by WGS of isogenic strains with incremental non-susceptibility to vancomycin and daptomycin, we successfully identified three frameshift mutations in **yycH**, an SNP in **mprF** and a non-synonymous mutation in each of two alleles that were potentially associated with the **VISA** and/or **DRSA** phenotypes. By screening the mutations in other successive strains, we identified a total of three evolutionary pathways during the phenotypic conversion from **VSSA/DSSA** into **VISA/DRSA** in ST239 strains. The capacity for developing drug resistance through multiple genetic pathways may have contributed to the failure of the initial glycopeptide monotherapy followed by daptomycin in this case. Additionally, the delayed bacterial clearance not only inevitably increased the medical costs, but also might have resulted in the death of this patient. A more effective therapeutic strategy against MRSA bacteraemia, such as initial regimens that combine different classes of antimicrobial agents or early transition to an alternative treatment, will be required, especially in the management of patients with complicated infections and in whom the primary source of infection cannot be readily eliminated.

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**Transparency declarations**

None to declare.

**Supplementary data**

Table S1, Table S2, Figure S1 and Figure S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
References


