Eravacycline susceptibility was impacted by genetic mutation of 30S ribosome subunits, and branched-chain amino acid transport system II carrier protein, Na/Pi cotransporter family protein in *Staphylococcus aureus*

Zhanwen Wang,† Zhiwei Lin,† Bing Bai,† Guangjian Xu, Peiyu Li, Zhijian Yu,‡ Qiwen Deng,‡ Yongpeng Shang,‡ and Jinxin Zheng,*

Abstract

**Background:** Our previous research indicated the excellent in vitro antibacterial activity of Eravacycline (Erava) and its heteroresistance frequency against clinical *Staphylococcus aureus* isolates. In this study, we further aimed to investigate the mechanisms of Erava resistance and heteroresistance in *S. aureus*. Eight parental *S. aureus* isolates were induced under Erava pressure in vitro and the Erava-resistant isolates were selected and identified. Then, the genetic mutations of 30S ribosomal subunits were analyzed by PCR and sequence alignment. RT-qPCR analysis were performed to compare the relative expression of eight candidate genes impacting the susceptibility of tetracycline (Tet) between the resistant or heteroresistant and parental isolates. Furthermore, the in vitro overexpression vectors of three selected candidate genes were constructed to test their impact on the heteroresistance and resistance of Erava in *S. aureus*.

**Results:** The MICs elevation in Erava-induced resistant *S. aureus* isolates were identified and the increasing MICs values of another two Tet class antibiotics, including both omadacycline (Omada) and tigecycline (Tige) were also tested. Genetic mutations in 30S ribosomal protein S10 were found frequently in Erava-derived resistant isolates. RT-qPCR analysis and the in vitro overexpression experiments indicated that *USA300HOU_RS00550* (an Na/Pi cotransporter family protein) and *USA300HOU_RS01625* (a branched-chain amino acid transport system II carrier protein) contributed to Erava heteroresistance in *S. aureus*.

(Continued on next page)
Background

Staphylococcus aureus is an important pathogen that leads to a wide spectrum of infectious diseases in humans, and the dissemination of methicillin-resistant S. aureus strains (MRSA) has posed a worldwide health threat in recent years [1, 2]. Recently, the emergence of multi-drug resistant S. aureus, including the vancomycin-intermediate or resistant, linezolid or daptomycin resistant isolates, has threatened the treatment options in clinics [3]. Thus, it’s urgent to develop new antimicrobials for the treatment of S. aureus infection.

Previous studies have indicated the high frequency of tetracycline resistance in S. aureus clinical isolates worldwide [4, 5]. The dissemination of tetracycline resistance mechanism also limited the application of tetracycline derivatives, including minocycline and doxycycline, in the treatment choices of the S. aureus infection. Recently, the optimization of tetracycline class drugs promote the development of several new-generation tetracycline derivatives, such as tigecycline (Tige), omadacycline (Omada) and eravacycline (Erava), and multiple reports have also indicated the robust antimicrobial activity of these new-generation tetracycline derivatives against S. aureus and other gram positive bacteria [6–8]. Our previous research demonstrated the excellent in vitro antibacterial activity of eravacycline (Erava) and its heteroresistance frequency against S. aureus clinical isolates, however, the mechanism of Erava resistance and heteroresistance in S. aureus still need to be further studied [7].

High level of transcriptional expression of tetracycline specific resistance factors, such as tet(M) and tet(K), have been found to mediate the Tige MIC creep in E. faecium isolates [8, 9]. Our previous data indicated that tet(K) was frequently detected in MSSA with Erava MIC ≥0.5mg/L and it’s still unknown whether tet(K) participated in the Erava heteroresistance or resistance [7]. The reduced susceptibility to Tige have been widely explained by the emergent mutations in genes encoding several 30S ribosome subunits, including 16S rRNA and 30S ribosome protein S10 [8–17]. The clinical significance of the genetic mutations of 30S ribosome subunits remains elusive should be further studied. Thus, the aim of this study was to examine the resistance mechanism in vitro by Erava-induced resistant S. aureus. The impact of the genetic mutations of 30S ribosome subunits on Erava susceptibility were also investigated. The transcriptional levels of eight candidate genes, which were previously reported to impact the Omada susceptibility, in the parental S. aureus isolates were compared with Erava heteroresistant and resistant derivative clones.

Results

Mechanism of Erava-induced resistance in S. aureus under Erava pressure

In order to investigate the relationship of Erava MICs creeps with the genetic mutations of 30S ribosome subunits, eight Erava susceptible S. aureus were selected in vitro under Erava pressure. The Erava resistant clones were identified and the MICs of Erava, Omada and Tige were measured as described in Table 1, indicating the cross resistance of Erava with Omada and Tige in these Erava-induced resistant S. aureus isolates. Genetic mutations of 30S ribosome subunits were determined in these Erava-induced resistant S. aureus isolates (Shown in Table 1), indicating the correlation of Erava MICs creep with the increasing copies numbers of five 16SrRNAs genes with genetic mutations. Whereas, the high frequency of T170G mutation in RR1, A1124G in RR2, C810T in RR3, G848C in RR4 and G1036A in RR5 was found in Erava-induced resistant S. aureus. The amino acid mutations Let47Let and Tyr87His in 30S ribosome protein S10 were found frequently in Erava-resistant S. aureus isolates. There were no mutations detected in the 30S ribosome protein S3 (data not shown). Importantly, increasing Erava MICs were accompanied by the increasing MICs values of both Omada and Tige in Erava-resistant S. aureus isolates, indicating that Erava cross-resistance with Omada and Tige can be induced under Erava pressure.

The transcriptional expression level of the candidate genes correlated with Erava susceptibility in S. aureus

The Erava MIC creep in heteroresistance clones could be previously reported to correlate with the Efflux pump, therefore we hypothesize that efflux pumps or membrane proteins might participate in the heteroresistance development. Our previous reported indicated the overexpression of eight candidate genes, including USA300HOU_RS00705, USA300HOU_RS03535, USA300HOU_RS10505, USA300HOU_RS13205, USA300HOU_RS13945, USA300HOU_RS10505, and USA300HOU_
Table 1 The MICs determination and genetic mutations of tetracycline target sites in Erava-induced resistant S. aureus isolates

| Strains            | MICs values (mg/L) | Mutation of 30S ribosome subunits |
|--------------------|--------------------|-----------------------------------|
|                    | Omada | Tige | Erava | RR1 | RR2 | RR3 | RR4 | RR5 | S10 |
| CHS221 (parental strain) | 0.5 | 0.5 | 0.25 | W   | W   | W   | W   | W   | W   |
| CHS221-E1a         | 16   | 32  | 16   | T170G| G77A; | A1124G | C810T| G848C| G185A; | G1036A | W   | W   |
| CHS221-E2a         | 32   | 32  | 16   | T170G| G77A; | A1124G | G742A; | C810T| G185A; | G1036A | W   | W   |
| CHS165 (parental strain) | 0.5 | 0.5 | 0.25 | W   | W   | W   | W   | W   | W   |
| CHS165-E1a         | 32   | 32  | 8    | T170G| A972T  | A1124G | C810T| G185A; | G1036A | W   | Tyr87His |
| CHS165-E2a         | 32   | 64  | 16   | T170G| G848C  | A1124G | C810T| G185A; | G1036A | G848A; | Let47Let; | Tyr87His |
| 149 (parental strain) | 0.5 | 0.25 | 0.06 | W   | W   | W   | W   | W   | W   |
| 149-E1a            | 16   | 8   | 8    | T170G| G77A; | A1124G | C810T| G185A; | G1036A | Let47His; |
| 149-E2a            | 32   | 32  | 32   | T170G| G77A; | AG847-848TA; A1124G | C810T| G185A; | G1036A | W   | Ile46ile |
| CHS5759 (parental strain) | 0.5 | 0.25 | 0.25 | W   | W   | W   | W   | W   | W   |
| CHS5759-E1a        | 16   | 8   | 8    | T170G| G77A; | A1124G | C810T| G185A; | G1036A | W   | W   |
| CHS5759-E2a        | 16   | 32  | 16   | T170G| G77A; | A1124G | C810T| G185A; | G1036A | T1257C; | W   |
| CHS810 (parental strain) | 0.5 | 0.25 | 0.25 | W   | W   | W   | W   | W   | W   |
| CHS810-E1a         | 16   | 32  | 8    | T170G| G77A; | A1124G | C810T| G185A; | G1036A | W   | Tyr87His |
| CHS810-E2a         | 16   | 32  | 8    | T170G| G848C  | G77A; | A1124G | C810T| G185A; | G1036A | W   | Tyr87His |
| CHS820 (parental strain) | 0.5 | 1   | 0.125 | W   | W   | W   | W   | W   | W   |
| CHS820-E1a         | 16   | 64  | 16   | T170G| G77A; | A1124G | C810T| G185A; | G1036A | C1254T; | Let47His |
| CHS820-E2a         | 16   | 64  | 16   | T170G| G77A; | A1124G | C810T| G185A; | G1036A | C1254T; | Let47His |
| N315 (parental strain) | 0.5 | 0.5 | 0.25 | W   | W   | W   | W   | W   | W   |
| N315-E1a           | 8    | 8   | 8    | T170G| G77A; | A1124G | C810T| G848C | G185A | T846A; | Tyr87His |
| N315-E2a           | 8    | 8   | 8    | T170G| G77A; | A1124G | C810T| G185A | W   | W   | Tyr87His |
| M54 (parental strain) | 0.5 | 0.25 | 0.25 | W   | W   | W   | W   | W   | W   |
| M54-E1a            | 16   | 32  | 16   | T170G| G77A; | A1124G | C810T| G185A; | G1036A | W   | Let47His |

*: Erava-induced resistant isolates, respectively; RR1–5: five separate copies of the 16S rRNA gene; W, wild-type (no mutation)

RS00660, might impact the Omada susceptibility in S. aureus. The profiles of the eight candidate genes were summarized in Table S1. Furthermore, high detection frequency of tet(K) and tet(L) in S. aureus with Erava MICs ≥0.5 mg/L, whereas whether the transcriptional level of tet(K) and tet(L) was correlated with Erava susceptibility need to be studied.

We have previously reported the heteroresistance occurrence of the parental isolates, including CHS237, CHS632, CHS62, CHS239 and its corresponding heteroresistance isolates in which the genetic mutation of 30S ribosome subunits was not found. Furthermore, the relative transcriptional expression of these eight candidate genes, tet(K) and tet(L) were investigated by RT-qPCR analysis. The relative transcriptional levels of these genes were compared between these four parental isolates and their heteroresistance and between two parental isolates, N315 and M54, and their Erava induced resistant isolates respectively. Interestingly, the transcriptional levels of USA300HOU_RS03535, USA300HOU_RS01625 (encodes a branched-chain amino acid transport system II carrier protein), USA300HOU_RS00550 (encodes a Na/ Pi cotransporter family protein) and tet(K) were shown with significant elevation in heteroresistance or Erava-resistant clones compared with that of the parental (Fig. 1). However, the other six candidate genes seemed no
significant elevation (Fig. 2), suggesting these four candidate genes (USA300HOU_RS03535, USA300HOU_RS01625, USA300HOU_RS00550 and tet(K)) might contribute to impact the Erava susceptibility and need to be further verified by the in vitro overexpression experiments.

**Fig. 1** Significant elevation of the relative transcriptional expression level of the three candidate genes, including USA300HOU_RS03535, USA300HOU_RS01625, USA300HOU_RS00550 and tet(K) in heteroresistance or Erava-resistant clones compared with that of the parental. The Origin represented the parental isolates including CHS237, CHS632, CHS62, CHS239, N315 and MS4; Resistance represented the corresponding heteroresistance isolates CHS237-E1, CHS632-E1, CHS62-E1, CHS239-E1, which was reported previously [6], and two Erava-resistant isolates shown in Table 1, including N315-E1 and MS4-E1. Relative expression of USA300HOU_RS03535 (a), USA300HOU_RS01625 (b), USA300HOU_RS00550 (c), tet(K) (d) were demonstrated by RT-qPCR analysis. The housekeeping gene gyrB was used as the endogenous reference gene. The original (parental) strain was used as the reference strain (expression = 1.0). All RT-qPCR were carried out in triplicate. **p < 0.01 (Student’s t-tests)**

**Relationship between three candidate genes overexpression and Erava susceptibility**

The impacts of four candidate genes, including USA300HOU_RS03535, USA300HOU_RS01625, USA300HOU_RS00550 and tet(K), were further tested by the in vitro overexpression experiments. The overexpression plasmids pRS00550, pRS01625, pRS03535, ptet(K) were successfully constructed and identified. Subsequently, the overexpression plasmids pRS00550, pRS01625, pRS03535, ptet(K) were transformed separately into clinical isolates SE4, SE7, SE13, CHS545 and CHS569, which have previously been reported with low expression level of three candidate genes [6]. The overexpression of the four genes in the five S. aureus isolates were confirmed by RT-qPCR and was shown in Fig. S1. The impact of the overexpression of these four candidate genes on Erava susceptibility was described in Table 2. Briefly to say, the protein encoded by these four candidate genes did not result in Erava MICs elevation, however, the transcriptional overexpression of USA300HOU_RS00550 and USA300HOU_RS01625 participate in the occurrence of Erava heteroresistance.

**Discussion**

Recent crystallographic analysis studies have revealed the same mechanism of the new-generation tetracycline class antibiotics, including Tige, Omada and Erava, to inhibit bacterial protein synthesis by binding to the 30S ribosomal subunits, including 16SrRNA and 30S ribosome protein S10 [8, 18]. Previous researches indicated that genetic mutations affecting the 30S ribosome subunits (i.e., 16SrRNA and ribosome proteins S10) have been shown to confer resistance to Tige and Omada in S. aureus [9–17, 18]. However, the characteristics of genetic mutation of the 30S ribosome subunits and cross resistance in Erava resistant S. aureus remains unknown. This study indicated that similar to other tetracycline class, Erava MIC elevation can be coupled with the increasing copies numbers of 16SrRNA genes with genetic
mutations. Meanwhile, our study revealed high frequency of genetic mutations in 30S ribosome protein S10 in Erava-derived resistant isolates, indicating this protein might be an important factor in Erava resistance evolution. Previous multiple reports have shown that the mutation in 30S ribosome protein S10 of \textit{S. aureus} can result in the MIC elevation of tetracycline, Omada and Tige, indicating its important role in the resistance evolution of tetracycline class drugs \cite{6, 9, 12, 13, 15, 17}. Therefore, the cross resistance under Erava pressure with Omada and Tige might be mainly explained by the genetic mutation of 30S ribosome subunits.

The frequent occurrence of antibiotics heteroresistance can result in the treatment failure. Our previous report indicated the occurrence of Erava heteroresistance in \textit{S. aureus} and this phenomenon would enhance the difficulty for the treatment of \textit{S. aureus} infection with Erava \cite{7}. Our previous indicated the transcriptional

---

**Fig. 2** No significant elevation of the relative transcriptional expression level of the five candidate genes, including USA300HOU_RS00705, USA300HOU_RS13205, USA300HOU_RS13945, USA300HOU_RS10505, USA300HOU_RS00660 and tetL in heteroresistance or Erava-resistant clones compared with that of the parental. The origin represented the parental isolates including CHS237, CHS632, CHS62, CHS239, N315 and MS4; Resistance represented the corresponding heteroresistance isolates CHS237-E1, CHS632-E1, CHS62-E1, CHS239-E1, which was reported previously \cite{6}, and two Erava-resistant isolates shown in Table 1, including N315-E1 and MS4-E1. Relative expression of USA300HOU_RS00705 (a), USA300HOU_RS13205 (b), USA300HOU_RS13945 (c), USA300HOU_RS10505 (d), USA300HOU_RS00660(e), tetL (f), were demonstrated by RT-qPCR analysis. The housekeeping gene \textit{gyrB} was used as the endogenous reference gene. The original (parental) strain was used as the reference strain (expression = 1.0). All RT-qPCR were carried out in triplicate. *\( p < 0.05 \); **\( p < 0.01 \) (Student’s t-tests).
expression level of the eight candidate genes, including \textit{USA300HOU\_RS00705, USA300HOU\_RS01625, USA300HOU\_RS03535, USA300HOU\_RS01625, USA300HOU\_RS00550, USA300HOU\_RS13205, USA300HOU\_RS13945, USA300HOU\_RS10505}, and \textit{USA300HOU\_RS00660}, which have been shown to be correlated with the Omada heteroresistance [6]. Our previous data indicated the cross resistance between Omada and Erava in \textit{S. aureus} and therefore, whether these eight candidate genes might contribute to impact the Erava susceptibility should be further evaluated. Although the high detection frequency of \textit{tet(K)} and \textit{tet(L)} in \textit{S. aureus} was reported in our previous study [7], it remained unknown that whether the overexpression of \textit{tet(K)} and \textit{tet(L)} would impact the Erava susceptibility. Our data indicated that the overexpression of \textit{USA300HOU\_RS00550} and \textit{USA300HOU\_RS01625} would enhance the occurrence of Erava heteroresistance but have no direct impact on Erava MIC creep, demonstrating their overexpression can result in Erava MIC elevation under Erava pressure in vitro. Our previous phylogenetic analysis have shown that both \textit{USA300HOU\_RS00550} and \textit{USA300HOU\_RS01625} encode efflux pump family proteins [6, 7], supporting our hypothesis that efflux pump or membrane proteins contribute to Erava or Omada heteroresistance and the cross-resistance between Erava and Omada in \textit{S. aureus} should arouse our attention.

**Table 2** Determination of Erava MIC value in the \textit{S. aureus} derivatives with the overexpression of the three candidate genes and \textit{tet(K)} and their impact on PAPs

| Vectors         | Strains      | Erava MIC (mg/L) | Parental isolates | Derivative isolates | PAP |
|----------------|--------------|------------------|-------------------|---------------------|-----|
| pRS00550       | SE7          | 0.25             | 0.125             | +                   |     |
|                | CHS545       | 0.125            | 0.125             | +                   |     |
|                | CHS569       | 0.125            | 0.125             | +                   |     |
| pRS01625       | SE4          | 0.125            | 0.125             | +                   |     |
|                | SE7          | 0.125            | 0.125             | +                   |     |
|                | SE13         | 0.125            | 0.125             | +                   |     |
|                | CHS545       | 0.125            | 0.125             | +                   |     |
|                | CHS569       | 0.0625           | 0.125             | +                   |     |
| pRS03535       | SE13         | 0.125            | 0.125             | –                   |     |
|                | CHS545       | 0.125            | 0.125             | –                   |     |
| ptetK           | SE7          | 0.125            | 0.125             | –                   |     |
|                | SE13         | 0.125            | 0.0625            | –                   |     |
|                | CHS545       | 0.25             | 0.125             | –                   |     |
|                | CHS569       | 0.125            | 0.125             | –                   |     |

**PAP** population analysis profiling

*The overexpression of \textit{USA300HOU\_RS03535, USA300HOU\_RS01625, USA300HOU\_RS00550 and tet(K)} in \textit{S. aureus} clinical isolates SE4, SE7, SE13, CHS545 and CHS569.*

In conclusion, Erava susceptibility in \textit{S. aureus} was impacted by genetic mutation of 30S ribosome subunits, including 16SrRNA and 30S ribosome protein S10. Furthermore, two efflux pump family proteins encoded by \textit{USA300HOU\_RS01625} (a branched-chain amino acid transport system II carrier protein) and \textit{USA300HOU\_RS00550} (an Na/Pi cotransporter family protein) contribute to Erava heteroresistance in \textit{S. aureus}.

**Methods**

**Bacterial isolates**

The \textit{S. aureus} clinical isolates used in this study were collected from the different inpatients of Shenzhen Nanshan People’s Hospital (a tertiary hospital with 1200 beds) between 2010 and 2016. The in vitro induction \textit{S. aureus} clinical isolates, including CHS221, CHS165, 149, CHS759, CHS810, CHS820, have been used in our previous study [6]. N315 (GenBank accession number: BA000018) and MS4 (GenBank accession number: CP009828) are two reference \textit{S. aureus} isolates reserved in our lab, which are MRSA and sensitive to Tigec, Omada. The parental MSSA isolates CHS237, CHS632, CHS62, CHS239 and their heteroresistant derived clones CHS237-E1, CHS6-32-E1, CHS62-E1, CHS239-E1, included in this study, have been described in our previous research for the investigation of Erava heteroresistance [7]. The \textit{S. aureus} isolates, including SE4, SE7, SE13, CHS545 and CHS569, were used as reference isolates for the overexpression experiments as described previously [6]. The minimum inhibitory concentration (MIC) of antimicrobials and multilocus sequence typing of the isolates used in this study were determined in our previous research [7], and were summarized in Table S2.

All procedures involving human participants were performed in accordance with the ethical standards of Shenzhen University and the 1964 Helsinki declaration and its later amendments, or comparable ethical standards. For this type of study, formal consent is not required.

**Antimicrobial susceptibility and population analysis profiling (PAP) development**

Omada, Tigec and Erava were purchased from the Medicines Company (Med Chem Express, Monmouth Junction, NJ) and their MICs were determined by the agar dilution method according to CLSI guidelines [19]. Because the CLSI guidelines provide no recommendation for the Erava MIC susceptibility breakpoints against \textit{S. aureus}, we adopted an MIC susceptibility breakpoint of 0.5 mg/L, the value recommended for tigecycline non-susceptible in Gram-positive bacteria and defined heteroresistance as growth in 0.5mg/L Erava [7, 20]. PAP experiments were conducted as previously [21, 22]. Briefly, 50μL aliquots (~ 10^8 colony forming units/ml) were spread onto Müller-Hinton agar plates containing serially diluted Erava (0.5, 1.0, 2.0, 3.0 mg/L). Colonies
were counted after 24h of incubation at 37°C. Erava heteroresistance was defined as the observation of subpopulations isolated from the Erava-containing plates able to grow in the presence of 0.5mg/L Erava (detection limit, ≥5 colony forming units/plate).

**Polymerase chain reaction (PCR) and sequence alignment**
Genomic DNA of all clinical isolates was extracted and used as templates for PCR amplification in lysis buffer for microorganisms to direct PCR (Takara Bio Inc., Japan). The presence of 3OS ribosomal subunit mutations, including five separate copies (RR1, RR2, RR3, RR4, RR5) of the 16S rRNA gene, the genes encoding the 3OS ribosomal proteins S3 and S10, were analyzed by PCR and sequence alignment as described previously [6], and the PCR primers were listed in Table S3.

**In vitro induction of Erava resistance**
Eight parental *S. aureus* isolates, including six clinical isolates (MSSA: CHS221, CHS165, 149; MRSA: CHS759, CHS810, CHS820) and two reference strains (MRSA: N315 and MS4), were used to select Erava-resistant isolates in vitro as described previously [6]. Briefly, these isolates were subcultured serially in Mueller-Hinton broth containing gradual increasing Erava concentrations with the initial concentration being MIC followed by successive increases to 2×, 4×, 8×, 16×, 32×MICs [23], with four passages at each concentration. Isolates from the passages of each concentration were stored at -80°C in Mueller-Hinton broth containing 40% glycerol for further analysis, including Tet-target site genetic mutation detection and MICs determination.

**Quantitative real time PCR (RT-qPCR) analysis**
Total RNA of *S. aureus* isolates was extracted with a RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) and RT-qPCR was performed with an SYBR Premix Ex Taq II kit in a Mastercycler EP Realplex system (Eppendorf, Hamburg, Germany) according to the manufacturer’s instructions (Takara Bio Inc., Shiga, Japan). The internal control gene *gyrB* was used to normalize gene expression. Threshold cycle (Ct) numbers were determined by detection system software and analyzed with the 2−ΔΔCt method. The RT-qPCR primers used were described previously and as shown in Table S4 [6]. RT-qPCR was performed in triplicate at least three times.

**Gene overexpression**
The overexpression vectors pRS00550, pRS1625, pRS03535; ptetK were constructed by introducing the candidate gene fragments, including *USA300HOU_RS00550* (encodes a Na/Pi cotransporter family protein), *USA300HOU_RS01625* (encodes a branched-chain amino acid transport system II carrier protein), *USA300HOU_RS03535* and *USA300HOU_Tet(K)*, each into separate pIB166 vectors as described previously [6]. Then, the vectors were transformed separately each into three to five Erava-sensitive isolates and confirmed by PCR and Sanger sequencing. The overexpression was induced by 2 μM CdCl2 and verified by RT-qPCR. All strains, plasmids, and primers used for overexpression analysis are listed in Tables S5 and S6.

**Statistical analysis**
Continuous data were analyzed by Student’s t-tests with SPSS software package (version 17.0, Chicago, IL). P values <0.05 were regarded as statistically significant.

**Supplementary information**
Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01869-6.

**Additional file 1 Table S1** The profiles of the eight candidate genes correlated with eravacycline susceptibility in *S. aureus*.

**Additional file 2 Table S2** MICs values and MLST of the *S. aureus* isolates used in this study.

**Additional file 3 Table S3** Primers used for the detection of Tet-resistance genes and Tet target sites in *S. aureus* by PCR.

**Additional file 4 Table S4** Primers used for RT-qPCR in this study.

**Additional file 5 Table S5** Strains and plasmids used for the overexpression test in this study.

**Additional file 6 Table S6** PCR primers used for the overexpression vector constructions in this study.

**Additional file 7 Figure S1** Overexpression of *USA300HOU_RS00550*, *USA300HOU_RS01625*, *USA300HOU_RS03535* and tetK in different Eravacycline-sensitive clinical *S. aureus* isolates. The RNA levels of *USA300HOU_RS00550* (A), *USA300HOU_RS01625* (B), *USA300HOU_RS03535* (C) and tetK (D) were determined by qRT-PCR. The wild-type isolates were used as the reference strain (mRNA level = 1.0).

**Abbreviations**
MRSA: Methicillin-resistant *S. aureus*; MSSA: Methicillin-sensitive *S. aureus*; PAP: Population analysis profiling; MIC: Minimal inhibitory concentration; RT-qPCR: Quantitative real time PCR

**Acknowledgments**
The authors thank Prof. Jingren Zhang (Center for Infectious Disease Research, School of Medicine, Tsinghua University, Beijing, China) for generously providing the pIB166 plasmid.

**Authors’ contributions**
ZW participated in the data analysis and drafted the manuscript. ZL performed the gene manipulation, stress tolerance experiments and RNA extraction, and RT-qPCR. BB participated in the gene manipulation and RT-qPCR. GX, PL conducted the RNA extraction and RT-qPCR. ZY, QD, YS, JZ performed the gene manipulation, stress tolerance experiments and RNA extraction, and RT-qPCR. BB participated in the gene manipulation and RT-qPCR. ZW participated in the data analysis and drafted the manuscript. ZL signed the study, participated in the data analysis, and provided critical revisions of the manuscript for valuable intellectual content. All authors have read and approved the manuscript.

**Funding**
This work was supported by the following grants: the collection of bacterial isolates, antimicrobials susceptibility tests, PAP development and PCR analyses were supported by the Sanming Project of Medicine in Shenzhen (SMGC201705029) and National Natural Science Foundation China (81902033); The in vitro induction of Erava resistance were supported by the National Science Foundation of Guangdong Province, China (2020A1515010052); The RT-qPCR analysis and gene overexpression were...
References

1. Caffee OP. Trends in community versus health care-acquired methicillin-resistant Staphylococcus aureus infections. Curr Infect Dis Rep. 2017;19(12):48.

2. Bal AM, David MZ, Garau J, et al. Future trends in the treatment of methicillin-resistant Staphylococcus aureus MRSA infection: an in-depth review of newer antibiotics active against an enduring pathogen. J Glob Antimicrob Resist. 2017;10:295–303.

3. Pfäffer MA, Rhomberg PR, Hubbard MD, Flamm RK. Activities of omadacycline and comparator agents against Staphylococcus aureus isolates from a surveillance program conducted in North America and Europe. Antimicrob Agents Chemother. 2017;61(3):e02411–6.

4. Pantosti A, Sanchini A, Monaco M. Mechanisms of antibiotic resistance in Staphylococcus aureus. Future Microbiol. 2007;2(3):323–34.

5. Gajdács M. The continuing threat of methicillin-resistant Staphylococcus aureus. Antibiotics (Basel). 2019;8(2):52.

6. Bai B, Lin Z, Pu Z, et al. In vitro activity and heteroresistance of Omadacycline against clinical Staphylococcus aureus isolates from China reveal the impact of Omadacycline susceptibility by branched-chain amino acid transport system II carrier protein, Na/Pi Co-transporter family protein, and Fibronectin-binding protein. Front Microbiol. 2019;10:2546.

7. Zhang F, Bai B, Xu GJ, et al. Eravacycline activity against clinical S. aureus with reduced susceptibility to Tigecycline. Antimicrob Agents Chemother. 2017;61(1):e01852–17.

8. Dabul ANG, Avoca-Crusca JS, Van Tynne D, Gilmore MS, Camargo ILBC. Resistance in vitro selected Tigecycline-resistant methicillin-resistant Staphylococcus aureus sequence type 5 is driven by mutations in mepR and mepA genes. Microb Drug Resist. 2018;24(3):19–26.

9. Seriki AT, Smith SJ, Adeleye AI, Fowora MA. Molecular analysis of low-level tetracycline resistance in clinical isolates of helicobacter pylori among dyspeptic patients in south West Nigeria. J Glob Antimicrob Resist. 2018;13:143–5.

10. Haim MS, Di Gregorio S, Galanternik L, et al. First description of rpsJ and mepA mutations associated with tigecycline resistance in Staphylococcus aureus isolated from a cystic fibrosis patient during antibiotic therapy. Int J Antimicrob Agents. 2017;50(6):739–41.

11. Niebel M, Quick J, Prieto AM, et al. Deletions in a ribosomal protein-coding gene are associated with tigecycline resistance in enterococcus faecium. Int J Antimicrob Agents. 2015;46(5):572–5.

12. Carro MJ, Lanard C, Coquelle T, et al. Genomic analysis of reduced susceptibility to tigecycline in enterococcus faecium. Antimicrob Agents Chemother. 2015;59(1):239–44.

13. Zhanel GG, Cheung D, Adam H, et al. Review of Eravacycline, a novel Fluorocycline antibacterial agent. Drugs. 2016;76(5):567–88.

14. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 24th informational supplement. Document M100-S26. Wayne: Clinical and Laboratory Standards Institute; 2016.

15. Pfäffer MA, Hubbard MD, Streit JM, Flamm RK, Sader HS. Surveillance of tigecycline activity tested against clinical isolates from a global (North America, Europe, Latin America and Asia-Pacific) collection (2016). Int J Antimicrob Agents. 2018;51(6):848–53.

16. Chen Y, Hu D, Zhang Q, Liao XP, Liu YH, Sun J. Efflux pump overexpression contributes to Tigecycline Heteroresistance in Salmonella enterica serovar Typhimurium. Front Cell Infect Microbiol. 2017;7:37.

17. Halaby T, Kucukkose E, Janssen AB, et al. Genomic characterization of Colistin Heteroresistance in Klebsiella pneumoniae during a nosocomial outbreak. Antimicrob Agents Chemother. 2016;60(11):6837–43.

18. Yao W, Xu G, Bai B, et al. In vitro-induced erythromycin resistance facilitates cross-resistance to the novel fluoroketolide, solithromycin, in Staphylococcus aureus. FEMS Microbiol Lett. 2018;365(12):10.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.