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Daptomycin resistance in methicillin-resistant *Staphylococcus aureus* is conferred by IS*256* insertion in the promoter of *mprF* along with mutations in *mprF* and *walK*

Yuyao Yin, Hongbin Chen, Shuguang Li, Hua Gao, Shijun Sun, Henan Li, Ruobing Wang, Longyang Jin, Yudong Liu, Hui Wang*

Department of Clinical Laboratory, Peking University People's Hospital, Beijing 100044, China

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ABSTRACT

Mechanisms underlying the emergence of daptomycin resistance in *Staphylococcus aureus* remain unclear. In this study, *Staphylococcus aureus* strain 3d0, isolated from a patient with bloodstream infection and belonging to the predominant Chinese hospital-associated methicillin-resistant *S. aureus* (MRSA) clone ST239, was serially passaged on gradient broth containing daptomycin for 34 days. The whole genomes of 3d0 and its serial passage strains were sequenced and compared. Five single nucleotide polymorphisms, four IS*256* insertions, and one 39-bp insert occurred in the progress of daptomycin resistance acquisition. IS*256* insertion in the *mprF* promoter region resulted in *mprF* overexpression. Two novel point mutations in *mprF* and *walK*, leading to amino acid substitutions in MprF (G299V and L473I) and WalK (L7Q and Y225N), were shown by allelic replacement experiments to increase the minimum inhibitory concentration (MIC) of daptomycin by 2–4 times. Allelic replacement of both *mprF* and *walK* in strain 3d0 increased the daptomycin MIC by 4–8-fold, indicating that *mprF* and *walK* mutations synergistically contribute to daptomycin non-susceptibility. Notably, these mutants acquired resistance without losing fitness and exhibited decreased expression of cell wall degradation-related genes. In conclusion, this study revealed novel mutations of MRSA daptomycin resistance acquisition in vitro as well as several novel mutations in *walK* and *mprF*, and includes the first in-depth analysis of the *mprF* promoter. This study sheds light on how MRSA may acquire daptomycin resistance during daptomycin treatment.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent pathogens responsible for hospital- and community-associated infections, with substantial morbidity and mortality [1]. Daptomycin, a lipopeptide antimicrobial agent active against Gram-positive bacteria, was first approved by the US Food and Drug Administration (FDA) for clinical use in the USA in 2004. Daptomycin inhibits cell wall synthesis by interacting with phosphatidylglycerol and interfering with fluid membrane microdomains [2]. Owing to its potent bactericidal activity and low rate of antimicrobial resistance, daptomycin has been recognised as the first-line antimicrobial agent for serious infections caused by multidrug-resistant Gram-positive pathogens [3]. Accumulating evidence indicates the development of daptomycin non-susceptibility in *S. aureus* owing to prolonged treatment courses [3].

There are multiple mechanisms leading to daptomycin non-susceptibility in *S. aureus*, and the mechanisms appear to be isolate-specific. It has been suggested that changes in the cell membrane and cell wall owing to adaptations in pathways involved in metabolic function and stress response may be associated with daptomycin non-susceptibility [4], but the mechanisms underlying daptomycin non-susceptibility remain incompletely understood. Mutations in MprF, a phosphatidylglycerol lysyltransferase, have been reported in several daptomycin-resistant *S. aureus* strains [5–10]. WalK (also known as YycG), a sensor protein kinase, colocalises with the cell wall biosynthesis complex, a target site for daptomycin insertion into the membrane during *S. aureus* division [11]. Increased cell wall thickness has also been observed in several daptomycin-non-susceptible *S. aureus* strains [6,12]. Furthermore, increased expression of genes involved in cell wall metabolism, such as *mecA*, *pbp2* and *sgtB*, in daptomycin-non-susceptible compared with daptomycin-susceptible *S. aureus* has been reported [4,6,13].

* Corresponding author. Tel.: +86 10 8832 6300.
E-mail address: wanghui@pkuph.edu.cn (H. Wang).

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Table 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source or reference
Strains		
Escherichia coli DH5α	Plasmid free, restriction deficient	Takara
Staphylococcus aureus		
RN4220	Restriction-negative derivative of NCTC 8325-4	[21]
3d0	ST239 clinical daptomycin-susceptible MRSA	This study
3d9	In vitro-selected daptomycin-resistant derivative of 3d0	This study
3d18	In vitro-selected daptomycin-resistant derivative of 3d0	This study
3d27	In vitro-selected daptomycin-resistant derivative of 3d0	This study
3d34	In vitro-selected daptomycin-resistant derivative of 3d0	This study
3d0W1	WalK L7Q derivative of 3d0	This study
3d0W2	WalK Y225N derivative of 3d0	This study
3d0M1	MprF G299V derivative of 3d0	This study
3d0M2	MprF L473I derivative of 3d0	This study
3d0M12	MprF G299V+L473I derivative of 3d0	This study
3d0W1M1	WalK L7Q and MprF G299V derivative of 3d0	This study
3d0W1M2	WalK L7Q and MprF L473I derivative of 3d0	This study
3d0W1M12	WalK L7Q and MprF G299V+L473I derivative of 3d0	This study
3d0W2M1	WalK Y225N and MprF G299V derivative of 3d0	This study
3d0W2M2	WalK Y225N and MprF L473I derivative of 3d0	This study
3d0W2M12	WalK Y225N and MprF G299V+L473I derivative of 3d0	This study
Plasmids		
pKOR1	*E. coli*/*S. aureus* shuttle vector; resistance to ampicillin and tetracycline	[23]
pKOR1_mprF_G896T+T1417A	The *mprF* fragment amplified from 3d34 and cloned into pKOR1 for allelic exchange in 3d0	This study
pKOR1_walK_T20A	The *walK* fragment amplified from 3d9 and cloned into pKOR1 for allelic exchange in 3d0	This study
pKOR1_walK_T673A	The *walK* fragment amplified from 3d34 and cloned into pKOR1 for allelic exchange in 3d0	This study

ST, sequence type; MRSA, methicillin-resistant *S. aureus*.

The insertion sequence IS*256* has been detected as part of the aminoglycoside resistance transposon Tn*4001* in the genomes of several clinical *S. aureus* isolates in variable copies [14,15] and transposes by a 'copy and paste' mechanism [16]. IS*256* has been shown to play roles in antibiotic resistance [17,18], virulence [19] and the formation of small-colony variants [20] in *S. aureus*. However, the role of the insertion in daptomycin non-susceptibility remains poorly understood.

We have previously shown that stable daptomycin-resistant mutants can be generated by a 34-day in vitro daptomycin selection process, with fitness cost [12]. Using comparative genomics, we identified several novel mutations during the evolution of in vitro-selected daptomycin resistance, including two amino acid substitutions in MprF (G299V and L473I), two amino acid substitutions in WalK (L7Q and Y225N), four IS*256* insertions, and one 39-bp insert in *pyk*. The aim of this study was to analyse the functional involvement of these novel mutations in daptomycin resistance. In addition, the effects of the novel mutations on bacterial growth and the expression of cell wall metabolism-related genes were assessed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. A daptomycin-susceptible ST239-t037 MRSA (strain 3d0, previously named pre3), was the parental strain in our previous study [12]. Details on the 34-day continuous passage selection process have been reported previously [12]. The serial passage strains 3d9, 3d18, 3d27 and 3d34 (previously named post3) were single colonies randomly picked from 9-, 18-, 27- and 34-day plate cultures, respectively. *Staphylococcus aureus* and *Escherichia coli* strains were routinely grown in tryptic soy broth (TSB) and Luria–Bertani medium, respectively. Growth media were supplemented as needed with antibiotics (100 μg/mL ampicillin, 10 μg/mL chloramphenicol and 1 μg/mL anhydrotetracycline).

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentration (MICs) of daptomycin, vancomycin, teicoplanin and oxacillin were determined by broth microdilution following Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. MIC assays were performed in triplicate.

2.3. Construction of mprF and walK mutant derivatives

Allelic replacement was used to construct *mprF* and *walK* mutants as described previously [23]. The primer pairs attB1_mprF_F/attB2_mprF_R and attB1_walK_F/attB2_walK_R were used to amplify point-mutant *mprF* and *walK* fragments from chromosomal DNA (Table 2) using a high-fidelity DNA polymerase (PrimeSTAR®; Takara, Shiga, Japan). Fragments were recombined into the pKOR1 shuttle vector using *E. coli* DH5α (Takara) and Gateway® BP Clonase II Enzyme Mix (Invitrogen, Waltham, MA, USA). Resultant plasmids (pKOR1_mprF_G896T+T1417A, pKOR1_walK_T20A and pKOR1_walK_T673A) were electroporated into *S. aureus* strain RN4220 using a Gene Pulser Electroporation System (Bio-Rad, Hercules, CA, USA). Recombinant plasmids were extracted from mutant *S. aureus* strain RN4220 using a Plasmid Midi Kit (QIAGEN, Hilden, Germany) and were subsequently electroporated into *S. aureus* 3d0. Finally, *mprF* and *walK* mutants were confirmed by PCR using EasyTaq PCR SuperMix (TransGen Biotech Co. Ltd., Beijing, China).

2.4. DNA and RNA extraction

Chromosomal DNA and total RNA were extracted using a TIANamp Bacteria DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China) and an RNeasy® Mini Kit (QIAGEN), respectively. Total RNA was treated with RNase-free DNase (QIAGEN). Unless stated otherwise, a Plasmid Mini Kit (Omega, Norcross, GA, USA) was used to extract plasmid DNA according to the manufacturer's instructions.

2.5. Whole-genome sequencing

Staphylococcus aureus 3d0 and its serial passage strains were sequenced using Illumina technology with a 150-bp paired-end

Table 2
PCR primers used in this study

Primer	Sequence (5′→3′)
Cloning of gap between high-copy-number regions	
1F600061+20	TTACAATGGTTGGAACAAGC
1R601058-21	GCACGATATGCGAGATTTTTA
2F1454209+20	GGCCTACTATAGCGATTTCT
2R1455188-21	AGTAACACTAAGGACATACGG
3F1826935+20	AGCAGCATCAATCGTAACTA
3R1827566-20	AATGCTATCGGTATTTCGGT
4F2077681+20	ACTTACGTTCTTCACCTAGC
4R2078441-20	GCCACACCTATGCAATAGAA
5F2811086+20	TATTTACACGCTTGTTGTGC
5R2811615-20	TTTTCTACGGCACGATACAT
Cloning of *mprF* gene for allelic exchange	
attB1_mprF_F	ggggacaagtttgtacaaaaaagcaggctTGTATCGGGAGTTATCTGGT
attB2_mprF_R	ggggaccactttgtacaagaaagctgggtCCTGAAGTTGAAAATTGCGT
Cloning of *walK* gene for allelic exchange	
attB1_walK_F	ggggacaagtttgtacaaaaaagcaggctAAAACGTGGCGAAGATATTG
attB2_walK_R	ggggaccactttgtacaagaaagctgggtTGTATCCATTTTGGCTGTCA
Real-time qPCR	
MprF_q_F	AGTTACAGATCAACACATGCCT
MprF_q_R	GCTCTAAATCCACGGCGTTT
lytM_q_F	TACAAGCAGGTTGGAGTAAC
lytM_q_R	GCTTTGACTTTATCACCAGC
isaA_q_F	GGTACTACATGGTCATGGAGCTATGAAGC
isaA_q_R	CTCACTGAACTTGAAGTAGTTGAAGTGCTG
ssaA_q_F	TGCTGTTTGGCTATTGTTTGT
ssaA_q_R	CTTGGACAGGAAATTGGAATC

protocol on an Illumina HiSeq instrument (Illumina Inc., San Diego, CA, USA). The metagenome distance identification tool Mash [24] was used to identify the best-matching chromosomal reference against an archive of RefSeq genomes by trimming and quality-filtering the raw sequencing reads. *Staphylococcus aureus* strain TW20 (GenBank accession no. **FN433596**) was the best-matching chromosomal reference. Reads were mapped to this reference with BWA-MEM v.0.7.12 [25], and single nucleotide polymorphisms (SNPs) were called for each sample independently using FreeBayes v.1.0.2 [26], with a minimum alternative fraction of 0.95 and otherwise default parameters. Additional SNP filtering was carried out in bcftools to extract those SNPs with a Phred-scaled quality score of ≥50. An in-house script was used for counting clip-reads to inform possible insertion or deletion sites. SNPs and potential insertion or deletion sites that were also present in the parental strain (3d0) were deleted to detect the differences between 3d0 and its serial passage strains. Finally, SNPs and potential insertion or deletion sites were examined in the genome data visualisation tool Integrative Genomics Viewer (IGV) v.2.4.14 [27]. Each insertion was confirmed by PCR and Sanger sequencing using unique primer pairs (Table 2) and was classified by ISfinder (https://www-is.biotoul.fr/index.php) using blastn [28]. Short read sequences were assembled using the SPAdes Genome Assembler v.3.13.0 [29]. The de novo assemblies were annotated automatically using the online Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org/). The promoter region of *mprF* was detected using SoftBerry BPROM software (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb).

2.6. Reverse transcription quantitative (RT-qPCR)

cDNA was generated from ~500 ng of total RNA using PrimeScript RT Master Mix (Takara). qPCR was run in triplicate using TB Green™ Premix Ex Taq™ II (Takara) in a MicroAmp® Optical 96-Well Reaction Plate (Thermo Fisher Scientific, Waltham, MA, USA) on a LightCycler® 480 System (Roche, Switzerland) according to the manufacturer's instructions. The primers used are listed in Table 2. RT-qPCR experiments were conducted using three biological repeats. Target gene expression was calculated from standard curves, and the *gyrB* gene was used as an internal control for normalisation.

2.7. Growth assay

Staphylococcus aureus isolates were cultured in TSB to log growth phase [optical density at 600 nm (OD_{600}) = 0.6]. Cultures were diluted to OD_{600} = 0.01 and were grown at 37 °C with agitation at 200 rpm for 24 h. Cell density was determined every 30 min by measuring the OD_{600}.

2.8. Cytochrome c binding assay

The cytochrome *c* binding assay was performed as described previously [30,31]. Briefly, *S. aureus* cells were cultured overnight in TSB and were washed with 20 mM MOPS buffer (pH 7.0) three times. Cells were then re-suspended in MOPS buffer at an OD_{600} of 1.5. Next, cells were incubated with 0.5 mg/mL cytochrome *c* (Solarbio, Beijing, China) for 15 min and the amount of cytochrome *c* remaining in the supernatant was measured by determining absorbance at 408 nm (absorption maximum in scanning spectrum). More unbound cytochrome *c* detected in the supernatant means more net positive charge on the bacterial surface. At least three independent runs in triplicate samples were performed on separate days.

2.9. Measurement of cell wall thickness

Cell wall thickness was measured by transmission electron microscopy (TEM). Sample preparation and TEM observation were performed at Peking University People's Hospital (Beijing, China) using a Tecnai™ Spirit (FEI, Hillsboro, OR, USA). Cell wall thickness was measured using ImageJ software.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism v.8.0.0 (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation and were compared by *t*-test. A *P*-value of <0.05 was considered statistically significant.

2.11. Data availability

The genome sequences of the parental strain 3d0 and its serial passage strains have been submitted to the Sequence Read Archive (SRA) under BioProject ID **PRJNA523976**, accession nos. **SAMN10993748** (3d0), **SAMN10993749** (3d9), **SAMN10993750** (3d18), **SAMN10993751** (3d27) and **SAMN10993752** (3d34).

3. Results

3.1. Selection of daptomycin-resistant Staphylococcus aureus mutants

Staphylococcus aureus strain 3d0, which belongs to the predominant Chinese hospital-associated MRSA clone ST239, was originally isolated from a patient with bloodstream infection. Following 34-day serial passage selection, the MIC of daptomycin increased from 0.5 μg/mL to 16 μg/mL. The MICs of vancomycin and teicoplanin also increased from 1 μg/mL to 4 μg/mL and from 1 μg/mL to 8 μg/mL, respectively (Table 3). Strain 3d34 remained tolerant to 4 μg/mL daptomycin for 210 generations [12]. Exposure to daptomycin did not affect the MIC of oxacillin, and the 'seesaw effect' in daptomycin and oxacillin sensitivity

Table 3
Antimicrobial susceptibility and gene mutations of *Staphylococcus aureus* strains in the serial passage progress

Strain	Length of serial passage (days)	MIC (μg/mL)				Gene mutations									
		DAP	VAN	TEC	OXA	*mprF*		*walK*		*nreB*		*pyk*	*yydJ*		Intergenic region
						nt	aa	nt	aa	nt	aa				
3d0	0	0.5	1	1	>256	–	–	–	–	–	–	–	–	–	–
3d9	9	4	4	8	>256	G896T	G299V	T20A	L7Q	–	–	–	–	–	2 IS*256* inserts
3d18	18	8	8	8	>256	G896T	G299V	T673A	Y225N	–	–	–	–	–	3 IS*256* inserts
						T1417A	L473I								
3d27	27	8	4	8	>256	G896T	G299V	T673A	Y225N	A270G	–	–	378_379insIS*256*		2 IS*256* inserts
						T1417A	L473I								
3d34	34	16	4	8	>256	G896T	G299V	T673A	Y225N	–	–	475_476ins(39)	378_379insIS*256*		2 IS*256* inserts
						T1417A	L473I								

MIC, minimum inhibitory concentration; DAP, daptomycin; VAN, vancomycin; TEC, teicoplanin; OXA, oxacillin; nt, nucleotide; aa, amino acid.

previously described for daptomycin-resistant *S. aureus* strains was not observed [32,33]. The maximum concentration of daptomycin at which the bacteria could sustain growth kept increasing steadily throughout the 34-day selection process (Supplementary Fig. S1).

3.2. walK *and* mprF *mutations in daptomycin-non-susceptible* Staphylococcus aureus

The serial passage strains 3d9, 3d18, 3d27 and 3d34 were single colonies randomly picked from 9-, 18-, 27- and 34-day plate cultures, respectively. Five SNPs were detected by sequencing in these four serial passage strains compared with the parental strain 3d0 (Table 3). Four of these SNPs were associated with amino acid substitutions in MprF (G299V and L473I) and WalK (L7Q and Y225N), and the fifth was a synonymous SNP in the oxygen sensor histidine kinase NreB. The emergence of mutations in *mprF* and *walK* genes was associated with gradually increasing daptomycin MICs (Table 3). WalK (T225N) replaced WalK (L7Q) in the later period, whilst MprF (G299V) changed to be MprF (G299V+L473I).

3.3. *Insertions detected in daptomycin-resistant* Staphylococcus aureus

Five high-copy-number regions were found by counting clip-reads in serial passage strains compared with the parental strain 3d0. PCR with five unique primer pairs was used in the four serial passage strains and the parental strain 3d0 to confirm this finding (Table 2). Four of the five PCR products from the serial passage strains were approximately 1.3 kb larger than that of 3d0. The products were identified as an IS*256* insertion by sequence analysis (Fig. 1). One IS*256* was inserted in *yydJ* (encoding YydJ, a putative permease for export of a regulatory peptide), and three IS*256* were inserted in intergenic regions (Table 3). The fifth high-copy-number region was identified by sequence analysis as a 39-bp insert in the *pyk* gene.

3.4. mprF *overexpression due to IS*256 *insertion may contribute to daptomycin resistance*

Serial passage strains 3d9, 3d18, 3d27 and 3d34 contained an IS*256* at position −55 from the *mprF* ATG start codon. The *mprF* promoter site was detected by SoftBerry BPROM software using a 400-bp sequence upstream of the open-reading frame (ORF) (Fig. 2). According to the predicted position of the promoter site, the IS*256* was inserted in the *mprF* promoter. Thus, *mprF* expression in 3d0 and the serial passage strains in log growth phase was compared by RT-qPCR. *mprF* expression in 3d9, 3d18, 3d27 and 3d34 was significantly increased (>5-fold; $P < 0.001$) compared with 3d0 (Fig. 3A). Compared with 3d0, strain 3d9 harboured two IS*256* insertions (one in the *mprF* promoter and one between the *veg* and *ispE* genes). In addition, strain 3d9 had mutations in *walK* (T20A) and *mprF* (G896T). Next, *mprF* expression in 3d0W1 (3d0 with the *walK* mutation), 3d0M1 (3d0 with the *mprF* mutation), 3d0W1M1 (3d0 with both the *walK* mutation and the *mprF* mutation) and 3d0 was compared and no increase was observed (Fig. 3B). Therefore, the IS*256* insertion between the −10 and −35 *mprF* promoter elements likely is the cause of high *mprF* expression.

3.5. walK *and* mprF *point mutations increase the MIC of daptomycin with no fitness cost*

Wild-type *mprF* and *walK* of the parental strain 3d0 were replaced with the mutant *mprF* (G896T, T1417A) from 3d18, *walK* (T20A) from 3d9, and *walK* (T673A) from 3d18 separately to generate a total of 11 mutant derivatives with different *mprF* and *walK* mutation profiles (Table 1). The daptomycin MICs of these mutants

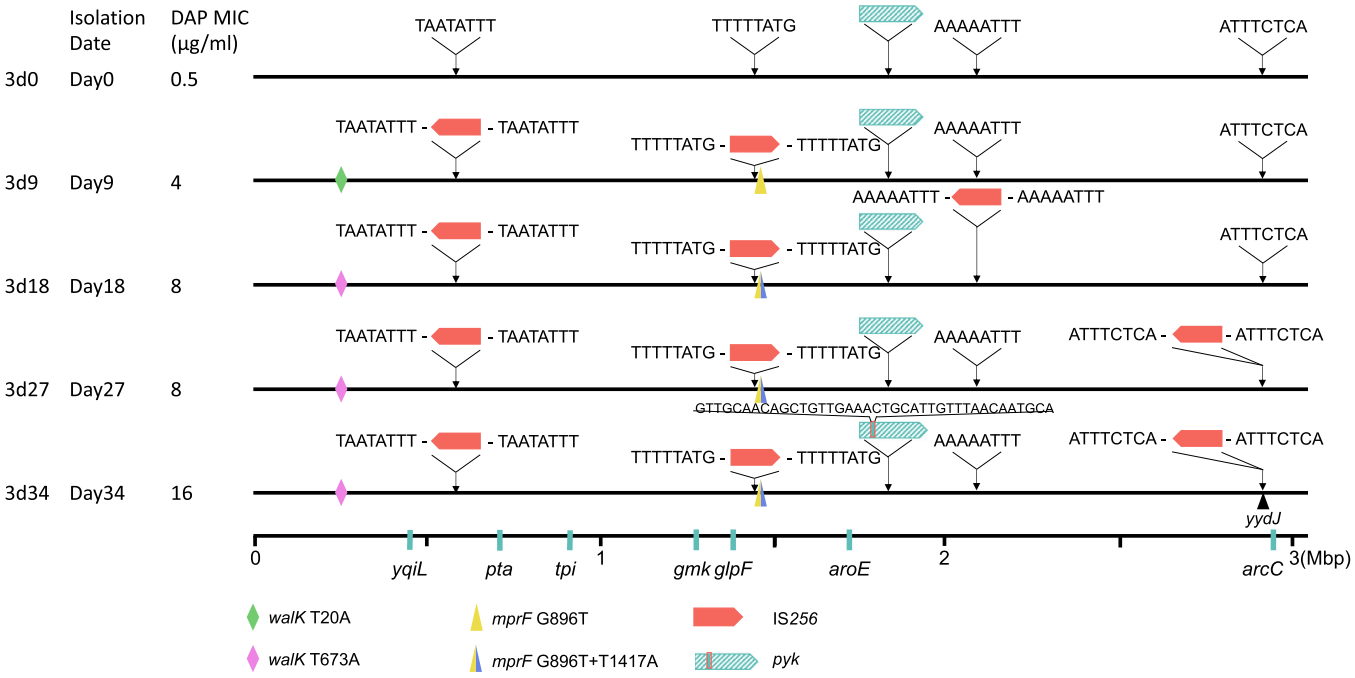

Fig. 1. Details of the genetic evolution in five methicillin-resistant *Staphylococcus aureus* strains during 34 days of daptomycin (DAP) selection passage. Strain 3d0 was the parental strain. The serial passage strains 3d9, 3d18, 3d27 and 3d34 were randomly picked single colonies isolated from the 9-, 18-, 27- and 34-day bacterial plate cultures, respectively. Minimum inhibitory concentration (MICs) of DAP for each strain were determined by broth microdilution. The orange arrows indicate IS*256*, and the sequences surrounding the arrows are direct repeats generated by the transposition event. Nucleotide mutations in *walK* are indicated by pink and green diamonds, and nucleotide mutations in *mprF* by yellow and blue triangles. Mint green hatched arrows with or without an orange box indicate a 39-bp insert fragment or no insert in *pyk*, respectively. The scale at the bottom indicates the relative position against the *S. aureus* TW20 reference genome. The seven mint green bars indicate the positions of the seven genes employed by the Oxford multilocus sequence typing (MLST) scheme.

were determined (Table 4). The daptomycin MICs for 3d0W1, 3d0W2, 3d0M1 and 3d0M2 were two-fold higher (1 μg/mL) than that of 3d0 (0.5 μg/mL). Interestingly, 3d0M12, which carried both *mprF* mutations (G896T and T1417A), displayed a daptomycin MIC of 2 μg/mL, indicating that the two point mutations may synergistically promote daptomycin resistance. In addition, strains with both *walK* and *mprF* mutations exhibited higher daptomycin MICs than strains with either *walK* or *mprF* mutations (Table 4). Interestingly, in addition to promoting non-susceptibility to daptomycin, *walK* single point mutation also contributed to vancomycin non-susceptibility (two-fold higher MIC than strains without such mutation), whereas *mprF* single point mutation did not affect the vancomycin MIC, which was consistent with a previous study [34]. Of note, the growth rates of the mutants were similar to that of 3d0 (Fig. 4), which is different to that of 3d0 and its serial passage strains (Supplementary Fig. S2).

3.6. walK *and* mprF *point mutations increase positive surface charge and cell wall thickness*

Cytochrome *c* binding assays revealed that 3d0W1, 3d0M1, 3d0M2 and 3d0M12 had significantly increased positive surface charge compared with the parental strain 3d0 (Fig. 5A). TEM images of 3d0W2, 3d0M1, 3d0M2 and 3d0M12 revealed an increased cell wall thickness compared with the parent strain 3d0 (Fig. 5B). Furthermore, the serial passage strains 3d9, 3d18, 3d27 and 3d34 presented significantly increased positive surface

Table 4
Antimicrobial susceptibility and gene mutations of strain 3d0 and its allelic replacement strains in this study

Strain	Gene mutation				MIC (μg/mL)			
	mprF		*walK*		DAP	VAN	TEC	OXA
	nt	aa	nt	aa				
3d0	–	–	–	–	0.5	1	1	>256
3d0W1	–	–	T20A	L7Q	1	2	4	>256
3d0W2	–	–	T673A	Y225N	1	2	4	>256
3d0M1	G896T	G299V	–	–	1	1	2	>256
3d0M2	T1417A	L473I	–	–	1	1	1	>256
3d0M12	G896TT1417A	G299VL473I	–	–	2	2	4	>256
3d0W1M1	G896T	G299V	T20A	L7Q	2	2	4	>256
3d0W1M2	T1417A	L473I	T20A	L7Q	2	2	4	>256
3d0W1M12	G896TT1417A	G299VL473I	T20A	L7Q	4	4	8	>256
3d0W2M1	G896T	G299V	T673A	Y225N	2	2	4	>256
3d0W2M2	T1417A	L473I	T673A	Y225N	2	2	4	>256
3d0W2M12	G896TT1417A	G299VL473I	T673A	Y225N	4	4	16	>256

nt, nucleotide; aa, amino acid; MIC, minimum inhibitory concentration; DAP, daptomycin; VAN, vancomycin; TEC, teicoplanin; OXA, oxacillin.

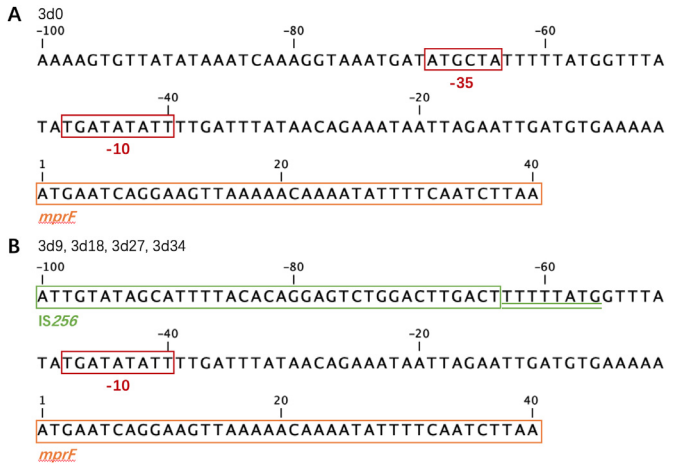

Fig. 2. Identification of the promoter of *mprF* in (A) parental strain 3d0 and (B) the serial passage strains 3d9, 3d18, 3d27 and 3d34. The insertion sequence IS*256* is boxed in green, and the direct repeats are double underlined in green. The predicted −35 and −10 motifs of the promoter are in red boxes, whereas open-reading frame (ORF) sequences are in orange boxes.

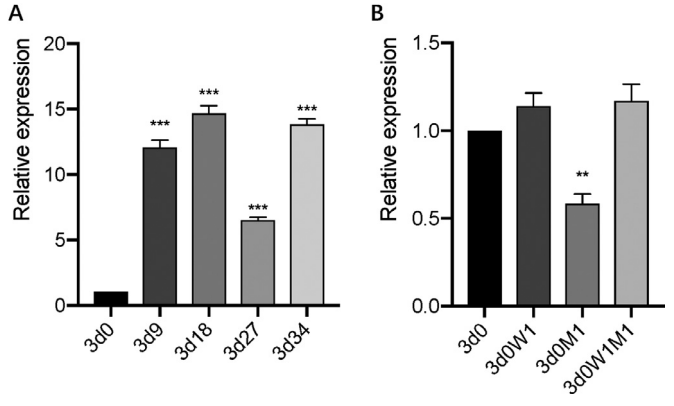

Fig. 3. Relative *mprF* gene transcript expression in (A) parental strain 3d0 and its daptomycin-resistant serial passage strains and (B) point mutant derivatives having the same mutation as 3d9 as determined by reverse transcription quantitative PCR, with *gyrB* as an internal control. Samples were prepared from cells in log growth phase ($OD_{600} = 0.6$) grown in tryptic soy broth. Data are the mean ± standard deviation of three biological repeats. ** $P < 0.01$ and *** $P < 0.001$ (vs. 3d0).

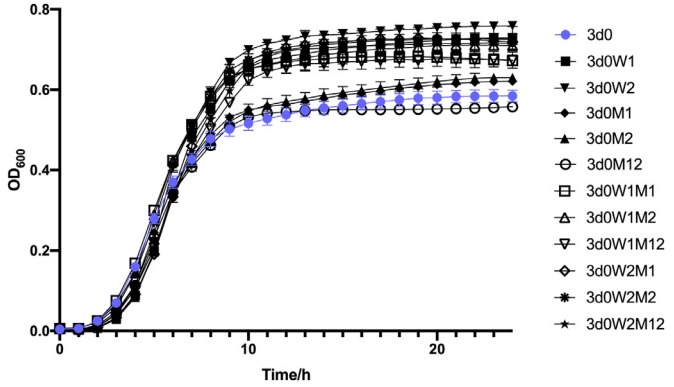

Fig. 4. Growth curves of parental strain 3d0 and its allelic replacement strains. *Staphylococcus aureus* strains were grown aerobically in tryptic soy broth at 37 °C. Data are the mean ± standard deviation of three independent experiments. OD_{600}, optical density at 600 nm.

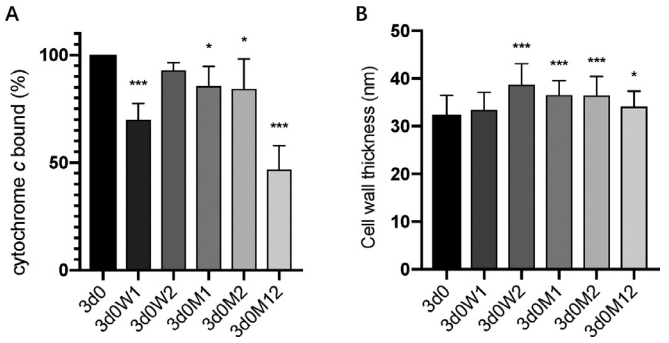

Fig. 5. (A) Relative positive surface charge by cytochrome *c* binding and (B) cell wall thickness by transmission electron microscopy of parental strain 3d0 and its allelic replacement strains. Percent of cytochrome *c* bound after 15 min of incubation with *Staphylococcus aureus* cells at room temperature was shown. Fifty cells were analysed for each strain. Data are the mean ± standard deviation. * $P < 0.05$ and *** $P < 0.001$ (vs. 3d0).

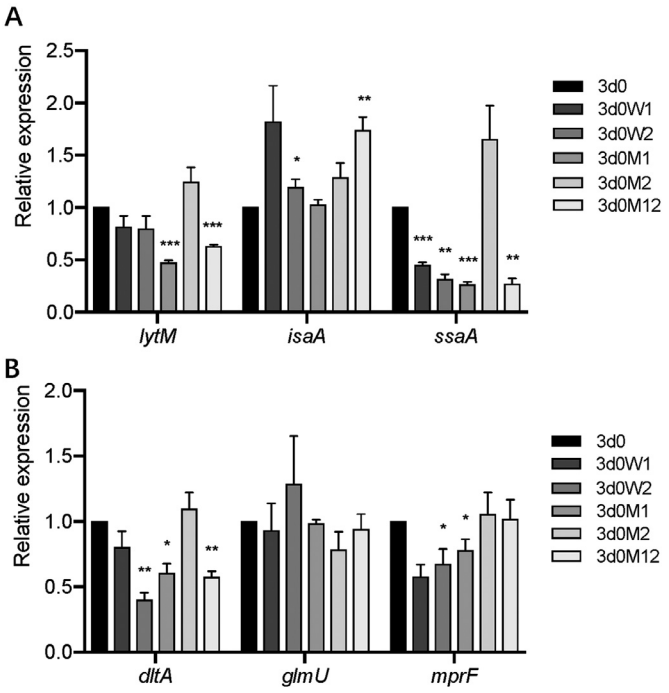

Fig. 6. Relative expression of genes involved in (A) cell wall degradation (*lytM, isaA* and *ssaA*) and (B) cell wall synthesis and modification (*dltA, glmU* and *mprF*) in parental strain 3d0 and its allelic replacement strains as determined by reverse transcription quantitative PCR (RT-qPCR) with *gyrB* as an internal control. Samples were prepared from cells in log growth phase ($OD_{600} = 0.6$) grown in tryptic soy broth. Data are the mean ± standard deviation of three biological repeats. * $P < 0.05$, ** $P < 0.01$ and ***$P < 0.001$ (vs. 3d0).

charge compared with the parent strain 3d0 (Supplementary Fig. S3).

3.7. walK *and* mprF *point mutations alter the expression of genes involved in cell wall degradation*

Genes involved in cell wall autolysis, such as *lytM, ssaA* and *isaA*, are positively controlled by the WalKR system [35]. To test the effect of *walK* and *mprF* point mutations on the expression of autolysis genes, the relative transcript levels of these genes in mutants were evaluated by RT-qPCR. As shown in Fig. 6A, expression of *lytM* and *ssaA* was significantly decreased in MprF (G299V and G299V+L473I) mutants compared with 3d0. *ssaA* expression was also significantly decreased in WalK (L7Q and Y225N) mutants

compared with 3d0. In contrast, *isaA* expression was significantly increased in WalK (Y225N) and MprF (G299V+L473I) mutants. Genes involved in cell wall biosynthesis, including *dltA, glmU* and *mprF*, did not show altered expression in any of the mutants analysed (Fig. 6B).

4. Discussion

Single point mutations in *mprF* and *walK* have been identified as contributing to daptomycin resistance [5,36–38]. However, few studies have characterised the genome evolution of daptomycin-non-susceptible MRSA [39,40]. The current study revealed, for the first time, dynamic details of the genome evolution of a clinical isolate acquiring non-susceptibility to daptomycin. The isolate used in this study was originally obtained from a patient with bloodstream infection and had a genetic background representing the predominant epidemic clone of MRSA in China (ST239). It was found that point mutations in *mprF* and *walK* along with IS*256* insertions may contribute to daptomycin non-susceptibility as observed in the in vitro selection system.

Although IS*256* has been shown to be implicated in daptomycin non-susceptibility [17], few studies, if any, have assessed its role in modulating *mprF* expression. In this study, IS*256* insertion in the *mprF* promotor region was detected, which resulted in higher expression of *mprF*. Previous reports have suggested that increased expression of a mutant *mprF* in *S. aureus* may be associated with daptomycin resistance [41–43]. Thus, IS*256* insertion between the −10 and −35 *mprF* promoter elements was likely the cause of daptomycin non-susceptibility in this study. The positive surface charge determined by cytochrome *c* binding assays also revealed that *mprF* overexpression actually translates to gain-in-phenotypic function.

Two novel point mutations in *mprF* (G896T, T1417A) and *walK* (T20A, T673A) were identified, which could independently or synergistically contribute to daptomycin non-susceptibility [5–10,37]. Interestingly, the T20A mutation in *walK* was detected in an early phase of the selection process (Day 9), whereas the *walK* T673A mutation was detected in a later phase (Day 18 to Day 34), and this mutation was retained throughout the selection (Table 3). No difference in growth ability between these two mutant strains was observed. The mechanism contributing to the change from T20A to T673A mutation remains to be determined.

mprF and *walK* point mutations displayed a synergistic effect in promoting daptomycin non-susceptibility, as strains harbouring both *walK* and *mprF* mutations exhibited higher daptomycin MICs than strains harbouring either of the mutations. Although *walK* and *mprF* mutations have been previously associated with daptomycin non-susceptibility [36,44], genetic manipulation of both *walK* and *mprF* mutations to confirm the contribution of these mutations to daptomycin resistance has not been reported. It is likely that *mprF* and *walK* mutations affect different pathways, resulting in the additive effect of these mutations in decreasing daptomycin susceptibility.

Previous studies have suggested that daptomycin non-susceptibility in MRSA is associated with a high fitness cost mediated by mutations in *mprF* [6,45]. Our previous study also revealed that 3d34 had decreased in vitro serum tolerance, weakened lethality and pathogenicity in mice, and a thicker cell wall than the parental strain 3d0 [12]. Interestingly, neither of the strains with point mutations in *mprF* or the strains with point mutations in *walK* showed decreased growth ability compared with the parental strain. Thus, the fitness cost of 3d34 may be caused by other insertions, mutations or transcriptome changes, which requires further investigation. *walK* and *mprF* point mutant strains showed a thicker cell wall and more positive surface charge, similar to previous studies [30,46]. It also means that the *mprF* point mutations translate to gain-in-phenotypic function.

Furthermore, the current results suggest a relationship between *mprF* and *walK* mutations and decreased expression of cell wall degradation-related genes. Part of the transcriptional analysis results differed from those in previous studies [34,47], which may partly be associated with the different backgrounds of the strains in the current study and the model strains used in previous studies. It may also be due to the expression of genes involved in the daptomycin-resistant phenotype not being reduced or increased, but due to dysregulation of genes such as *dltA* that may not be 'shut off' during the stationary growth phase.

One of the limitations of this study is that only the reference strain *S. aureus* TW20 was used in genome sequence comparison and thus genes that are not present in *S. aureus* TW20 may have been missed. Strain TW20, which was sequenced in 2010, is widely used as a reference strains because of the high quality of the genome sequence [48–50]. It contains resistance genes encoded on mobile genetic elements as well as mutations in housekeeping genes [51], therefore the possibility that we missed any gene is low and can almost be ignored.

In conclusion, this study revealed critical details of the genetic evolution during in vitro MRSA daptomycin resistance acquisition. This is the first report that IS*256* insertion in the *mprF* promoter region promotes MprF overexpression. Two novel point mutations in *mprF* and *walK* were identified, which could independently or synergistically contribute to daptomycin non-susceptibility without sacrificing fitness. Thus, this study may have significant clinical relevance as it sheds light on the possible mechanisms underlying daptomycin resistance acquisition in MRSA in the clinic.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.08.021.

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