Mechanisms of high-level fosfomycin resistance in *Staphylococcus aureus* epidemic lineage ST5

Tianchi Chen[1]†, Lin Zhao[1]†, Yao Liu[1], Ya'nan Wang[1], Ying Jian[1], Na Zhao[1], Ziyu Yang[1], Xi Wang[1], Qian Liu[1]
and Min Li[1,2]*

[1]*Department of Laboratory Medicine, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China;*
[2]*Faculty of Medical Laboratory Science, College of Health Science and Technology, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China*

*Corresponding author. E-mail: ruth_limin@126.com
†These authors contributed equally to this paper.

Objectives: Fosfomycin resistance has become a clinical concern. In this study, we analysed the dynamic change of fosfomycin MIC in the epidemic *Staphylococcus aureus* lineages in a teaching hospital in Shanghai for 12 years and sought to elucidate the major underlying mechanisms.

Methods: MLST was conducted for 4580 *S. aureus* isolates recovered from 2008 to 2019. Fosfomycin MIC was determined by the agar dilution method. The genome data of 230 *S. aureus* epidemic lineage isolates were acquired from a next-generation sequencing (NGS) platform. Gene deletion and corresponding complementation mutants were constructed to confirm the mechanism of fosfomycin resistance.

Results: The predominant *S. aureus* lineages during the past 12 years were ST5 and ST239 (45.6%; 2090/4580). However, ST5 has been spreading clinically, while ST239 has gradually disappeared recently. Consistent with epidemic trends, fosfomycin-resistant ST5 increased from 19.5% to 67.3%. Most fosfomycin-resistant ST5 isolates (92.7%; 647/698) possessed high-level resistance (MIC > 1024 mg/L) with combined mutations mainly in *glpT* and *uhpT*. In contrast, fosfomycin-resistant ST239 isolates (76.8%; 149/194) mainly acquired low-level resistance (MIC = 64–128 mg/L) with mutation primarily in *hptA*. Deletion of a single resistant gene merely resulted in low-level fosfomycin resistance, while double-gene mutants Δ*glpT*Δ*uhpT*, Δ*glpT*Δ*hptA* and Δ*glpT*Δ*hptR* acquired high-level fosfomycin resistance.

Conclusions: The high-level fosfomycin resistance of *S. aureus* epidemic lineage ST5 is mainly due to the accumulation of mutations in the resistant genes related to membrane transporter systems, and partly contributes to its persistent prevalence under clinical antibiotic pressure.

Introduction

Staphylococcus aureus is an infamous pathogen that poses a great threat in both community and healthcare settings. As the third most prevalent pathogen, accounting for 9.38% of clinical isolates in China in 2021 (CHINET; http://www.chinets.com/Data/AntibioticDrugFast), *S. aureus* can cause severe or complicated infections, such as endocarditis, bacteraemia, deep-seated skin and soft-tissue infections and osteoarticular infections.[1,2] Moreover, *S. aureus* is an adaptable bacterium that can survive in harsh environments and acquire resistance to multiple clinically available antibiotics through chromosomal gene mutations and/or horizontal gene transfer.[3–5] With the extensive use of antibiotics, MDR *S. aureus* strains exist, among which MRSA is

one of the most notorious cases.[6,7] Vancomycin, which serves as both the first treatment option and the last resort, has been increasingly applied for MRSA infections.[8,9] Subsequently, the first vancomycin-resistant *S. aureus* was reported by Hiramatsu *et al.*[10] in Japan and a phenomenon of sluggish increase in vancomycin MIC, called 'MIC creep', was also reported.[11] Taking both the lack of effective clinical treatment and the difficulty to develop new antibiotics into consideration, the re-evaluation of older antimicrobial agents appears fascinating.[12,13] Recently, fosfomycin has been assessed and gained considerable attention because of its relatively broad antibacterial spectrum.

The broad-spectrum antimicrobial ability of fosfomycin largely benefits from its unique mechanism of action. Fosfomycin uses two membrane transporter systems, the L-α-glycerol-3-phosphate

transporter (GlpT) and the glucose-6-phosphate (G-6-P) transporter (UhpT), to invade the bacterium.[12] The UhpT system is induced in the presence of G-6-P and serves as an alternative pathway of the GlpT system.[14] Subsequently, fosfomycin inhibits the synthesis of the bacterial cell wall by covalently binding to the thiol group on a cysteine of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), leading to the loss of peptidoglycan layer integrity and cell lysis.[12,15–18] Deficiency in humans and little homology with human proteins make MurA an ideal target for treatment of infections.[19] Fosfomycin can penetrate biofilms and acquire intracellular bactericidal activity owing to its low molecular weight. Afterwards, fosfomycin may support other antibiotics to reach targets more efficiently, improving therapeutic effects of combined antibiotics.

Since guidelines for the treatment of uncomplicated lower urinary tract infections were modified in 2010 to recommend fosfomycin as a first-line agent,[20] its usage has increased exponentially,[21] which has inevitably led to the emergence of fosfomycin-resistant *S. aureus*. Research concerning MRSA isolated from a university hospital in China from 2013 to 2015 demonstrated that the overall fosfomycin resistance rate reached 53.2%,[22] and Lee *et al.*[23] reported a significant increase in the fosfomycin resistance rate, from 3.4% in 2002 to 11.0% in 2012. Therefore, numerous studies have been devoted to discovering the mechanisms conferring resistance to fosfomycin. First, mutations concerning two transporters prevent fosfomycin from invading the bacterium, including: mutations in *uhpT* and *glpT*, affecting the function of transporters; mutations in the *hptRS* regulon (*hptA-hptRS-uhpT*), interfering with the expression of *uhpT*;[24] and mutations in *cyaA* and *ptsI*, lowering the cAMP level and down-regulating two transporters.[25,26] Additionally, fosfomycin resistance resulted from mutations in *murA*, overexpression of the Tet38 efflux pump, which might be regulated by MgrA, and carriage of fosfomycin-modifying enzyme genes, *fosA*, *fosB* and *fosX* (*Staphylococcus* spp. mainly possess *fosB*).[27–31]

The prevalence of *S. aureus* has depended mainly on lineage spread. According to epidemiological reports in China, the major epidemic *S. aureus* lineages include ST5 and ST239, which have gained great resistance to commonly used antibiotics.[32–35] However, research on the dynamic change of fosfomycin resistance in these lineages is still scarce. Furthermore, we previously discovered gradual alteration of isolation rates in ST5 and ST239 lineages during the past decade.[7,32] Whether this alteration was associated with antibiotic selection pressure was also ambiguous. In this study, we conducted epidemiological analysis on 4580 non-repetitive successive *S. aureus* isolates collected in a Chinese teaching hospital from 2008 to 2019. After confirming ST5 and ST239 as major epidemic lineages, we explored the dynamic changes of fosfomycin resistance and the underlying mechanisms to elucidate that *S. aureus* lineage ST5 can be highly resistant to fosfomycin through mutation accumulation in resistance-related genes, contributing to its prevalence under antibiotic pressure.

Materials and methods

Ethics

This study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China (approval number: 2019016). This project was a retrospective study. The *S. aureus* isolates from de-identified patient samples were cultured and identified in routine microbiology laboratories. Patients were not involved in any way in the study and only molecular analysis of the bacteria was performed. Therefore, informed consent was not required for participation in this research.

Bacterial isolates and growth conditions

A total of 4580 sequential and non-repetitive clinical *S. aureus* isolates were collected and stored as described before[32] from the university-affiliated tertiary hospital, which is a centrally located and a particularly representative teaching hospital in Shanghai, with 2000 beds and 10 000 admissions per day. Detailed information of the isolates involved in each trial is shown in Figure S1, available as Supplementary data at *JAC* Online. *S. aureus* isolates were grown in tryptic soy broth (TSB, Oxoid) or on sheep blood agar plates, and *Escherichia coli* was grown in LB medium (Oxoid) at 37°C. Antibiotics were added at the following concentrations: ampicillin, 100 mg/L; chloramphenicol 10 mg/L, when necessary.

Molecular typing

MLST of *S. aureus* isolates was carried out by detecting seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) as described previously.[36] The sequences were then submitted to the *S. aureus* MLST database for ST type confirmation (https://pubmlst.org).

Antimicrobial susceptibility testing

The MIC of fosfomycin was determined by the agar dilution method. Mueller–Hinton agar was supplemented with 25 mg/L G-6-P, as recommended by EUCAST (https://www.eucast.org/clinical_breakpoints/). Result interpretation was also based on the clinical breakpoints guideline of EUCAST (susceptible, MIC ≤32 mg/L; resistant, MIC >32 mg/L). *S. aureus* ATCC 29213 was used as the control. Other antimicrobial susceptibility was evaluated by the bioMérieux VITEK 2 system following the manufacturer's instructions, and results were interpreted according to CLSI guidelines.

WGS and phylogenetic analysis based on multi-locus sequence analysis (MLSA)

As listed in Table S2, a total of 230 *S. aureus* isolates were randomly selected by the random selection module in Microsoft Office Excel for WGS, which was performed on the NovaSeq 6000 or HiSeq 4000 sequencing platform (Illumina Inc., San Diego, CA, USA). The sequences are available in the Sequence Read Archive (BioProject: PRJNA803935 and PRJNA660290). Read quality control, trimming of the raw data, *de novo* assembly, whole-genome alignment and mutation calling were all conducted in CLC Genomics Workbench 20.0 (QIAGEN, Aarhus, Denmark) with the default option. The chromosomal sequence of *S. aureus* N315 (ST5, fosfomycin susceptible, NCBI accession code: NC_002745.2) was used as the reference for read mapping. The process of mutation screening is shown in Figure S3. The sequences of five major fosfomycin resistance-related genes were extracted separately from the assembled contigs. The following phylogenetic analyses were all conducted in Phylosuite.[37] The progressive multiple sequence alignments were accomplished by MAFFT, and the aligned sequences were concatenated into a single dataset with default options (~6024 bp). PartitionFinder2 was then used for selection of the best-fit partitioning scheme. IQ-TREE directly recognized the results of PartitionFinder2 as the run parameters to construct a maximum-likelihood (ML) tree, with bootstrap choosing ultrafast. The trees in this research were annotated and managed using iTOL (https://itol.embl.de/).[38]

Allelic gene replacement by homologous recombination and genetic complementation

Strains and plasmids used for mutant construction are listed in Table 1 and oligonucleotides used in this study are listed in Table S1. Gene deletion and complementation mutants were constructed as previously described.[39] The concise description is available in the Supplementary data.

Statistical analysis

Chi-squared tests were performed to analyse statistical significance. Linear trends were tested using linear correlation and regression or the chi-squared test for trend. All data were analysed using SPSS 26 and GraphPad Prism 8.0. Error bars indicate the standard deviation (mean ± SD), and $P < 0.05$ was considered as statistically significant.

Results

ST5 has replaced ST239 to become the current major epidemic lineage

A total of 4580 non-repetitive *S. aureus* isolates were recovered for MLST typing. Figure 1a exhibits the distribution of major clonotypes and reveals ST5 and ST239 (45.6%; 2090/4580) as the predominant clones during the past 12 years. These data, in combination with those in Figure 1b, which emphasizes isolation rates of ST5 and ST239 separately, disclosed to us an interesting clinical phenomenon: that the isolation rate of *S. aureus* ST5 had been at a stable high level during the study period (from 41.9% in 2008 to 32.0% in 2019), while the isolation rate of ST239 was decreasing gradually (from 40.6% in 2008 to 0.61% in 2019; $P < 0.001$). In other words, ST5 and ST239 had distinct epidemiology trends in which ST5 maintained its prevalence while ST239 gradually faded out in predominance from 2008 to 2019, indicating that ST5 replaced ST239 as the major prevalent lineage.

Fosfomycin resistance rate was consistent with the epidemiology trends in ST5 and ST239

Focusing on these two clinically predominant lineages (2090 altogether, involving 1433 ST5 and 657 ST239 isolates), we analysed the resistance rate of antibiotics frequently used in clinical practice, including penicillin, cefazolin, cefoxitin, erythromycin, fosfomycin, gentamicin, levofloxacin, linezolid and vancomycin. All isolates were susceptible to linezolid and vancomycin, suggesting their superior antimicrobial activity. It is obvious that resistance rates of *S. aureus* ST5 were lower than or similar to those of ST239 for most selected antibiotics, but in fosfomycin the trend was reversed. The fosfomycin resistance rate of *S. aureus* ST5 increased significantly from 19.5% (63/323) in 2008 to 67.3% (105/156) in 2019 ($P < 0.001$) and surpassed ST239 around 2010, as shown in Figure 2. Concisely, the dynamic change of fosfomycin resistance was consistent with ST5 and ST239 epidemiology trends.

Fosfomycin resistance level of S. aureus ST5 was higher than that of ST239

To further investigate the resistance variance between ST5 and ST239, the distribution of MICs for 892 fosfomycin-resistant isolates was arranged and shown in the form of a heatmap (Figure 3). Of interest was that the proportion of fosfomycin MIC > 1024 mg/L in *S. aureus* ST5 remained substantial from 2008 to 2019 (647/698; 92.7% on average), indicating that most ST5 isolates were highly resistant to fosfomycin. In contrast, the majority of ST239 isolates gathered in MIC = 64 mg/L and MIC = 128 mg/L groups (149/194; 76.8% on average), representing a relatively lower resistance level. In summary, we observed a significant difference in fosfomycin resistance level between the current epidemic *S. aureus* ST5 and the previously prevalent ST239 isolates (>1024 mg/L, high resistance level: 92.7% in ST5 versus 11.5% in ST239, $P < 0.001$; and 64 and 128 mg/L, low resistance level: 6.4% in ST5 versus 76.8% in ST239, $P < 0.001$).

Most frequent mutations related to fosfomycin resistance were dissimilar between ST5 and ST239 fosfomycin-resistant isolates

In order to disclose the molecular mechanisms underlying the difference of fosfomycin resistance level between the two STs, 230 *S. aureus* isolates (168 ST5 and 62 ST239 isolates) were randomly selected and are listed in Table S2. The fosfomycin resistance rates of sequenced *S. aureus* ST5 and ST239 isolates were 73.2% (123/168) and 48.4% (30/62), respectively. Similar to our aforementioned results, the MICs of most sequenced ST5 isolates were >1024 mg/L (105/123; 85.4%), while ST239 isolates were mainly distributed across MIC = 64 mg/L and MIC = 128 mg/L (21/32; 65.6%), as shown in Figure S2.

Additionally, 11 fosfomycin resistance-related genes were acquired through literature retrieval, including *uhpT*, *glpT*, *hptR*, *hptS*, *hptA*, *ptsI*, *cyaA*, *murA*, *fosB*, *tet38* and *mgrA* (described in the Introduction). Mutations within these 11 selected fosfomycin resistance-related genes were acquired from mutation-calling of CLC workbench for further investigation. After excluding synonymous and benign mutations, we found that *glpT*, *hptA*, *hptS*, *hptR* and *uhpT* were the five major genes with superior mutation rates in fosfomycin-resistant isolates (shown in Figure 4a and Figure S3). These five fosfomycin resistance-related genes mainly involved two membrane transporter systems, GlpT and UhpT (including the UhpT regulon). It is noteworthy that mutations mostly occurred in *glpT* (103/123; 83.7%) and *uhpT* (79/123; 64.2%) in ST5 fosfomycin-resistant isolates, but in *hptA* (24/30; 80.0%) in ST239 fosfomycin-resistant isolates. Figure 4b exhibits nonsynonymous mutation types and positions in five fosfomycin resistance-related genes of ST5 and ST239. A total of 14 mutation types of *uhpT* and five mutation types of *glpT* were detected among ST5 fosfomycin-resistant isolates, while merely two different mutation types were spotted in the *hptA* gene in ST239. The mutation types, amino acid substitution and mutation frequency (rate) of five major fosfomycin resistance-related genes are elaborated in Table 2. Despite numerous kinds of mutations in fosfomycin resistance-related genes, no specific predominant mutation type was detected as being responsible for the fosfomycin resistance. In summary, the discrepancy detected between ST5 and ST239 fosfomycin-resistant *S. aureus* was that of the genes with the highest mutation rates (*glpT* and *uhpT* in ST5, but *hptA* in ST239).

Phylogenetic analysis based on MLSA assisted in exploration of the major mechanism of fosfomycin resistance

In order to elucidate the impact of the aforementioned five major genes on fosfomycin resistance, *S. aureus* ST5 isolates were

Table 1. Bacterial strains and plasmids used in this study

| Strain and plasmids	Description	Source	Fosfomycin MIC (mg/L)
S. aureus			
RN4220	derived from NCTC8325-4; r−m+	de Azavedo *et al.*[47]	
N315	ST5 reference sequence used for mapping	NC_002745.2	4
2018140	ST5 clinical isolates	This study	4
2018140-Δ*uhpT*	2018140 *uhpT* deletion mutant	This study	64
2018140-Δ*uhpT*±pOS1-*uhpT*	2018140 *uhpT* deletion mutant with pOS1-*uhpT*	This study	16
2018140-Δ*glpT*	2018140 *glpT* deletion mutant	This study	8
2018140-Δ*glpT*±pOS1-*glpT*	2018140 *glpT* deletion mutant with pOS1-*glpT*	This study	4
2018140-Δ*hptA*	2018140 *hptA* deletion mutant	This study	64
2018140-Δ*hptA*±pOS1-*hptA*	2018140 *hptA* deletion mutant with pOS1-*hptA*	This study	32
2018140-Δ*hptR*	2018140 *hptR* deletion mutant	This study	64
2018140-Δ*hptR*±pOS1-*hptR*	2018140 *hptR* deletion mutant with pOS1-*hptR*	This study	16
2018140-Δ*glpT*Δ*uhpT*	2018140 *glpT*/*uhpT* double mutant	This study	>1024
2018140-Δ*glpT*Δ*hptA*	2018140 *glpT*/*hptA* double mutant	This study	>1024
2018140-Δ*glpT*Δ*hptR*	20167 *glpT*/*hptR* double mutant	This study	>1024
2018140-Δ*uhpT*Δ*hptA*	20167 *uhpT*/*hptA* double mutant	This study	64
2018140-Δ*uhpT*Δ*hptR*	20167 *uhpT*/*hptR* double mutant	This study	64
2018140-Δ*hptA*Δ*hptR*	20167 *hptA*/*hptR* double mutant	This study	64
20167	ST239 clinical isolates	This study	4
20167-Δ*uhpT*	20167 *uhpT* deletion mutant	This study	128
20167-Δ*uhpT*±pOS1-*uhpT*	20167-Δ*uhpT* complemented with *uhpT* by plasmid pOS1	This study	32
20167-Δ*glpT*	20167 *glpT* deletion mutant	This study	8
20167-Δ*glpT*±pOS1-*glpT*	20167-Δ*glpT* complemented with *glpT* by plasmid pOS1	This study	4
20167-Δ*hptA*	20167 *hptA* deletion mutant	This study	64
20167-Δ*hptA*±pOS1-*hptA*	20167-Δ*hptA* complemented with *hptA* by plasmid pOS1	This study	32
20167-Δ*hptR*	20167 *hptR* deletion mutant	This study	64
20167-Δ*hptR*±pOS1-*hptR*	20167-Δ*hptR* complemented with *hptR* by plasmid pOS1	This study	16
20167-Δ*glpT*Δ*uhpT*	20167 *glpT*/*uhpT* double mutant	This study	>1024
20167-Δ*glpT*Δ*hptA*	20167 *glpT*/*hptA* double mutant	This study	>1024
20167-Δ*glpT*Δ*hptR*	20167 *glpT*/*hptR* double mutant	This study	>1024
20167-Δ*uhpT*Δ*hptA*	20167 *uhpT*/*hptA* double mutant	This study	128
20167-Δ*uhpT*Δ*hptR*	20167 *uhpT*/*hptR* double mutant	This study	128
20167-Δ*hptA*Δ*hptR*	20167 *hptA*/*hptR* double mutant	This study	64
E. coli			
Top10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80 *lacZ*ΔM15Δ *lacX74 recA1 ara*Δ139Δ(*ara-leu*) 7697 *galU galK rpsL* (*StrR*) *endA1 nupG*	TIANGEN Biotech	
Plasmids			
pKOR1	*E. coli*/*Staphylococcus* shuttle cloning plasmid used for allelic replacement, *cmR*, *ampR*	Bae *et al.*[48]	
pKOR1-Δ*uhpT* (ST5/ST239)	pKOR1 for deletion of *uhpT* in ST5 and ST239	This study	
pKOR1-Δ*glpT* (ST5/ST239)	pKOR1 for deletion of *glpT* in ST5 and ST239	This study	
pKOR1-Δ*hptA* (ST5/ST239)	pKOR1 for deletion of *hptA* in ST5 and ST239	This study	
pKOR1-Δ*hptR* (ST5/ST239)	pKOR1 for deletion of *hptR* in ST5 and ST239	This study	
pOS1	*E. coli*/*Staphylococcus* shuttle cloning plasmid used for complementation, *cmR*, *ampR*	Bubeck *et al.*[49]	
pOS1-*uhpT* (ST5/ST239)	pOS1 with insertion of *uhpT* in ST5 and ST239	This study	
pOS1-*glpT* (ST5/ST239)	pOS1 with insertion of *glpT* in ST5 and ST239	This study	
pOS1-*hptA* (ST5/ST239)	pOS1 with insertion of *hptA* in ST5 and ST239	This study	
pOS1-*hptR* (ST5/ST239)	pOS1 with insertion of *hptR* in ST5 and ST239	This study	

S. aureus isolates with underlining indicate those complemented with pOS1.

selected for further investigation on account of the superior fosfomycin resistance level and rate. Figure 5a demonstrated that the five-gene scheme could approximately divide 168 isolates into susceptible, low-level (clade I and clade II) and high-level resistant categories, implying the close association of these genes with fosfomycin resistance. What interested us was that most
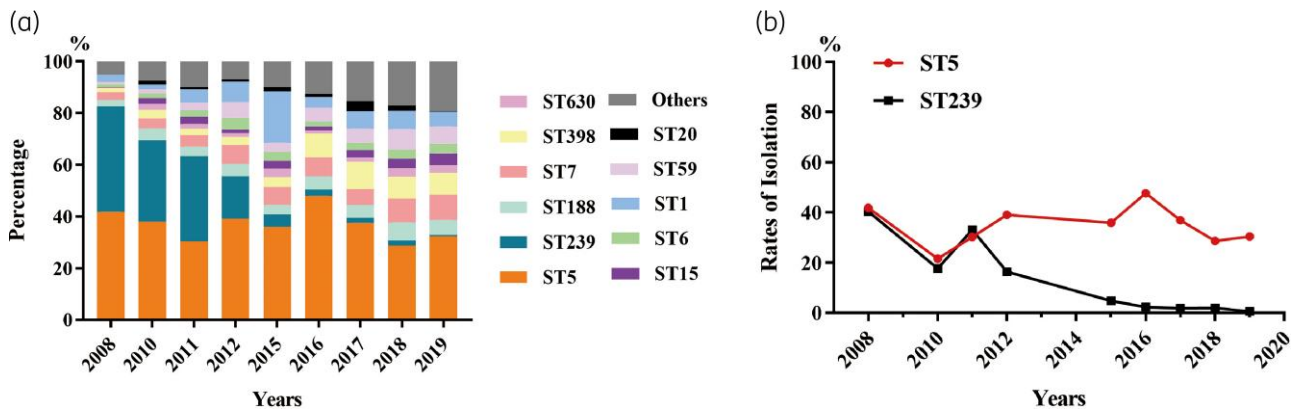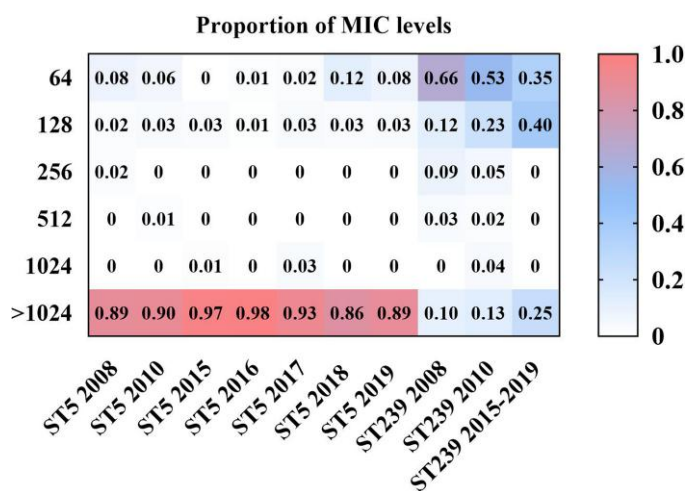
Figure 1. The epidemiology characteristics of *S. aureus* lineages, from 2008 to 2019. (a) The dynamic changes of lineages of *S. aureus* recovered from the study period. Blocks are coloured to reflect the proportion of corresponding lineages. (b) Isolation rates of two epidemic *S. aureus* lineages, ST5 and ST239. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.
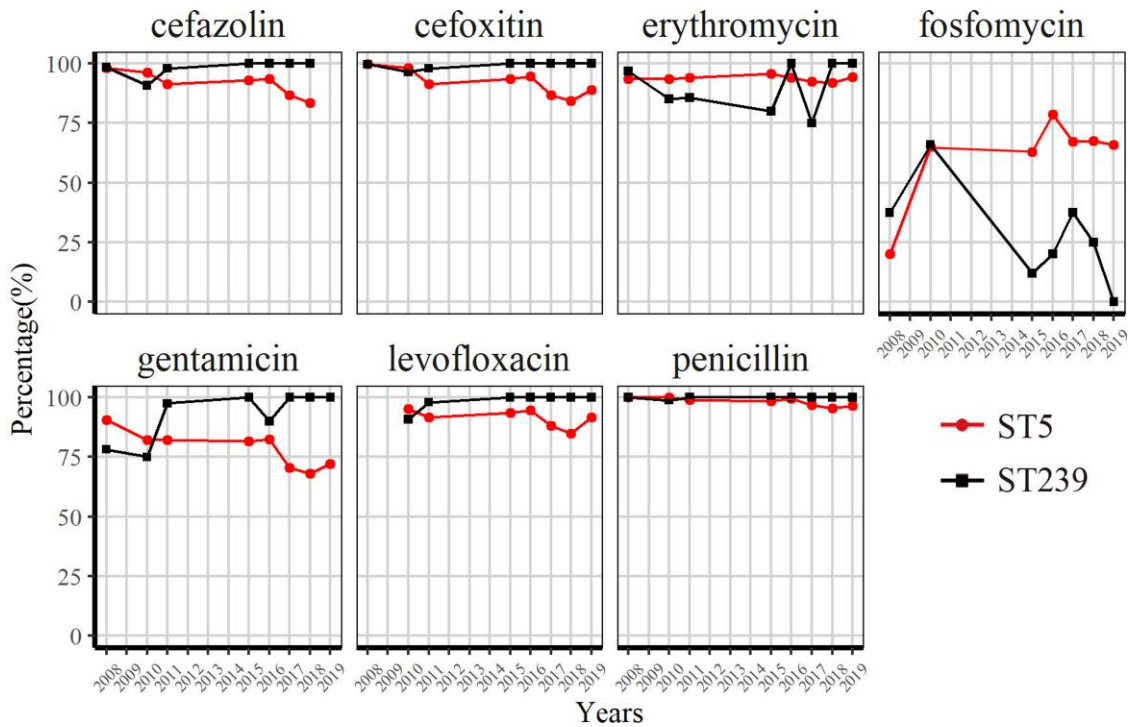
Figure 2. Resistance rates of commonly used antibiotics for *S. aureus* ST5 (red circles) and ST239 (black squares). The chosen antibiotics included penicillin, cefazolin, cefoxitin, erythromycin, fosfomycin, gentamicin, levofloxacin, linezolid and vancomycin. All isolates were susceptible to linezolid and vancomycin (not shown here). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

isolates in low-level fosfomycin-resistant clades had fewer mutated genes, indicating the probability that the accumulated mutations of multiple genes could lead to high-level fosfomycin resistance. Nevertheless, the existence of isolates with high-level fosfomycin resistance but only one mutated gene suggested the irrationality of excluding the hypothesis that fosfomycin resistance resulted from one critical gene mutation.

Since many isolates shared identical concatenated sequences, we de-duplicated the repetitive genotypes, shown in Figure 5b. As a result, a total of 21 distinct genotypes were attained, among which 16 genotypes were associated with high-level fosfomycin resistance (MIC > 1024 mg/L). The mutated genes and the number of every genotype are shown on the right side of Figure 5b. Most genotypes (12/21; 57.1%) had mutations

Proportion of MIC levels

	ST5 2008	ST5 2010	ST5 2015	ST5 2016	ST5 2017	ST5 2018	ST5 2019	ST239 2008	ST239 2010	ST239 2015-2019
64	0.08	0.06	0	0.01	0.02	0.12	0.08	0.66	0.53	0.35
128	0.02	0.03	0.03	0.01	0.03	0.03	0.03	0.12	0.23	0.40
256	0.02	0	0	0	0	0	0	0.09	0.05	0
512	0	0.01	0	0	0	0	0	0.03	0.02	0
1024	0	0	0.01	0	0.03	0	0	0	0.04	0
>1024	0.89	0.90	0.97	0.98	0.93	0.86	0.89	0.10	0.13	0.25

Figure 3. Comparison of fosfomycin MICs between ST5 and ST239 fosfomycin-resistant *S. aureus* isolates. Six different MIC levels are included. The number in each grid represents the proportion of different MIC levels in ST5 and ST239 during the study period. The numbers of cases of fosfomycin-resistant ST239 *S. aureus* isolates after 2010 were <5 (0 in 2019) and are stated altogether. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

in both *glpT* and *uhpT*, but the genotype with mutations in *glpT* and *hptR* was the most predominant (27/168; 16.1%). Two low-level fosfomycin-resistant clades were represented by 2005-02 and 2012-349. In addition, the high-level resistant isolates with one mutated gene were characterized by 2005-08. Similar to the conclusion drawn from Figure 5a, more trials should be performed to confirm the mechanism of high-level fosfomycin resistance.

Gene deletion and complementation verified contribution of five selected genes to fosfomycin resistance

In order to clarify whether high-level fosfomycin resistance resulted from mutation of multiple genes or one critical gene, single- and double-gene mutants were constructed, whose fosfomycin MICs are shown in Table 1. Two fosfomycin-susceptible *S. aureus* clinical isolates with no mutation in 11 selected genes, 2018-140 (ST5) and 2016-7 (ST239), were chosen as WT for gene deletion and their fosfomycin MICs were both 4 mg/L, i.e. they were fosfomycin susceptible. First, *glpT*, *hptA*, *hptR* and *uhpT* single-gene deletion mutants were constructed in both 2018-140 and 2016-7. Of the isogenic deletion mutants, the knockout of *uhpT*, *hptR* and *hptA* genes resulted in a phenotype change of the clinical isolates from susceptibility to low-level resistance (from 4 to 64/128 mg/L in 2018-140 and 2016-7). Even though no MIC increase was observed in the *glpT* mutant, the combined mutants Δ*glpT*Δ*uhpT*, Δ*glpT*Δ*hptA* and Δ*glpT*Δ*hptR* in both 2018-140 and 2016-7 were highly resistant to fosfomycin (>1024 mg/L). As we expected, Δ*uhpT*Δ*hptA*, Δ*uhpT*Δ*hptR* and Δ*hptA*Δ*hptR*, in which only the UhpT transporter system was

influenced despite double-gene deletion, were still resistant to fosfomycin at a low level. The complementation of deleted genes partly restored the phenotype. Briefly, our gene deletion trial indicated that both ST5 and ST239 *S. aureus* would reach a high fosfomycin resistance level when the function of two transporter systems was affected together.

Discussion

Fosfomycin is a broad-spectrum antibiotic with a unique mechanism, which requires membrane transporter systems to invade bacteria and inhibit the first step of bacterial cell wall synthesis. Recently, however, fosfomycin resistance has gradually appeared, meriting increasing clinical concerns. Regarding fosfomycin resistance in Gram-negative and -positive bacteria, researchers have demonstrated their rigorous opinions in specific articles. For instance, Xu *et al.*[14] found that mutations of *uhpT* and *glpT* could lead to fosfomycin resistance in *S. aureus* strain Newman. Furthermore, Fu *et al.*[40] demonstrated that the *fosB* genes might not be the major mechanism of fosfomycin resistance in *S. aureus* but *uhpT*, *glpT* and *murA* mutations were more likely to contribute to fosfomycin resistance. When characterizing the HPT system in USA300 strains of *S. aureus*, Park *et al.*[24] demonstrated that disruption of *hptA* or *hptRS* increased resistance to fosfomycin.

In our study, we focused on clinical isolates instead of laboratory strains. First, an interesting clinical phenomenon was observed: the ST5 lineage spread clinically, while ST239 decreased gradually during the study period. This epidemiology characteristic coincided with the resistance rates of fosfomycin. Our finding that higher fosfomycin resistance levels existed in the ST5 lineage was consistent with the findings of Wu *et al.*[22] that ST5 MRSA isolates obtained from 2013 to 2015 showed higher fosfomycin resistance levels (MIC$_{50}$ > 1024 mg/L) in comparison with ST239 MRSA isolates (MIC$_{50/90}$ = 64/512 mg/L). Furthermore, we discovered for the first time through WGS data analysis that most mutations occurred in *glpT* and *uhpT* in ST5 fosfomycin-resistant isolates, but in *hptA* in ST239 isolates. Phylogenetic analysis based on MLSA suggested strong correlation between the genes and fosfomycin resistance. Afterwards, gene deletion trials were conducted to explore whether high-level fosfomycin resistance resulted from mutations of multiple genes or one critical gene. Isogenic deletion only resulted in low-level fosfomycin resistance, while double-gene mutants Δ*glpT*Δ*uhpT*, Δ*glpT*Δ*hptR* and Δ*glpT*Δ*hptA* proved to be highly resistant to fosfomycin. It is noteworthy that the Δ*glpT* mutant was still susceptible to fosfomycin (MIC < 32 mg/L), but combined with *uhpT*, *hptR* and *hptA* deletion, its MIC went straight up to >1024 mg/L. In our research, the high-level fosfomycin resistance was not caused by single-gene mutation but mutations in multiple genes, consistent with the conclusion drawn by Téllez *et al.*[25] in *E. coli*. To our knowledge, we were the first to: (1) notice the discrepancy of mutated genes between different clonotypes of epidemic *S. aureus* by utilizing NGS sequencing data of a large quantity of isolates; (2) elucidate the close relationship between genotypes and fosfomycin resistance using phylogenetic analysis based on concatenated sequences of five major genes; and (3) identify mutations of multiple related genes as key to fosfomycin resistance in *S. aureus* epidemic strains.
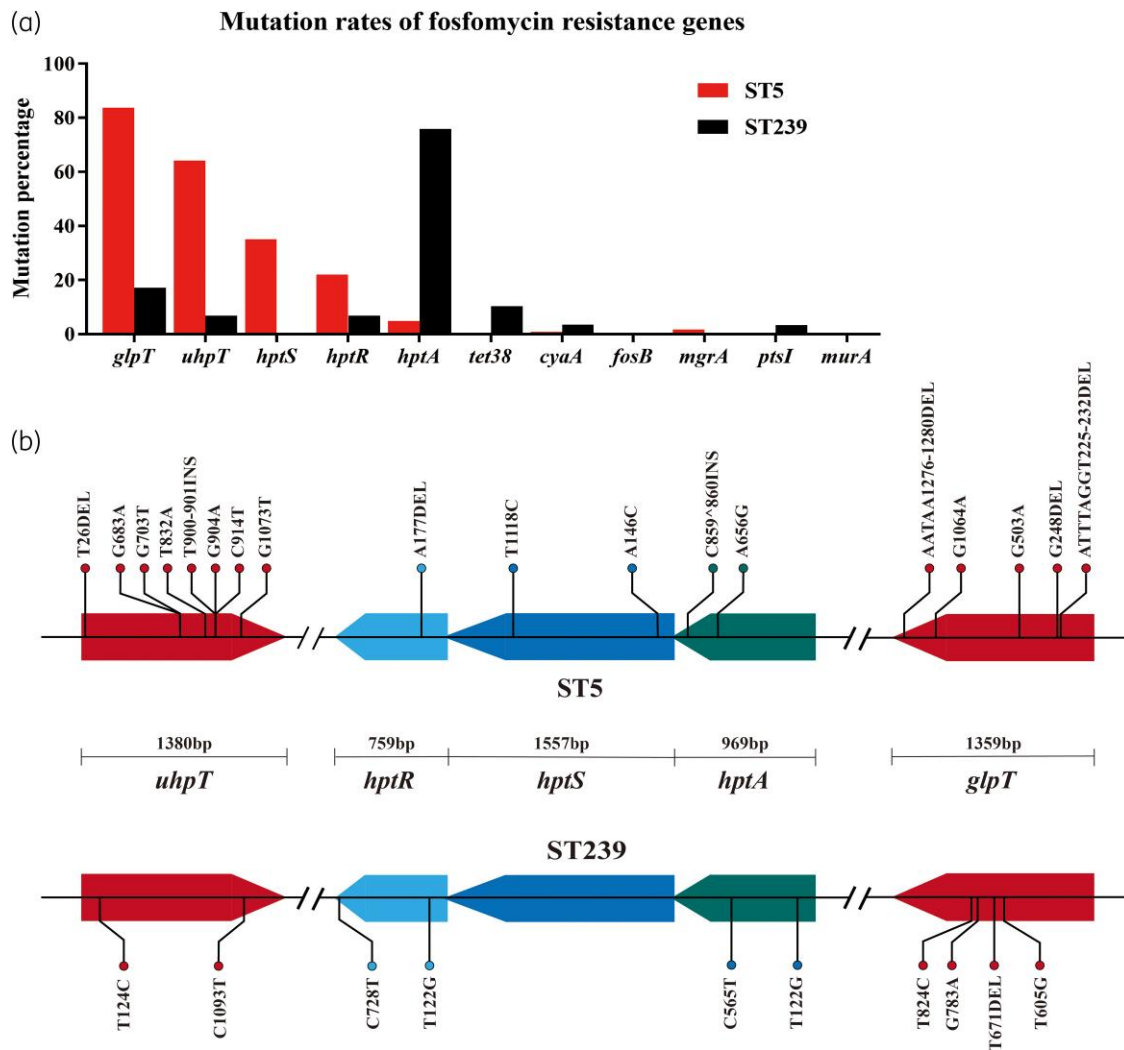
Figure 4. Major mutations in ST5 and ST239 fosfomycin-resistant *S. aureus* isolates. (a) Mutation rates of fosfomycin resistance genes in ST5 and ST239 isolates. Most mutations appeared in *glpT*, *hptA*, *hptS*, *hptR* and *uhpT*. (b) Major mutation types and positions in ST5 and ST239 isolates. A total of 14 mutation types were discovered in *uhpT* of ST5 but only 8 major mutation types are presented in the figure. The lengths of five genes are shown in the middle. DEL, deletion; INS, insertion. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Owing partly to limited use since its approval, fosfomycin is still effective against bacteria with resistance to commonly used antibiotics.[41] Fosfomycin has been recommended for cystitis in immunocompetent patients in America from 2010.[20] Moreover, fosfomycin was considered the first-choice drug for therapy of uncomplicated cystitis and used for women who developed catheter-associated urinary tract infections without upper urinary tract symptoms after removal of an indwelling catheter in Spain,[42] indicating increasing attention attracted by fosfomycin in clinical settings. Additionally, fosfomycin resistance rates and WGS data demonstrated that the resistance rate of ST5 exceeded that of ST239 around 2010, but the mutations of fosfomycin resistance-related genes had already appeared before that, suggesting the possibility that this lineage of *S. aureus*, ST5,

was inherently more resistant to fosfomycin. In this situation, *S. aureus* ST5 with more mutated genes than ST239 (shown in Figure S4) can possess a higher fosfomycin resistance level, which might provide a growth advantage for ST5 and eventually lead to a hospital epidemic. Therefore, more attention should be paid to surveillance and prevention of fosfomycin-resistant *S. aureus*.

With the rapid development of sequencing technology, WGS was extensively applied to *S. aureus* for antimicrobial resistance prediction.[43–45] However, research on fosfomycin resistance prediction has been relatively scarce. Here, we have presented numerous non-synonymous mutations detected in fosfomycin-resistant *S. aureus* isolates, which partly contribute to fosfomycin resistance prediction. Deletion of ATTTAGGT from 225 to 232, G1064A and deletion of 248G in *glpT*, together with

Table 2. The mutation types, amino acid substitution and mutation frequencies (rates) of five selected genes in this study

| ST	Fosfomycin resistance genes	Nucleotide mutation	Amino acid substitution	Number of isolates (%)
ST5 (*n* = 123)	**glpT**	**G1064A**	**Trp355***	**40 (32.5)**
		G248DEL	Delete	27 (22.0)
		ATTTAGGT225-232DEL	Delete	20 (16.3)
		ATTTAGGT225-232DEL/ TTATT1276-1280DEL	Delete	14 (11.4)
		G503A/G1064A	Gly168Glu/Trp355*	2 (1.6)
	hptA	**A656G**	**Asp219Gly**	**5 (4.1)**
		C859-860INS	Insert	1 (0.8)
	hptR	**A177DEL**	**Delete**	**27 (22.0)**
	hptS	**A146C**	**His49Pro**	**42 (34.1)**
		T1118C	Leu373Ser	1 (0.8)
	uhpT	**T900-901INS/G904A**	**Insert/Glu302Lys**	**32 (26.0)**
		T26DEL	Delete	13 (10.6)
		G1073T	Gly358Val	12 (9.8)
		C914T	Ala305Val	6 (4.9)
		G703T	Glu235*	4 (3.3)
		G683A	Trp228*	2 (1.6)
		T832A	Trp278Arg	2 (1.6)
		T900-901INS/G904A/C301T	Insert/Glu302Lys/ Leu101Phe	2 (1.6)
		G418A	Gly140Arg	1 (0.8)
		G172T	Glu58*	1 (0.8)
		G200C	Gly67Ala	1 (0.8)
		C406A	Gln136Lys	1 (0.8)
		A636DEL/A641G	Delete/Asp214Gly	1 (0.8)
		G949T	Asp317Tyr	1 (0.8)
ST239 (*n* = 32)	**glpT**	**G783A**	**Trp261***	**2 (6.3)**
		T824C	Val275Ala	1 (3.1)
		T671DEL	Delete	1 (3.1)
		T605G	Leu202Arg	1 (3.1)
	hptA	**C565T**	**Gln189***	**20 (62.5)**
		T44DEL	Delete	4 (12.5)
	hptR	C728T	Ser243Leu	1 (3.1)
		T122G	Leu41*	1 (3.1)
	hptS			0 (0.0)
	uhpT	T124C	Tyr42His	1 (3.1)
		C1093T	Gln365*	1 (3.1)

DEL, deletion; INS, insertion; *, substitution of a stop codon. The items in bold indicate the most frequent nucleotide mutation among the five fosfomycin resistance-related genes in ST5 and ST239, the corresponding amino acid substitution and mutation rates.

G1073T and deletion of 27T in *uhpT* were also confirmed in fosfomycin-resistant *S. aureus* by Xu *et al.*[13] and Fu *et al.*[40] These mutations of *glpT* and *uhpT* were verified to affect the gene function,[14] suggesting that other mutations listed in Table 2 might disturb corresponding gene function and result in fosfomycin resistance. It is also worth noting that 14 isolates that were highly resistant to fosfomycin (MIC > 1024 mg/L) only had mutations in *uhpT*, indicating the existence of additional mechanism in these isolates.

Overall, our research demonstrated the discrepancy of fosfomycin resistance levels between two clinical epidemic *S.* *aureus* lineages, in that ST5, with more mutated genes (*glpT* and *uhpT*), had a higher fosfomycin resistance level than ST239 (mainly *hptA*). Phylogenetic analysis and gene deletion trials revealed that fosfomycin resistance in clinical epidemic lineages probably resulted from the accumulation of mutations, mainly in genes concerning two membrane transporter systems. Of course, our research had some limitations. We only focused on gene mutations to elucidate the mechanism of fosfomycin resistance and did not take transcription data into account. Another limitation was the use of gene deletion instead of point mutation. Although some studies confirmed
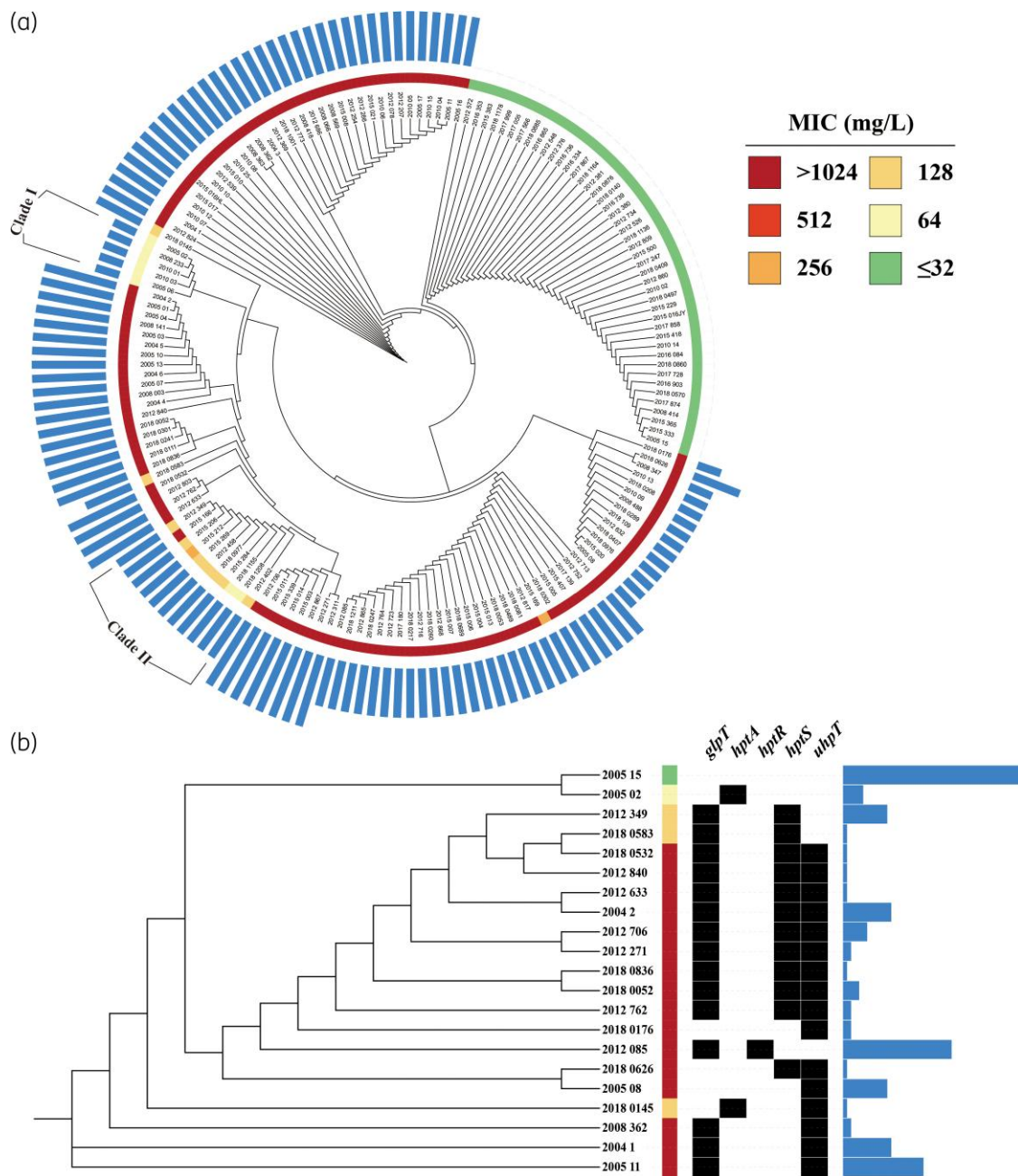
Figure 5. Phylogenetic analysis based on MLSA and the de-duplicated tree. (a) Phylogenetic tree of 168 *S. aureus* ST5 isolates, including both susceptible (≤32 mg/L, the green part of the circular multi-coloured strip) and resistant phenotypes, was built with IQ-TREE. The circular multi-coloured strip reflects the MIC levels (shown in the MIC legend). The blue bars outside the strip indicate the number of the mutated genes in every isolate (from 0 to 3). Two clades with low fosfomycin resistance are characterized by clade I and clade II. (b) The de-duplicated phylogenetic tree of 21 distinct genotypes. The multi-coloured strip reflects the MIC levels (shown in the MIC legend). The heatmap reveals the genes mutated in the genotypes (WT in white and mutant in black). The blue bars on the far right indicate the number of each genotype. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

certain gene function in fosfomycin resistance through gene deletion and complementation,[14,25,46] point mutation might be a better choice to clearly define the effect of these mutations on gene function.

Supplementary data

Supplementary methods, Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online.

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