CusS-CusR Two-Component System Mediates Tigecycline Resistance in Carbapenem-Resistant Klebsiella pneumoniae

Dongjie Chen1,2†, Yunan Zhao1†, Yanqin Qiu1, Liying Xiao1, Huaqiang He1, Dongmei Zheng1, Xiaqin Li3, Xiaoli Yu3, Nengluan Xu3, Xinlan Hu2, Falin Chen2, Hongru Li1,3* and Yusheng Chen1,3*

1 Shengli Clinical Medical College of Fujian Medical University, Fuzhou, China, 2 Clinical Microbiology Laboratory, Fujian Provincial Hospital, Fuzhou, China, 3 Department of Pulmonary and Critical Care Medicine, Fujian Provincial Hospital, Fuzhou, China

Background: The increase in carbapenem-resistant Klebsiella pneumoniae (CRKP), especially the emergence of tigecycline-resistant K. pneumoniae (KP), is a serious public health concern. However, the underlying mechanism of tigecycline resistance is unclear. In this study, we evaluated the role of the CusS-CusR two-component system (TCS), which is associated with copper/silver resistance, in tigecycline resistance in CRKP.

Methods: Following the in vitro evolution of tigecycline-resistant KP, the minimum inhibitory concentrations of tigecycline were determined using the micro-broth dilution method. RNA sequencing and data analysis were performed to identify differentially expressed genes. Quantitative PCR (qPCR) was performed to verify the genes of interest. Genes associated with tigecycline resistance, such as ramR, tex (T), and tet (A), were detected by PCR, and then mutants were confirmed by sequencing. Additionally, the efflux pump-associated genes soxS, oqxA, oqxB, acrE, and acrF were also analyzed by qPCR. CusR was deleted and complemented by the suicide vector pKO3-Km plasmid and pGEM-T-easy plasmid, respectively.

Results: Nine strains of KP were evaluated in our study. Strains A2 and A3 were evolved from A1, B2, and B3 were evolved from B1, and C2 and C3 were evolved from C1. The tigecycline minimum inhibitory concentration for A1, B1, and C1 was 0.5 µg/mL; that for A2, B2, and C2 was 16.0 µg/mL; and that for A3, B3, and C3 was 32.0 µg/mL. RNA-sequencing and qPCR confirmed that the differentially expressed genes cusE, cusS, cusR, cusC, cusF, cusB, and cusA showed higher expression in C2 and C3 than in C1. Genes related to the efflux pump AcrAB-ToIC showed higher expression in B2 and B3 than in B1. No mutants of ramR, tex (T), or tet (A) were detected. SoxS, oqxA, oqxB, acrE, and acrF did not show increased expression in any group. After deletion and...
complementation of cusR among C3, the MIC of tigecycline decreased to 4 \( \mu \)g/mL, and then recovered to 32 \( \mu \)g/mL. The expression of cusFBCA, correspondingly decreased and increased significantly.

**Conclusion:** In addition to its primary function in resistance to copper/silver, the CusS-CusR two-component system is associated with CRKP resistance to tigecycline.

**Keywords:** Klebsiella pneumoniae, carbapenem resistance, tigecycline resistance, CusS-CusR two-component system, RNA sequencing

**INTRODUCTION**

In recent decades, multiple drug-resistant and carbapenem-resistant Enterobacteriaceae have increased in abundance, particularly Klebsiella pneumoniae (KP), Escherichia coli, Enterobacteriaceae spp., Proteus spp., and Serratia mucosa, greatly limiting the efficacy of antibiotic treatment. Tigecycline is currently widely used in clinical practice as an anti-infective treatment and has become the most important antibiotic currently widely used in clinical practice. Tigecycline is effective in treating carbapenem-resistant Acinetobacter baumannii and carbapenem-resistant Enterobacteriaceae. However, A. baumannii, E. coli, and K. pneumoniae have shown resistance to tigecycline. Additionally, various mechanisms have been reported to confer tigecycline resistance, with most studies focusing on resistance-nodulation cell division transporters, mainly among AcrAB-ToLC efflux pumps, while other pumps and various control pathways are also reported to be associated with tigecycline resistance (Pouranaras et al., 2016; Chiu et al., 2017; Ye et al., 2017; He et al., 2018).

Sheng et al. (2014) determined the tigecycline resistance mechanism in 26 strains of tigecycline-non-susceptible KP and found that the resistance of nine strains could not be explained by existing mechanisms. Recent studies revealed a co-regulatory effect between antibiotic resistance and metal resistance. Wang et al. (2017) reported that the CzcR-CzcS two-component system (TCS) in Pseudomonas aeruginosa participates in metal detoxification and antibiotic resistance, mainly by co-regulating cross-resistance between Zn (II) and carbapenem antibiotics. Perron et al. (2004) reported that in P. aeruginosa, the CzcR-CzcS TCS induced resistance to cadmium, zinc, and cobalt through over-expression of the efflux pump system, resulting in an increase in the MICs of carbapenem. In a study of metal resistance and its relationship with antibiotic resistance, Pal et al. (2017) found that metal resistance and antibiotic resistance genes were located at the same site in the cell, and a single resistance mechanism (such as the efflux pump) could result in resistance to either antibiotics or metal. Hölzel et al. (2012) studied the relationship between heavy metals and bacterial antibiotic resistance in pig manure, and revealed that the presence of copper and zinc was significantly correlated with the increased drug resistance rate of \( \beta \)-lactam antibiotics, while the presence of mercury was significantly correlated with the low drug resistance of E. coli to \( \beta \)-lactams, aminoglycosides, and other antibiotics. These studies suggest that bacterial resistance to metals is associated with antibiotic resistance.

The CusS-CusR TCS is associated with bacteria resistant to the heavy metals copper/silver, in which copper/silver ions stimulate the phosphorylation of the outer membrane protein, CusS; the phosphorylated CusS phosphorylates the translation regulator, CusR. The phosphorylated CusR promotes higher expression of the genes encoding the efflux pump CusCFBA, which pumps the copper/silver out of the bacteria, thus leading to bacteria that are resistant to heavy metals. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of CusS-CusR TCS is shown in Figure 1.

However, only a few studies have focused on the tolerance of K. pneumoniae to heavy metals and its association with antibiotic resistance. Accordingly, in this study, we induced three forms of carbapenem-resistant Klebsiella pneumoniae (CRKP) resistance to tigecycline in vitro and identified the differentially expressed genes using transcriptome sequencing. In particular, we focused on the CusS-CusR TCS to determine its potential role in tigecycline resistance in nine CRKP strains that evolved from the three parental strains KPN222 (A), KPN114 (B), and KPN315 (C).

**MATERIALS AND METHODS**

**Antimicrobial Susceptibility Testing**

Antibiotic susceptibility testing was performed using the Vitek 2 system (BioMérieux, Marcy-l’Étoile, France). The MICs of tigecycline and polymyxin B were determined using the micro-broth dilution method (BIO-KONT Co., Ltd., Wenzhou, China). The breakpoints of tigecycline and polymyxin B were interpreted according to the Clinical and Laboratory Standards Institute guidelines (Kuti et al., 2018). E. coli ATCC25922 and P. aeruginosa ATCC27853 were used as quality controls.

**Laboratory Evolution of Tigecycline-Resistant Mutants**

Independent single colonies of the CRKP strains KPN222 (A), KPN114 (B), and KPN315 (C) were grown overnight at 37°C. The cultures were then inoculated into Luria–Bertani (LB) agar with serially increasing concentrations of tigecycline, starting at a final concentration of tigecycline and polymyxin B were determined using the micro-broth dilution method (BIO-KONT Co., Ltd., Wenzhou, China). The breakpoints of tigecycline and polymyxin B were interpreted according to the Clinical and Laboratory Standards Institute guidelines (Kuti et al., 2018). E. coli ATCC25922 and P. aeruginosa ATCC27853 were used as quality controls.
RNA Extraction, Sequencing, and Data Analysis

The isolates were grown in high-osmolality LB broth at 37°C until the early exponential phase, and then RNA was extracted using the RNPure Bacteria kit (CWBio Co., Beijing, China) according to the manufacturer’s protocol. The concentration of RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RNA sequencing was performed using a HiSeq 2000 sequencer (Illumina, San Diego, CA, United States) and de novo assembly was carried out using Trinity software1. BLASTn (Altschul et al., 1990) was used to analyze Unigene NT with a Blastx (Altschul et al., 1990) or Blast2GO (Conesa et al., 2005) and the NR annotation results for Gene Ontology (GO) annotation. Bowtie 2 (Langmead and Salzberg, 2012) was used to compare the clean reads with the corresponding sequences in Unigene, and RSEM (Li and Dewey, 2011) was then used to calculate the gene expression levels of each sample. The PossionDis method was used for differential gene detection.

Quantitative PCR and Standard PCR

The expression levels of the CusS-CusR TCS were determined using quantitative qPCR. The primers used for the CusS-CusR TCS and other genes associated with tigecycline resistance are shown in Table 1. The primers used for the AcrAB-ToIC efflux pump-associated genes soxS, acrB, rarA, rama, ramR, mraR, toIC, and acrA were obtained from the literature (He et al., 2015). The primers associated with the SdiA-AcrEF and RarA-OqxAB efflux pump were also shown in Table 2. Total bacterial RNA was extracted using the RNA Pure Bacteria kit (CWBio Co., Beijing, China). cDNA was then synthesized using a Revert Aid First Strand cDNA Synthesis kit (CWBIO Co., Beijing, China). cDNA was then measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific) was used and the reaction system contained 25.0 µL of 2 × Dream Taq PCR Master Mix, 0.4 µL of forward and reverse primers (10 mol/L), and 4.0 µL of DNA template supplemented with 50.0 µL ddH2O. The reaction conditions were as follows: 95°C for 1 min; followed by 30 cycles of 95°C at 30 s, 50°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 2 min. The threshold cycle (CT) numbers were confirmed using the 2ΔΔCT method. Gene expression levels were determined and compared with those of wild-type strains (A1, B1, and C1, expression = 1).

The mutations of tex (T) and tet (A), which are associated with tetracycline resistance, were detected using the standard PCR. The DreamTaq PCR Master Mix kit (Thermo Fisher Scientific) was used and the reaction system contained 25.0 µL of 2 × Dream Taq PCR Master Mix, 0.4 µL of forward and reverse primers (10 mol/L), and 4.0 µL of DNA template supplemented with 50.0 µL ddH2O. The reaction conditions were as follows: 95°C for 1 min; followed by 30 cycles of 95°C at 30 s, 50°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 2 min. The mutations of tex (T) and tet (A), which are associated with tetracycline resistance, were detected using the standard PCR. The DreamTaq PCR Master Mix kit (Thermo Fisher Scientific) was used and the reaction system contained 25.0 µL of 2 × Dream Taq PCR Master Mix, 0.4 µL of forward and reverse primers (10 mol/L), and 4.0 µL of DNA template supplemented with 50.0 µL ddH2O. The reaction conditions were as follows: 95°C for 1 min; followed by 30 cycles of 95°C at 30 s, 50°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 2 min.

Table 1: Primers of CusS-CusR TCS and other genes associated with tigecycline resistance.

| Genes | Primers F/R (5′ → 3′) | Length (nt) | Annealing temperature (°C) |
|-------|---------------------|-------------|---------------------------|
| cusE  | CCGATTGCCCCTTATTCC | 230         | 55                        |
| cusS  | GCAAAATCGACACCCAC  | 148         | 55                        |
| cusR  | GCGAAGATTGACACAGGG  | 168         | 58                        |
| cusC  | TGACAGGCGACGCCCCA   | 257         | 55                        |
| cusF  | TGGTGCTTCCTGTCGCA   | 162         | 56                        |
| rsaB  | CAGAGCGCCGTCCGACCTTCT | 103       | 56                        |
| rsaA  | GTTGCCATGTTGCACTCTGCTTG | 223   | 57                        |
| tex(T)*| GAGCGGATTGACGACTGCTA | 193       | 55                        |
| tet(A)*| CAAGGAGCTGGAAGGAGGAA | 173       | 58                        |
| acrE  | ATGCCCTGCGATGTTG    | 175         | 56                        |
| acrF  | GCGATGTGCGTACCTTG   | 162         | 55                        |
| oqxA  | CAGCGTAACTTGAGCCTCAC | 168       | 57                        |
| oqxB  | CAGAAAGAGACCTCCCTTAC  | 178     | 58                        |

1https://github.com/trinityrnaseq/trinityrnaseq/wiki/Output-of-Trinity-Assembly

* tex (T) and tet (A) were detected by standard PCR and confirmed the mutant by Sanger sequencing.
TABLE 2 | Antibiotics susceptibility of primary strains and induced strains.

| Antibiotics                  | MIC (µg/mL) S/I/R |
|------------------------------|-------------------|
|                              | A1    | A2    | A3    | B1    | B2    | B3    | C1    | C2    | C3    |
| Amikacin                     | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   | ≤2 S   |
| Tobramycin                   | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  |
| Minocycline                  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  |
| Trimethoprim–Sulfamethoxazole| ≥320 R | ≥320 R | ≥320 R | ≥320 R | ≥320 R | ≥320 R | ≥320 R | ≥320 R | ≥320 R |
| Cefoperazone/sulbactam       | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  |
| Levofloxacin                 | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   | ≤8 R   |
| Ciprofloxacxin               | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   | ≥4 R   |
| Cefepime                     | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  | ≥32 R  |
| Aztreonam                    | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  |
| Piperacillin–Tazobactam      | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R |
| Ceftazidime                  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  | ≥64 R  |
| Amoxicillin/clavulanic- acid | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R | ≥128 R |
| Imipenem                     | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  |
| Meropenem                    | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  | ≥16 R  |
| Polymyxin B*                 | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S | ≤0.5 S |
| Tigecycline*                 | 0.5 S  | 16 R   | 32 R   | 16 R   | 32 R   | 0.5 S  | 16 R   | 32 R   | 16 S   |

MIC, minimal inhibitory concentration; S, sensitive; I, intermediary; R, resistance; *: tigecycline and polymyxin B antibiotic susceptibility were determined by micro-broth dilution method.

TABLE 3 | Differential expression genes come from RNA sequencing.

| Gene ID         | log2Fold change |
|-----------------|-----------------|
| Unigene 28_All  | 13.8 (C2/C1)    |
| Unigene274_All  | 10.59 (C2/C1)   |
| Unigene1733_All | 6.32 (C2/C1)    |
| Unigene1142_All | 3.31 (B2/B1)    |

8 min. All qPCR and standard PCR products were confirmed using agarose gel electrophoresis and sequencing.

Deletion and Complementation of the Gene cusR

Based on the homologous recombination technology, the deletion strain of the ΔcusR gene was constructed using the suicide vector pKO3-Km plasmid, and the gene fragment containing the activation region and the transcriptional termination region of the coding region of the C-cusR gene was amplified. The gene fragment was cloned into the pGEM-T-Easy plasmid and transferred to the deletion strain to construct the complemented strain of the C-ΔcusR gene. The primers are shown in Table 4.

RESULTS

Antibiotic Susceptibility of the Strains

All strains were resistant to tobramycin, minocycline, trimethoprim–sulfamethoxazole, cefoperazone/sulbactam, levofloxacin, ciprofloxacin, cefepime, aztreonam, piperacillin–tazobactam, ceftazidime, amoxicillin/clavulanic acid, imipenem, and meropenem. The strains were only sensitive to amikacin and polymyxin B with MICs of 2 and 0.5 µg/mL, respectively. The tigecycline MIC of A1, B1, and C1 was 0.5 µg/mL; that of A2, B2, and C2 was 16 µg/mL; and that of A3, B3, and C3 was 32 µg/mL. Antibiotic susceptibility of the primary strains and induced strains is shown in Table 2.
RNA Sequencing

The differentially expressed genes identified by RNA-sequencing are shown in Table 3 and Supplementary Material. Unigene 28_All was 4997 bp long and the NCBI BLAST revealed that it contained cusE, cusS, cusR, cusC, and cusF. Unigene274_All was 1090 bp long and contained cusA. Unigene1733_All was 1111 bp long and contained cusB. These three genes showed higher expression in C2 and C3 than in C1. Unigene1142_All was 2137 bp long, annotated to the transcriptional regulatory gene ramA in the genes encoding the AcrAB-TolC efflux pump, and highly expressed in B2 and B3, with B2/B1 log2 fold-change = 3.31 and B3/B1 log2 fold-change = 3.61.

Quantitative PCR

The relative expression levels (fold-change) of genes related to the AcrAB-TolC efflux pump (soxS, acrB, rarA, ramA, ramR, marA, tolC, and acrA) in A2/A1 and A3/A1, B2/B1 and B3/B1, and C2/C1 and C3/C1 are shown in Figure 2. The expression level of AcrAB-TolC efflux pump-related factors was the highest in group B. The relative expression levels (fold-change) of genes related to the CusS-CusR TCS (cusF, cusB, cusC, cusA, cusS, cusR, and cusE) in A2/A1 and A3/A1, B2/B1 and B3/B1, and C2/C1 and C3/C1 are shown in Figure 3. CusS-CusR TCS showed the highest expression in group C.

Tigecycline Resistance-Associated Genes

We detected the AcrEF-TolC efflux pump and OqxAB-TolC efflux pump in A2, A3, B2, B3, C2, and C3. In contrast, the acrE, acrF, oqxA, and oqxB genes did not show higher expression than A1, B1, and C1. The ramR, tex (T), and tet (A) genes contained no mutations.

Effect of Deletion and Complementation

Successfully built a non-trace cusS gene deletion strain ΔC3 and the complemented strain C-ΔC3, and then verified by RT-PCR of the cusR gene in ΔC3 showing no expression. The tigecycline MIC of ΔC3 was decreased to 4 μg/mL and that of C-ΔC3 was recovered to 32–4 μg/mL, and the expression of cusFBCA correspondingly decreased and increased significantly.

DISCUSSION

In the present study, we successfully screened three groups of tigecycline-resistant strains by RNA-sequencing, followed by annotation with biological information. We found that the AcrAB-TolC efflux pump system was highly expressed in group B, indicating that this system plays an essential role in the development of CRKP resistance to tigecycline. The AcrAB-TolC efflux pump is involved in resistance-nodulation cell division and has been reported to be resistant to several types of antibiotics (Zhong et al., 2014; Castanheira et al., 2016; Yuhan et al., 2016). However, Nielsen et al. (2014) reported that the IS5 insertion element increased the MIC of tigecycline by four-fold and was not dependent on the functional AcrAB-TolC efflux pump, suggesting that the AcrAB-TolC efflux pump is not the only mechanism causing tigecycline resistance. Hladicz et al. (2017) reported that RamR is a repressor that prevents the overexpression of ramA; when ramR is mutated, ramA is overexpressed, leading to tigecycline resistance; this mechanism was confirmed by Li et al. (2017). However, in the current study, no mutation in ramR was detected.

Zhang et al. (2018) reported that impairment of AcrAB-ToIC function upregulates acrEF quinolone resistance in E. coli. Thus, we also detected acrE and acrF, but no increase in expression was observed. Juan et al. (2016) evaluated 43 strains each of tigecycline-non-susceptible KP and tigecycline-susceptible KP, and found that the overexpression of OqxAB efflux pumps was a major cause of tigecycline-non-susceptible KP resistance to tigecycline. However, the evolved strains in this study did not show an increase in their expression. Furthermore, the genes...
tex(T) and tet(A), which are associated with tigecycline resistance, also did not contain mutations, indicating that these genes were not involved in tigecycline resistance in groups A, B, and C.

Andrade (Andrade et al., 2018) showed that multidrug-resistant CTX-M-(15, 9, and 2) – and KPC-2-producing Enterobacter hormaechei and Enterobacter asburiae isolates possessed a set of acquired heavy metal tolerance genes including a chromosomal sil operon (for acquired silver resistance). Yuan et al. (2019) also indicated that mercury/silver resistance genes were associated with antibiotic resistance. Elkrewi et al. (2017) reported that cryptic Ag + resistance pertaining to the sil operon is prevalent and readily activated in particular genera (Enterobacter and Klebsiella). Conroy et al. (2010) reported that CusCFBA had a narrow substrate spectrum transporting Cu (I) and Ag (I); in this research, the MIC of tigecycline and the expression of cua operon is prevalent and readily activated in particular genera (Enterobacter and Klebsiella). Conroy et al. (2010) reported that CusCFBA had a narrow substrate spectrum transporting Cu (I) and Ag (I); in this research, the MIC of tigecycline and the expression of cua operon was clearly elaborated by Pal et al. (2017), however, further research is necessary to verify whether it is involved in cross-resistance or co-resistance to metal and resistance to antibiotics. In conclusion, we considered that in group C, the CusS-CusR TCS was activated and resistance was induced in C2/C3 strains to tigecycline. We recognize some limitations to our study. The strains in group A did not show any changes in AcrAB-TolC efflux pump-related and CusS-CusR TCS-related gene expression. Additionally, genes associated with tigecycline resistance such as acrE, acrF, oqxA, and oqxB did not show increased expression, and tex(T) and tet(A) did not contain any mutations. Thus, other mechanisms may have caused A2 and A3 resistance to tigecycline, which requires additional analysis.

CONCLUSION

Despite these limitations, we found that the increased expression of the CusS-CusR TCS, which is associated with Cu and Ag resistance, mediated CRKP resistance to tigecycline, which may become a novel target of antibiotics. Thus, in cases where the common mechanisms are not identified as mediators of tigecycline resistance, the presence of high CusS-CusR TCS expression may be considered.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J. Mol. Biol. 215, 403–410. doi: 10.1016/S0022-2836(98)00360-2

Andrade, L. N., Siqueira, T. E. S., Martinez, R., and Darini, A. L. C. (2018). Multidrug-Resistant CTX-M-(15, 9, 2) and KPC-2-Producing Enterobacter hormaechei and Enterobacter asburiae Isolates Possessed a Set of Acquired Heavy Metal Tolerance Genes Including a Chromosomal sil Operon (for Acquired Silver Resistance). Front. Microbiol. 9, 339. doi: 10.3389/fmicb.2018.00339

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60. doi: 10.1038/nmeth.3176

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI SRA https://www.ncbi.nlm.nih.gov/sra/PRJNA596084.

AUTHOR CONTRIBUTIONS

YC, DC, and HL designed the experiments, analyzed the data, and wrote the manuscript. YZ, LX, YQ, HH, and DZ performed the experiments and analyzed the data. XL, XY, NX, XH, and FC analyzed the data.

FUNDING

This work was supported by the Scientific Innovation Project of Fujian Provincial Health and Family Planning Commission (Grant No. 2017-CX-3), the National Major Science and Technology Project for the Control and Prevention of Major Infectious Diseases of China (Grant No. 2017ZX10103004), the Fujian Medical Science and Technology Innovation Project (Grant No. 2016Y9005), the Fujian Provincial Natural Science Foundation Project (Grant No. 2019J01178), and the Startup Fund for scientific research, Fujian Medical University (Grant No. 2016QH116).

ACKNOWLEDGMENTS

We thank Prof. Bei Li from Basic Medical College of Hubei University of Medicine for providing the pKO3-Km plasmid and pGEM-T-easy plasmid.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.03159/full#supplementary-material

DATA SHEET S1 | Transcriptome sequencing results of groups B and C.
He, F., Fu, Y., Chen, Q., Ruan, Z., Hua, X., Zhou, H., et al. (2015). Tigecycline resistance in Klebsiella pneumoniae. *Antimicrob. Agents Chemother.* 58, 115–122. doi: 10.1128/AAC.03053-14

Nielsen, L. E., Snersud, E. C., Ornus-Leone, F., Kwak, Y. I., Avilés, R., Steele, E. D., et al. (2014). ISS element integration, a novel mechanism for rapid in vivo emergence of tigecycline nonsusceptibility in Klebsiella pneumoniae. *Antimicrob. Agents Chemother.* 58, 6151–6156. doi: 10.1128/AAC.03053-14

Pal, C., Asiani, K., Arya, S., Rensing, C., Stelk, D. J., Larsson, D. G. J., et al. (2017). Metal Resistance and Its Association with Antibiotic Resistance. *Adv. Microb. Physiol.* 70, 261–313. doi: 10.1016/bs.ampbs.2017.02.001

Copyright © 2020 Chen, Zhao, Qiu, Xia, He, Zheng, Li, Yu, Xu, Hu, Chen, Li and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.