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ORIGINAL RESEARCH

Efflux Pump AcrAB Confers Decreased Susceptibility to Piperacillin–Tazobactam and Ceftolozane–Tazobactam in Tigecycline-Non-Susceptible *Klebsiella pneumoniae*

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Introduction: Drug efflux pumps are critical for resistance in Gram-negative organisms, but there are limited data on the role they play in decreased susceptibility to β-lactam/β-lactamase inhibitor combinations. In this study, we aimed to investigate the impact of efflux pump AcrAB on piperacillin–tazobactam (TZP) and ceftolozane–tazobactam (C/T) susceptibility in tigecycline-non-susceptible *Klebsiella pneumoniae* (TNSKP) strains.

Methods: A tigecycline gradient was used to obtain various TNSKP strains, and in conjunction with the gradient derived strains, a TNSKP clinical strain (TNSKP24) was also included. Minimum inhibitory concentrations (MICs) of antibiotics were determined by the broth microdilution method, and whole-genome sequencing (WGS) was carried out to analyze genomic changes. PCR and sequencing were performed to confirm mutations in *ramR, acrR*, and the intergenic region of *ramR-romA*, and qRT-PCR was applied to evaluate levels of gene expression. In-frame *acrB* knockout and complementation were performed in 3 TNSKP strains.

Results: Two derivatives of *K. pneumoniae* K2606 (K2606-4 and K2606-16) and TNSKP24 overexpressed efflux pump AcrAB were obtained for further study. The MICs of TZP and C/T exhibited a 4- to 8-fold increase in K2606-4 and K2606-16, respectively, when compared with K2606 (TZP, 2/4 μg/mL; C/T, 0.25/4 μg/mL). Deletion of *acrB* decreased the MICs of TZP and C/T by 4- to 16-fold in TNSKP24, K2606-4, and K2606-16, respectively, and complementation of *acrB* increased the MICs of these agents. MICs of clavulanate, sulbactam, and avibactam in the presence of β-lactam compounds did not change after *acrB* deletion and subsequent introduction of complementation mutants.

Conclusion: This study highlights that decreased susceptibility to TZP and C/T could be caused by the multidrug efflux pump AcrAB in TNSKP strains.

Keywords: drug efflux pump, *Enterobacteriaceae*, multidrug resistance, β-lactam/β-lactamase inhibitor combinations

Introduction

Over the last decade, Gram-negative bacterial resistance has increased worldwide, and the growing threat of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is of great concern. With increased resistance to carbapenems, carbapenem-sparing strategies have been proposed,[1,2] and potential alternative agents have been investigated.[2,3] β-lactam/β-lactamase inhibitor combinations (BL/BLIs) are

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considered to be promising candidates for the treatment of mild to moderate infections caused by extended-spectrum-β-lactamase (ESBL)-producing *K. pneumoniae*.[1,3,4] The widely available piperacillin–tazobactam (TZP) and another newly released tazobactam combination, ceftolozane–tazobactam (C/T), both demonstrate excellent in vitro activity against ESBL-producing Gram-negative bacteria, including *K. pneumoniae*.[2,4–8]

However, attention should be paid to the development of bacterial resistance to these two tazobactam combinations. Bacterial resistance against TZP is mainly characterized by the following mechanisms used in *Enterobacteriaceae*: 1) hyper-production of β-lactamases such as TEM-1 in *K. pneumoniae*,[9] and the presence of TEM, SHV, and CTX-M variants in *Escherichia coli* and *K. pneumoniae*,[7,10,11] and 2) production of β-lactamases that are not readily inhibited by tazobactam (eg, carbapenemases, AmpC β-lactamases, and OXA-group β-lactamases).[12] It has been reported that a deficiency of the OmpK36 porin likely results in *K. pneumoniae* resistance to TZP.[13] Another study has also suggested that *E. coli* may potentially leverage a multidrug efflux pump to promote TZP resistance.[14] Lastly, investigations in *Pseudomonas aeruginosa* have found that an intrinsic AmpC mutation and acquired β-lactamases appear to be the main mechanisms of bacterial resistance to C/T, a new-generation BL/BLI that is tazobactam combined with a novel cephalosporin ceftolozane.[15–17] However, it is important to note that the novel tazobactam combination can also be hydrolyzed by carbapenemase enzymes and is considered not to be affected by efflux pumps or porin loss.[18] Therefore, the exact role that efflux pumps play in *K. pneumoniae* susceptibility to TZP and C/T requires further investigation.

Overexpression of efflux pump AcrAB, a member of the resistance-nodulation-division (RND) superfamily, is one of the main mechanisms mediating tigecycline resistance in tigecycline-non-susceptible *K. pneumoniae* (TNSKP). Upregulation of *acrAB* can be activated by a global transcriptional activator RamA, and increased expression of *ramA* occurs mainly because *ramR*, the gene encoding a local transcriptional repressor of RamA, is mutated.[19] While studying tigecycline resistance mechanisms of *K. pneumoniae* in vitro, we observed that laboratory TNSKP strains simultaneously showed decreased susceptibility to TZP and C/T. This interesting result led us to examine how efflux pump AcrAB affects the susceptibility of clinical and laboratory-derived TNSKP strains to BL/BLIs, including tazobactam, sulbactam, clavulanate, and avibactam combined with their β-lactam partners.

Materials and Methods

Bacterial Strains and Antimicrobial Susceptibility Testing

TNSKP24 is a clinical isolate with a tigecycline MIC 4 μg/mL tested by broth microdilution method as described previously.[19] K2606 is a clinical isolate with a tigecycline MIC 2 μg/mL that was randomly selected for tigecycline resistance induction experiment. TNSKP24 and K2606 were obtained from sputum and blood samples, respectively, of two hospitalized patients in Huashan Hospital in 2012. The clinical samples were part of the routine hospital laboratory procedure. In this study, all *K. pneumoniae* strains used were maintained at −80°C in 20% (vol/vol) glycerol for cryoprotection.

The MICs of tigecycline were determined as described previously[20] and interpreted based on the breakpoint for *Enterobacteriaceae* endorsed by the US Food and Drug Administration (≤2.0 μg/mL is susceptible, 4.0 μg/mL is intermediate, and ≥8.0 μg/mL is resistant). MICs of other antimicrobial agents were also determined by broth microdilution methods and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[21] MIC results were considered significant when there was a ≥ 4-fold difference between the parental strains and their derivatives. *E. coli* ATCC25922 was used as the quality control strain.

Selection of Tigecycline-Resistant Mutants by Serial Passage

To explore the potential mechanisms causing reduced susceptibility to BL/BLIs in TNSKP strains, resistance induction with tigecycline gradient was carried out in vitro as described previously with minor modifications.[22] Briefly, the parental K2606 strain was statically cultured overnight in 2 mL Muller-Hinton (MH) broth, starting with 0.5× tigecycline MIC of the parental strain. And then, 20 μL of the overnight inoculum was suspended in another 2 mL fresh MH broth containing a two-fold tigecycline concentration of the former, with this process repeated every 24 h until the MH broth reaching a tigecycline concentration of 16× MIC of the original strain after 6 days (ie, reaching 16× MIC of the original strains). The daily subculture (10 μL) was propagated on MH agar plates without antibiotics,

and a single colony from each plate was randomly selected and stored at −80°C for further study.

Efflux Pump Inhibition Test

To assess the role of efflux pump in tigecycline MIC alteration, efflux pump inhibition test was performed using Phe-Arg-β-naphthylamide (PAβN) as previously described.[23] Briefly, MICs of tigecycline alone or in combination with PAβN (25 μg/mL) were determined in TNSKP24, K2606, and derivatives of K2606.

Whole Genome Sequencing (WGS) and Analysis

To identify the genomic alterations of K2606 after exposure to tigecycline, we sequenced the whole genomes of K2606 and its two derivatives (K2606-4 and K2606-16). Genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacture's protocol. Genomes were sequenced using a combination of PacBio *RS* II Single Molecule Real Time (SMRT) and Illumina sequencing platforms, and the resulting data were used for bioinformatics analysis. Glimmer was used for CDS prediction, tRNA-scan-SE was used for tRNA prediction and Barrnap was used for rRNA prediction. The predicted CDSs were annotated from NR, Swiss-Prot, Pfam, GO, COG and KEGG database using sequence alignment tools such as BLAST, Diamond and HMMER. Briefly, each set of query proteins was aligned with the databases, and annotations of best-matched subjects (e-value $<10^{-5}$) were obtained for gene annotation.

Genome of K2606 was used as a reference. Variants (SNPs, short indels, and large deletions) of K2606-4 and K2606-16 were called based on the Bowtie2 assembled data using GATK toolkit by Broad Institute. The variants were verified by universal PCR and sequencing.

Universal PCR and Quantitative Real-Time PCR (qRT-PCR)

To detect the presence of mutations in *ramR, acrR*, and the intergenic region of *ramR-romA*, universal PCR and sequencing were applied as described previously.[19] Genome of *K. pneumoniae* MGH78578 (tigecycline MIC, 1 μg/mL) was used as a reference for gene mutation analysis. To evaluate the transcriptional expression levels of efflux pump genes *acrB* and *oqxB*, and the regulatory genes *ramA* and *rarA* in TNSKP strains, qRT-PCR was performed as described previously.[19] The normalized relative expression of genes was determined by the $2^{-\Delta\Delta CT}$ method. A tigecycline-susceptible *K. pneumoniae* clinical isolate (TSKP1; tigecycline MIC, 0.5 μg/mL) and K2606 were used as a reference isolate for the gene expression analysis of TNSKP24 and the two derivatives of K2606 (K2606-4 and K2606-16), respectively.

Construction of *acrB* Deletion Mutants

To investigate the role of the efflux pump AcrAB in the susceptibility to BL/BLIs in *K. pneumoniae* strains, TNSKP24 and two derivatives of K2606 (K2606-4 and K2606-16) were used for *acrB* gene knockout. Lambda Red recombination and Flp-FRT recombination were used to construct markerless in-frame gene deletion mutants as described previously.[24] Finally, 3123-bp of *acrB* was replaced by an 84-bp segment, and markerless in-frame indel mutants TNSKP24Δ*acrB*, K2606-4Δ*acrB*, and K2606-16Δ*acrB* were obtained, and confirmed by PCR and sequencing. The primers used in this study are listed in Table 1.

Complementation with the Wild-Type *acrB* Gene

To establish the influence of *acrB* knockout on the susceptibility to TZP and C/T, complementation with the wild-type *acrB* gene was performed in TNSKP24Δ*acrB*, K2606-4Δ*acrB*, and K2606-16Δ*acrB*. Briefly, the 3147-bp *acrB* gene of strain TNSKP24 and K2606 was, respectively, amplified using primers Hindш-*acrB*-F and BamH Ⅰ-*acrB*-R (Table 1), digested with Hindш and BamH Ⅰ, and ligated to vector pHSG396 to generate the recombinant vector (pHSG396*acrB*). pHSG396*acrB* was verified by sequencing, and then it was electroporated into competent strains. And the transformants were selected on Luria Bertani (LB) agar plates with 50 μg/mL chloramphenicol and were verified by sequencing. Empty plasmid pHSG396 was used as a negative control.

Results

Susceptibility Profiles of TNSKP24, K2606, and Their Derivatives

The MICs of 18 compounds for *K. pneumoniae* TNSKP24, K2606, and their derivatives are summarized in Table 2. K2606-4 and K2606-16 were derived from K2606 by gradient exposure at 4× and 16× tigecycline MIC of K2606, respectively. The two derivatives not only

Table 1 Primers Used in This Study

Primers	Sequence (5′ to 3′)	Product (bp)	Reference
GmF	CGAATTAGCTTCAAAAGCGCTCTGA	1649	[24]
Gm-R2	AATTGGGGATCTTGAAGTTCCT		
EBGNHe-5	CCCGCTAGCGAAAAGATGTTTCGTGAAGC	1960	[24]
EBGh3-3	GGGAAGCTTATTATCGTGAGGATGCGTCA		
acrB-UF-F	CGGCAAAGCGAAAGTGGAG	512	This study
acrB-UF-R	TCAGAGCGCTTTTGAAGCTAATTCG[a] GAAATTAGGCATGTCTTAACGGC		
acrB-DF-F	AGGAACTTCAAGATCCCCAATT[b] GAGCATCATTAATCTTCACTCC	509	This study
acrB-DF-R	GTA TTC GTC CAT AAC GCA TC		
acrB-internal-F	TATCGGCTACGACTGGACC	317	This study
acrB-internal-R	GCCCTTTGCCCTCTTTCT		
acrB-external-F	TGATTTCCTGCGCCTGAAG	4247	This study
acrB-external-R	AGCGTTTCG CAG AGAAAG C		
ramR-romA-F	GATGGCGACCACGCTGAA	305	This study
ramR-romA-R	TATGCCGACTGGGCGAAA		
HindIII-*acrB*-F	ATGACCATGATTACGCCAAGCTTATGCCTAATTTCTTTATCGATCG	3192	This study
BamH I -*acrB*-R	GTACCCCATCGATGGGGGATCCTTAATGATGCTCAACCTGATGGC		

Notes: [a]Reverse-complement to Gm-F; [b]reverse-complement to Gm-R; Gm-F/R for hygromycin B resistance gene *hph* cassette flanked by the Flp recombinase target sites; EBGNHe-5/EBGh3-3 for the recombination vector pKOBEG; *acrB-UF*-F/R for 5′ upstream flanking of *acrB* fragment; *acrB-DF*-F/R for 3′ downstream flanking of *acrB* fragment; *acrB*-internal-F/R for a fragment inside *acrB*; *acrB*-external-F/R for a fragment including *UF, DF,* and *acrB*.

exhibited a significant increase in the MICs of tigecycline and ciprofloxacin but also in TZP and C/T when compared with K2606 (Table 2). Interestingly, MICs of other BL/BLIs (sulbactam, clavulanate, and avibactam combinations) tested showed either a marginal increase (2-fold) or none at all (Table 2).

The tigecycline MIC of TNSKP24 was 4 μg/mL and decreased to 1 μg/mL in the presence of 25 μg/mL PAβN. Additionally, the MICs of K2606-4 and K2606-16 were >128 μg/mL, and both were reduced to 16 μg/mL in the presence of 25 μg/mL PAβN. On the other hand, the tigecycline MIC of K2606 was not significantly decreased (2 μg/mL to 1μg/mL) in the presence of PAβN.

Genomic Changes of K2606 After Exposure to Tigecycline

We obtained complete genome sequences and summarized all mutations of K2606-4 and K2606-16 (Table 3, Figure S1). When compared with K2606, nonsynonymous mutations occurred in five genes (K2606-4, n=3; K2606-16, n=3). Among the five genes, only one (*tet*(A), encoding tetracycline efflux pump) exhibited a mutation (S251A) in both of the derivatives (Table 3, Figure S1A). Of the four remaining genes with nonsynonymous mutations, two were mutated in each of the derivatives. *dksA*, a gene encoding a transcriptional regulator, and *rpsJ*, a gene encoding ribosomal protein RpsJ, were found to be mutated in K2606-4 and K2606-16, respectively. The other two genes encode hypothetical proteins (Table 3, Figure S1B, C). It is important to note that genes encoding β-lactamases and their corresponding promoters did not exhibit mutations in the two derivatives of K2606.

Multiple intergenic point mutations were detected in K2606-4 (n=9) and K2606-16 (n=8) (Table 3). Of these mutations, six (T843830G, A1127404G, A1127631G, A1127769G, A1128074G, and C3872385T) were concurrent in both of the two strains (Table 3, Figure S1A). These six mutations occurred up or downstream of six distinct genes, and four of the genes (*fimB, csrA*, gene1123, and *ramR*) were associated with bacterial virulence, metabolism, signal transduction, and resistance, respectively. Five derivative specific intergenic point

Table 2 Antimicrobial Susceptibility Patterns of the *K. pneumoniae* Clinical Parental Strains and Their Derivatives

Strains	MIC (μg/mL)																	
	PIP	TZP	C/T	SAM	CFZ	CXM	CAZ	CCV	CZA	SCF	FEP	MEM	IPM	ATM	AMK	TGC	CIP	CST
TNSKP24[a]	256	4/4	1/4	32/16	>32	>64	0.5	0.5/0.25	0.25/4	16/8	8	≤0.06	0.125	2	0.5	4	0.5	0.5
TNSKP24Δ*acrB*[b]	256	0.25/4	0.25/4	32/16	>32	>64	0.5	0.5/0.25	0.25/4	16/8	8	≤0.06	0.125	2	0.5	0.5	≤0.06	0.5
TNSKP24Δ*acrB*/pHSG396	256	0.25/4	0.25/4	32/16	>32	>64	0.5	0.5/0.25	0.25/4	16/8	8	≤0.06	0.125	2	0.5	0.5	≤0.06	0.5
TNSKP24Δ*acrB*/pHSG396*acrB*	256	2/4	0.5/4	32/16	>32	>64	0.5	0.5/0.25	0.25/4	16/8	8	≤0.06	0.125	2	0.5	2	0.5	0.5
K2606[c]	512	2/4	0.25/4	32/16	>32	>64	1	0.5/0.25	0.25/4	16/8	4	≤0.06	≤0.06	4	2	2	1	1
K2606-4[d]	512	16/4	1/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	>128	8	1
K2606-4Δ*acrB*[e]	256	2/4	0.25/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	2	≤0.06	1
K2606-4Δ*acrB*/pHSG396	256	2/4	0.25/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	2	≤0.06	1
K2606-4Δ*acrB*/pHSG396*acrB*	512	8/4	0.5/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	16	16	1
K2606-16[f]	512	16/4	1/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	>128	8	1
K2606-16Δ*acrB*[g]	256	2/4	0.25/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	2	≤0.06	1
K2606-16Δ*acrB*/pHSG396	256	2/4	0.25/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	2	≤0.06	1
K2606-16Δ*acrB*/pHSG396*acrB*	512	8/4	0.5/4	32/16	>32	>64	2	0.5/0.25	0.25/4	32/16	8	≤0.06	≤0.06	8	2	16	16	1
ATCC25922	2	2/4	0.125/4	4/2	4	4	0.125	0.25/0.125	≤0.25/4	0.25/0.125	≤0.06	≤0.06	≤0.06	≤0.06	2	0.125	≤0.06	0.5

Notes: [a]TNSKP24, a clinical *K. pneumoniae* strain with tigecycline MIC 4 μg/mL. [b]TNSKP24Δ*acrB*, TNSKP24 without *acrB*. [c]K2606, a clinical *K. pneumoniae* strain with tigecycline MIC 2 μg/mL. [d]K2606-4, K2606 exposure to tigecycline with a concentration 4× MIC of K2606. [e]K2606-4Δ*acrB*, K2606-4 without *acrB*. [f]K2606-16, K2606 exposure to tigecycline with a concentration 16× MIC of K2606. [g]K2606-16Δ*acrB*, K2606-16 without *acrB*.

Abbreviations: PIP, piperacillin; TZP, piperacillin–tazobactam; C/T, ceftolozane–tazobactam; SAM, ampicillin-sulbactam; CFZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidime; SCF, cefoperazone-sulbactam; CCV, ceftazidime-clavulanate; CZA, ceftazidime-avibactam; FEP, cefepime; MEM, meropenem; IPM, imipenem; ATM, aztreonam; AMK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; CST, colistin.

Table 3 Genomic Changes of K2606 After Exposure to Tigecycline

Location	Mutation	Position	Mutation Type	Information	Annotation	Gene Description
SNP[a] in both K2606-4 and K2606-16						
Chr[b]	T843830G	Intergenic of gene0838 and gene0839	-	-	*fimB*;-	type 1 fimbriae regulatory protein; hypothetical protein
Chr	A1127404G, A1127631G A1127769G, A1128074G	Intergenic of gene1117 and gene1123	-	-	*csrA*;-	carbon storage regulator; phospholipid-binding domain protein
Chr	C3872385 T	Intergenic of gene3810 and gene3811	-	-	*romA; ramR*	beta-lactamase domain protein; TetR family transcriptional regulator
pA[c]	A90269C	Internal of gene0095	Nonsynonymous	c.T751G p.S251A	*tetA*	tetracycline efflux MFS transporter
SNP in K2606-4 only						
Chr	G303519A	Intergenic of gene0279 and gene0278	-	-	*tusA*;-	tRNA 2-thiouridine (34) synthase; hypothetical protein
Chr	G586814C	Intergenic of gene0574 and gene0575	-		-;-	hypothetical protein; hypothetical protein
Chr	G4324175T	Internal of gene4263	Nonsynonymous	c.G386T, p.R129L	*dksA*	transcriptional regulator
Chr	C4350054A	Internal of gene4291	Nonsynonymous	c.G59T, p.C20F	-	hypothetical protein
pB[d]	C125391G	Intergenic of gene0112 and gene0113	-		-;*higA*	hypothetical protein; transcriptional regulator
SNP in K2606-16 only						
Chr	C427705A	gene0395	Nonsynonymous	c. C116A, p.P39Q	*rpsJ*	ribosome protein RpsJ
Chr	C2049592G	Intergenic of gene1998 and gene1999	-	-	-;-	GNAT family acetyltransferase; transcriptional regulator
Chr	A4052373C	Internal of gene3986	Nonsynonymous	c. A101C, p.Y34S,	-	hypothetical protein
pD[e]	C29141G	Intergenic of gene0036 and gene0037	-	-	-;-	hypothetical protein; hypothetical protein
[f]INDEL in K2606-4						
Chr	GCTATACCAAA deleted between 586,798 and 586,808	Intergenic of gene0572 and gene0573	Deletion	-	*glxK*;-	glycerate 2-kinase; hypothetical protein

Notes: [a]SNP, single nucleotide polymorphism; [b]Chr, chromosome; [c]pA, plasmid A; [d]pB, plasmid B; [e]pD, plasmid D; [f]INDEL, insertion-deletion.

mutations, three in K2606-4 and two in K2606-16, were also noted (Table 3, Figure S1B and S1C). Lastly, only one 11-bp fragment deletion was detected in K2606-4, and none were found in K2606-16 (Table 3, Figure S1D).

Mutations of *ramR, acrR*, the Intergenic Region of *ramR-romA*, and Increased Transcriptional Level of *acrB, oqxB, ramA*, and *rarA*

No mutations were detected in *ramR, acrR*, and intergenic regions of *ramR-romA* in TNSKP24 when compared with *K. pneumoniae* MGH78578. Also, no mutations were detected in *acrR* in K2606, K2606-4, and K2606-16. Interestingly, two mutations that were found in the K2606 parent strain persisted in its derivatives. A missense mutation (D77H) in *ramR* and a point mutation (C111T) in the intergenic region of *ramR-romA* were detected in K2606 and its two derivatives (K2606-4 and K2606-16) when compared with MGH78578. However, a novel point mutation (C133T, mentioned above C3872385T) was found in K2606-4 and K2606-16 (Figure 1A) but was absent in K2606.

The TNSKP24 clinical strain showed slightly altered expression levels of *acrB, oqxB, ramA*, and *rarA* when compared with TSKP1 (Figure 1B). K2606-4 and K2606-16 possessed relatively high transcriptional expression levels of *ramA* (13- and 11-fold), while those of *acrB, rarA* and *oqxB* had little to no increase (Figure 1C).

Antimicrobial Susceptibility Patterns of *acrB* Deletion and Complementation Mutants

The efflux pump gene *acrB* was deleted in-frame in three strains (TNSKP24, K2606-4, and K2606-16), and the corresponding mutants were designated as TNSKP24Δ*acrB*, K2606-4Δ*acrB*, and K2606-16Δ*acrB*, respectively. The TZP MICs of TNSKP24Δ*acrB*, K2606-4Δ*acrB*, and K2606-16Δ*acrB* were 0.25/4 μg/mL, 2/4 μg/mL, and 2/4 μg/mL (Table 2), respectively, which were reduced remarkably (16-fold or 8-fold) compared with the corresponding original strains. Similarly, the C/T MICs of the three knockout mutants were all 0.25/4 μg/mL and were also significantly reduced (4-fold) compared with the original strains. The MICs of piperacillin and other BL/BLIs

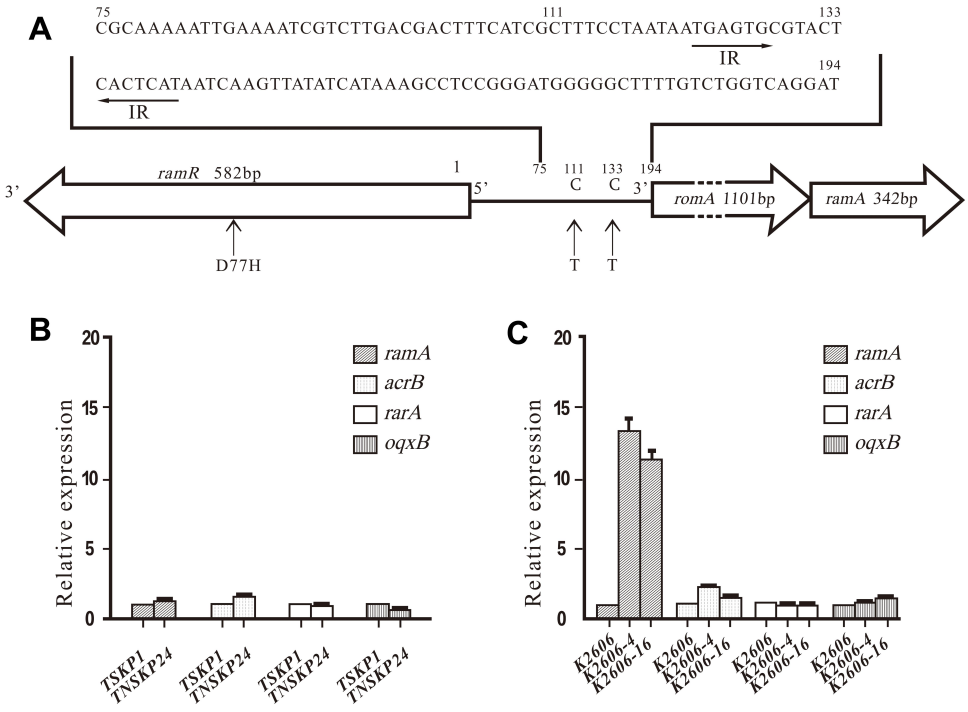

Figure 1 Gene mutation and expression in TNSKP24, K2606 K2606-4, and K2606-16. (**A**) Mutations in *ramR* and the intergenic region of *ramR-romA* in *K. pneumoniae*K2606 and its two derivatives when compared with *K. pneumoniae* MGH78578 (GenBank: CP000647.1). The arrow segments in the intergenic region of *romR-romA* are the inverted repeat (IR) sequences recognized by the RamR protein (24). The numbering system is based on the "t" prior to *romA*'s start codon ATG as the 194. Missense mutation (D77H) in *ramR*, and point mutation (C111T) in the intergenic region of *ramR-romA* was detected in K2606, K2606-4, and K2606-16, and an additional novel point mutation (C133T) was found between the palindromic repeats in the 2 derivatives. (**B**) and (**C**) Transcriptional expression (mean±standard deviation) of *ramA, acrB, rarA*, and *oqxB* in TNSKP24, K2606 K2606-4, and K2606-16.

tested, however, were only marginally reduced and in some cases, exhibited no reduction at all. These results suggest that functional AcrAB contributes to reduced TZP and C/T susceptibility in these strains.

As expected, the MICs of tigecycline and ciprofloxacin were also significantly decreased among the *acrB* knockout mutants compared with the corresponding original strains (Table 2), suggesting that AcrAB can efficiently pump out tigecycline and ciprofloxacin as well. On the other hand, the MICs of certain cephalosporins, carbapenems, aztreonam, aminoglycosides, and colistin were unaffected, indicating that these compounds are likely not good substrates of AcrAB.

Complementation with wild-type *acrB* was successfully constructed in TNSKP24Δ*acrB*, K2606-4Δ*acrB*, and K2606-16Δ*acrB* (designated as TNSKP24Δ*acrB*/pHSG396*acrB*, K2606-4Δ*acrB*/pHSG396*acrB*, and K2606-16Δ*acrB*/pHSG396*acrB*, respectively). The complementation of *acrB* increased the MICs of TZP, C/T, tigecycline, and ciprofloxacin, confirming that *acrB* plays a role in decreasing susceptibilities to these agents in TNSKP (Table 2).

Discussion

Multidrug efflux pumps, especially RND family transporters such as AcrAB, are critical for antibiotic resistance in Gram-negative bacteria, including the intrinsic and acquired resistance to structurally diverse agents. Identifying the actual role of efflux pumps in β-lactam susceptibility, however, is difficult due to complicated resistance backgrounds in clinical isolates. In this study, one clinical and two laboratory-derived tigecycline-non-susceptible *K. pneumoniae* strains simultaneously showed decreased susceptibility to two tazobactam-containing compounds, TZP and C/T. A study by Nicolas-Chanoine et al suggested that piperacillin, ceftolozane, and tazobactam may be substrates of AcrAB.[25] Tam et al confirmed that AcrB can bind and extrude carboxylated β-lactams.[26] To this end, our study recapitulates results from the aforementioned studies and concludes that the mechanism underlying MIC changes in TZP and C/T is the extrusion of carboxylated β-lactams by AcrAB in *K. pneumoniae*. Very recently, a study by Suzuki et al indicated that AcrAB also contributes to TZP non-susceptibility in *E. coli*.[27] Although piperacillin and ceftazidime are considered substrates of AcrAB,[25,28] our study showed that the MICs of these substances exhibited marginal change, if at all, in *acrB* deletion mutants of TNSKP24, K2606-4, and K2606-16, and the minimal changes in MICs may be due to β-lactamase production in these strains ($bla_{SHV-168}$ and $bla_{CTX-M-14}$ existed in TNSKP24; bla_{SHV-27} and $bla_{CTX-M-14}$ detected in K2606, K2606-4, and K2606-16, data not shown).

C/T is a second-generation BL/BLI that was recently approved by the US Food and Drug Administration.[6] The emergence of C/T resistance is rare and mainly reported in *P. aeruginosa* due to AmpC structural modification.[15–17] However, our study is the first to show that the overexpression of efflux pump AcrAB contributes to decreased susceptibility to C/T in *K. pneumoniae*.

After K2606 exposure to tigecycline, the derivatives acquired tigecycline resistance expectedly. Surprisingly, tigecycline MICs were extremely high (>128 μg/mL) in the two derivatives (K2606-4 and K2606-16). The high MICs were likely a result of the synergistic effects of an efflux pump TetA mutation and efflux pump AcrAB overexpression, and the synergic effect of efflux pump TetA and RND transporters on conferring higher tigecycline resistance has been observed in *Acinetobacter baumannii* recently.[29] However, tigecycline MIC returned to basal level when *acrB* was deleted in K2606-4 and K2606-16, suggesting AcrAB plays a critical role in mediating tigecycline resistance. Moreover, when wild-type *acrB* was introduced into K2606-4Δ*acrB* and K2606-16Δ*acrB*, tigecycline MICs increased to 16 μg/mL in the mutants, indicating that other factors may be contributing to high tigecycline MICs (>128 μg/mL) in the parental strains. Du et al reported that *tet*(A) mutation can confer a relatively high-level of tigecycline resistance (32 μg/mL) in vivo in *K. pneumoniae* strain,[30] but further study is needed to elucidate how *tet*(A) mutation and synergy between efflux pumps TetA and AcrAB affect tigecycline resistance in *K. pneumoniae*.

Next, we observed transcriptional expression levels of *acrB* in the clinical strain, TNSKP24, and the two laboratory-derived strains. Although the transcriptional expression levels of *acrB* were not markedly increased, MICs of TZP and C/T were significantly decreased in the corresponding *acrB* deletion mutants, suggesting that overexpression of efflux pump AcrAB contributes to reduced susceptibility to these two tazobactam combinations. Complementation with the *acrB* gene partially restored the MICs of TZP and C/T. As a result, this finding supports the notion that AcrAB functions to alter the MICs of the two antibiotics. Although the MICs were only partially

restored, this may be due, in part, to the insufficient restoration of *acrB* expression as well as other factors.

RamR has been shown to indirectly affect transcriptional levels of *acrAB* in *K. pneumoniae*. When RamR binds the palindromic repeats in the intergenic region of *ramR-romA, romA* and *ramA* are transcriptionally repressed.[31] Additionally, another study showed that increased expression of *ramA* can up-regulate the expression of *acrAB*.[19] In our study, two derivatives of K2606 exhibited a novel point mutation in the palindromic repeats of *ramR-romA* after exposure to tigecycline, and this likely contributed to the up-regulation of *ramA* in the two strains. Previously, we also identified that a 12-bp deletion in the intergenic region of *ramR-romA* caused the elevated expression of *ramA* and *acrB* which also conferred tigecycline resistance in vivo in *K. pneumoniae*.[32] In summary, these results indicate that mutations in the intergenic region of *ramR-romA* may help promote tigecycline resistance by upregulating expression of *acrB* in *K. pneumoniae.*

The present study shows that tigecycline and ciprofloxacin can be efficiently expelled by efflux pump AcrAB. However, carbapenems, certain cephalosporins, aztreonam, and aminoglycosides cannot be removed by the pump, and this is consistent with what has been shown in previous studies.[19,28,33,34] Previous studies have also reported that efflux played a primary role in resistance to cefepime in *P. aeruginosa*.[35,36] Consistent with our study, however, the studies also found that the efflux pump did not contribute to cefepime resistance in *K. pneumoniae* strains.[36] It is also worth mentioning that the net contribution of AcrAB to the MICs of β-lactams was likely masked by the presence of various β-lactamases, especially in clinical isolates. Overall, our study supports the notion that efflux pump AcrAB can significantly reduce the susceptibility of *K. pneumoniae* strains to tazobactam-containing compounds.

Our study also showed that the MICs of TZP and C/T were still in the susceptible range in TNSKP24, K2606-4, and K2606-16. In many cases, however, TZP resistance can be promoted in vitro when exposed to *E. coli*, which may leverage a multidrug efflux pump system to help augment resistance.[14] The efflux pump AcrAB may reduce the BL/BLIs susceptibility only below the clinical breakpoint, and resistance may only become obvious when reduction in membrane permeability and production of β-lactamases occur simultaneously. For instance, high-level ceftazidime-avibactam resistance can be mediated in vitro by a combination of increased β-lactamase expression, loss of functional outer membrane proteins, and activated efflux in *K. pneumoniae*.[37]

Juan et al reported that prior fluoroquinolone use was an independent risk factor that promoted *K. pneumoniae* non-susceptibility to tigecycline.[38] The underlying mechanism may be due to the overexpression of AcrAB, which mediates non-susceptibility to both fluoroquinolone and tigecycline. AcrAB is widely distributed in *K. pneumoniae* and helps mediate cross-resistance to various antimicrobials. Due to the aforementioned mechanisms, the simultaneous or sequential use of various AcrAB substrates requires caution and should be avoided if possible.

It is important to note that this study has a few limitations. First, the two parental clinical strains, TNSKP24 and K2606, are both β-lactamase producers. β-lactamases have the potential to mask the role of efflux pumps, including AcrAB, and thus cause the effect of efflux to be underestimated in clinical strains. Despite this setback, the present study clearly supports that efflux pump AcrAB plays a significant role in reducing TZP and C/T susceptibilities in *K. pneumoniae*. Secondly, although the present study supports tazobactam as a likely substrate of AcrAB, it was not confirmed to be one in *K. pneumoniae*, and this could be partially attributed to β-lactamases produced by our strains. Irrespective of this limitation, the conclusion that efflux pump AcrAB contributes to reducing the susceptibilities of the two tazobactam combinations as a whole is still strongly supported. Lastly, the present study mainly evaluated AcrAB contribution via *acrB* deletion and complementation in TNSKP clinical and laboratory strains, but the impact of β-lactamase and other mutated genes (such as *tet* (A)) was not confirmed by gene knockout. The MICs of TZP and C/T were still significantly reduced in *acrB*-deletion mutants, which strongly suggests *acrB* plays an important role in bacterial susceptibility to the two tazobactam combinations. In order to address the limitations presented in this work, further study is needed to clarify the synergistic effects of efflux pump AcrAB and various other proteins, such as efflux pump TetA and β-lactamases, on BL/BLIs in *K. pneumoniae* strains.

Conclusions

Efflux pump AcrAB can remarkably reduce susceptibilities to two tazobactam-containing combinations (piperacillin–tazobactam and ceftolozane–tazobactam) in TNSKP strains. This study also highlights how the often overlooked and underestimated role of efflux is critical to resistance in *K. pneumoniae* clinical strains. In addition, simultaneous decreases in susceptibility to various other

antimicrobials could be caused by efflux pump AcrAB. In turn, this could impact the combination or sequential use of these agents which would ultimately have serious clinical implications. To this end, it is important to be cognizant of the far-reaching impacts that efflux pumps have on bacterial susceptibility, especially as *K. pneumoniae* drug resistance continues to increase.

Abbreviations

TZP, piperacillin–tazobactam; C/T, ceftolozane–tazobactam; TNSKP, tigecycline-non-susceptible *Klebsiella pneumoniae*; MIC, minimum inhibitory concentration; WGS, whole-genome sequencing; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; BL/BLIs, β-lactam/β-lactamase inhibitor combinations; ESBL, extended-spectrum-β-lactamase; RND, resistance-nodulation-division; CLSI, Clinical and Laboratory Standards Institute; MH, Muller-Hinton; PAβN, Phe-Arg-β-naphthylamide; SMRT, Single Molecule Real Time; qRT-PCR, quantitative real-time PCR; TSKP, tigecycline-susceptible K. pneumoniae clinical isolate; LB, Luria Bertani.

Data Sharing Statement

The complete genome sequence of K2606 is available at US National Center for Biotechnology Information (NCBI) website under the following GenBank accession numbers: CP047633 for chromosome, CP047634 for plasmid A, CP047635 for plasmid B, CP047636 for plasmid C, CP047637 for plasmid D, and CP047638 for plasmid E.

Supplemental material is available online in Figure S1.

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Disclosure

The authors report no conflicts of interest in this work.

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