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Short Communication

Efflux pumps AcrAB and OqxAB contribute to nitrofurantoin resistance in an uropathogenic *Klebsiella pneumoniae* isolate

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ABSTRACT

Klebsiella pneumoniae is a common cause of urinary tract infections (UTIs). Nitrofurantoin (NIT), with high therapeutic concentrations in urine, is recommended as the first-line drug for both empiric treatment and chemoprophylaxis of UTIs. Although NIT resistance in *K. pneumoniae* is relatively high, the resistance mechanism is not well understood. This study collected a NIT-resistant *K. pneumoniae* [NRKP, minimum inhibitory concentration (MIC)=128 mg/L] and investigated the resistance mechanism. Addition of efflux pump inhibitors increased the susceptibility of NRKP to NIT (MIC decreased from 128 to 32 mg/L), implying the important role of efflux pumps in NIT resistance. Quantitative reverse transcriptase polymerase chain reaction analysis showed that NRKP had >100-fold increased expression of *ramA*, which was demonstrated to be caused by *ramR* mutation. Deletion of *ramA* led to a four-fold decrease in the MIC of NIT, and the expression levels of efflux pumps *acrB* and *oqxB* were downregulated by four- to seven-fold. Complementation of *ramA* restored both the MIC value and the expression level of *acrB* and *oqxB* in the *ramA* mutant strain. In order to confirm the role of *acrB* and *oqxB* in NIT resistance, gene knockout strains were constructed. Deletion of *acrB* or *oqxB* alone led to a four-fold decrease in the MIC of NIT, and deletion of *acrB* and *oqxB* simultaneously led to a 16-fold decrease in the MIC of NIT. These results demonstrate that AcrAB and OqxAB contribute to NIT resistance in *K. pneumoniae.*

1. Introduction

Uncomplicated urinary tract infections (UTIs), including cystitis and pyelonephritis, are some of the most common infectious diseases in the outpatient setting. Gram-negative bacteria, especially *Escherichia coli* and *Klebsiella pneumoniae,* are the predominant aetiological agents of UTIs in both adults and children [1,2]. For many years, trimethoprim-sulfamethoxazole (TMP-SMX) and the fluoroquinolones were the preferred antibiotics for the treatment of UTIs [3]. However, with the increasing prevalence of resistance, the clinical use of these two drug types has become limited [4]. Nitrofurantoin (NIT), one of the synthetic nitrofuran compounds, is active against both Gram-positive and -negative pathogens by inhibiting bacterial enzymes involved in DNA and RNA synthesis, carbohydrate metabolism, and other metabolic enzyme proteins [5,6]. NIT has been prescribed as an oral antimicrobial agent since 1952. It can be rapidly absorbed and excreted, and generates high therapeutic concentrations in the urine [6]. In addition, NIT is inexpensive and does not predispose to antimicrobial resistance [5]. These properties make NIT advantageous for the treatment of UTIs. The 2017 German clinical guidelines for management of UTIs recommended NIT as the first-line drug for the empiric treatment of uncomplicated UTIs, and chemoprophylaxis for postmenopausal women with recurrent UTIs [7].

However, resistance to NIT has emerged, especially among Enterobacteriaceae. By far the most reported mechanisms of NIT resistance in *E. coli* were mutations in chromosomal nitroreductase encoding genes (*nfsA* and *nfsB*), which metabolize NIT into toxic intermediate compounds [6]. Overexpression of the efflux pump OqxAB, located on a transferable plasmid, was also reported to contribute to NIT resistance in *E. coli* [8]. Moreover, deletion of *ribE*, which encodes a lumazine synthase, led to NIT resistance in *E. coli*, although *ribE* mutations have not been found in clinical *E. coli* isolates to date [9]. However, the NIT resistance mechanisms

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Table 1
Strains and plasmids used in this study.

Strains/plasmids	Description	Source
Strains		
ATCC 13883	Standard susceptible *K. pneumoniae*	Laboratory collection
E. coli DH5a	Competent cell strain used for transformation	Purchased from TIANGEN Biotech
ATCC 25922	Standard susceptible *E. coli*	Laboratory collection
NRKP	NIT-resistant clinical *K. pneumoniae* isolate	This study
NRKP△*ramA*	Derivative of NRKP that lacks *ramA* gene	This study
NRKP△*ramA*/pHSG396*ramA*	NRKP△*ramA* with wild-type *ramA*	This study
NRKP/pHSG396*ramR*	NRKP with wild-type *ramR*	This study
NRKP△*acrB*	Derivative of NRKP that lacks *acrB* gene	This study
NRKP△*oqxB*	Derivative of NRKP that lacks *oqxB* gene	This study
NRKP△*acrB*△*oqxB*	Derivative of NRKP that lacks *acrB* and *oqxB* genes	This study
NRKP△*ramA*/pHSG396	NRKP△*ramA* with expression vector pHSG396	This study
NRKP/pHSG396	NRKP with expression vector pHSG396	This study
Plasmids		
pKOBEG	Mediates λRed homologous recombination induced by L-arabinose (0.5% w/v), with apramycin	This study
pMD-18T-hph	A gene replacement vector, with hygromycin	This study
pHSG396	Expression vector; chloramphenicol	Purchased from TaKaRa Biotech

K. pneumoniae, Klebsiella pneumoniae; E. coli, Escherichia coli.

described above were mainly limited to *E. coli*. Little is known about the mechanism of NIT resistance in *K. pneumoniae*.

This study collected an uropathogenic NIT-resistant *K. pneumoniae* clinical isolate (NRKP) and investigated the underlying resistance mechanism. RamA-dependent overexpression of efflux pumps AcrAB and OqxAB was shown to play an important role in NIT resistance. Discoveries in this study may help expand our knowledge of NIT resistance in *K. pneumoniae*.

2. Materials and methods

2.1. Bacterial strains and plasmids

NRKP was obtained from the urine of a patient with UTI at Huashan Hospital in Shanghai, China. NRKP was identified by VITEK2 Compact System (bioMérieux, Lyon, France). Information about the strains and plasmids used in this study is given in Table 1.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using both the broth microdilution method and the disc diffusion method on Mueller-Hinton agar plates according to the guidelines of the Clinical and Laboratory Standards Institute [10]. The breakpoints of NIT for the broth microdilution method were: susceptible, 32 mg/L; intermediate, 64 mg/L; and resistant, 128 mg/L. The breakpoints of NIT for the disc diffusion method were: susceptible, ≥17 mm; intermediate, 15–16 mm; and resistant, ≤14mm. All of the minimum inhibitory concentrations (MICs) for the various strains were tested in triplicate. *E. coli* ATCC 25922 served as the quality control strain.

2.3. Whole-genome sequencing and sequence alignment

Whole-genome sequencing (WGS) was performed as described previously [11]. Sequences of *nfsA, nfsB, ribE, ramR* and *oqxR* were extracted from the WGS data and aligned with that in ATCC 13883. The accession numbers for the NRKP sequenced in this study are available at DDBJ/ENA/GenBank under PRJNA499050.

2.4. Efflux pump inhibition test

MICs of NIT alone or in combination with phenyl-arginine-β-naphthylamide (PAβN 25 mg/L) and carbonyl cyanide m-chlorophenylhydrazone (CCCP 25 mg/L), inhibitors of resistance–nodulation–division pumps in Enterobacteriaceae, were determined [12,13]. The efflux pump inhibitor test was performed in triplicate.

2.5. Deletion of ramA, oqxB and acrB

Knockout of chromosomal *ramA* was achieved following a strategy adjusted from Datsenko and Wanner [14]. Briefly, the plasmid pKOBEG, which carries the λRed homologous recombination-induced system under the induction of L-arabinose (0.5% w/v), was transformed by electroporation into NRKP to generate NRKP/pKOBEG. Homology fragments of *ramA* (∼500 base pairs upstream and downstream) were amplified by polymerase chain reaction (PCR) using the primers listed in Table S1 (see online supplementary material). The fragments were inserted into the plasmid pMD-18T-hph on either side of the hygromycin-resistance gene. The recombinant plasmid was then digested by enzymes KpnI and HindIII (TaKaRa, Dalian, China) to obtain the final linear fragment that contained the *ramA* homology fragments with the hygromycin gene in the configuration 'upstream–hygromycin–downstream'. The final fragment was transformed by electroporation into NRKP/pKOBEG. Mutant clones were confirmed by PCR (Figure S1, see online supplementary material). The same method was applied to obtain *acrB* and *oqxB* deletion.

2.6. Complementation of ramA and ramR

The wild-type *ramA* of ATCC 13883 was amplified by PCR using primers *ramA*-XbaI-F/*ramA*-SacI-R (Table S1, see online supplementary material). The products were cloned into the vector pHSG396 (TaKaRa, Dalian, China) at XbaI and SacI sites to generate the recombinant vector pHSG396*ramA*. pHSG396*ramA* was confirmed by both chloramphenicol selection and sequencing. Subsequently, pHSG396*ramA* was transformed electrically into NRKP△*ramA*. The positive clone was designated as NRKP△*ramA*/pHSG396*ramA*. The same procedure was used to perform complementation of wild-type *ramR* of ATCC 13883 into the NRKP named NRKP/pHSG396*ramR*. The empty plasmid pHSG396 was used as a negative control.

2.7. Gene expression testing by quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was used to detect the transcriptional expression levels of the efflux pump genes *acrB* and *oqxB*, as well as the regulator genes *ramA* and

Table 2
Minimum inhibitory concentrations (MICs) of nitrofurantoin (NIT) and other antibiotics for strains used in this study.

Strains	MIC[a] of NIT (mg/L)	MIC fold change[b]	Diameter of inhibition (mm)
NRKP	128	1	13
NRKP+PAβN	32	4	19
NRKP+CCCP	32	4	17
ATCC 25922	8	-	21

	MIC							
	NIT	CIP	NOX	OLX	NAL	CHL	TET	TGC
NRKP	128	2	2	128	128	32	16	8
NRKPΔ*ramA*	32	0.25	1	4	4	4	2	0.5
NRKPΔ*ramA*/pHSG396*ramA*	128	2	4	256	256	128	16	16
NRKP/pHSG396*ramR*	32	0.125	1	64	16	8	4	2
NRKPΔ*acrB*	32	0.25	1	64	64	8	2	0.5
NRKPΔ*oqxB*	32	0.125	0.5	32	32	16	1	8
NRKPΔ*acrB*Δ*oqxB*	8	0.125	0.5	4	2	8	1	0.5
NRKPΔ*ramA*/pHSG396	32	0.25	1	4	4	4	2	0.5
NRKP/pHSG396	128	2	2	128	128	32	16	8
ATCC 25922	8	<0.06	0.125	8	2	4	0.5	<0.06

NRKP, NIT-resistant *Klebsiella pneumoniae*; PAβN, phenylalanine-arginine-β-naphthylamide; CCCP, carbonyl cyanide m-chlorophenylhydrazone; NOX, norfloxacin; CIP, ciprofloxacin; OLX, olaquindox; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; TGC, tigecycline.
[a] Nitrofurantoin sensitivity changes of NRKP upon addition of efflux pump inhibitors.
[b] 1 represents no MIC fold change; the final concentration of PAβN/CCCP was 25 mg/L.

rarA. The primers used were described in previous studies [15,16]. *rrsE* was selected as the endogenous reference gene. Total RNA was prepared as described previously [16], and RT-PCR was performed using SYBR Premix *Ex Taq* (TaKaRa, Dalian, China) on the ABI ViiA 7 real-time PCR system (Thermo Fisher Scientific, Colorado Springs, CO, USA). Reactions were repeated in triplicate, and the fold changes in expression of these genes were calculated as described previously [16]. *K. pneumoniae* ATCC 13883 was used as a reference strain for the gene expression analysis.

3. Results

3.1. NIT susceptibility of NRKP was increased by efflux pump inhibitors

NIT susceptibility of NRKP was measured by both the broth microdilution and disc agar diffusion methods. Results showed that NRKP was resistant to NIT, with an MIC of 128 mg/L and a diameter of inhibition of 13 mm (Table 2). No mutations of NIT-resistance-associated genes (*nfsA, nfsB* and *ribE*) were observed in NRKP. Thus, an efflux pump inhibition test was performed. Addition of the efflux pump inhibitors PAβN and CCCP increased the susceptibility of NRKP to NIT, leading to a four-fold decrease in the MIC (Table 2). Similar results were observed by disc agar diffusion test. The addition of PAβN and CCCP with NIT increased the diameter of inhibition of NRKP by 4–6 mm (Table 2). These results imply the important role of efflux pumps in NIT resistance.

3.2. Overexpression of ramA contributes to NIT resistance in NRKP

As RamA is the primary regulator of efflux pumps in most enterobacteria, qRT-PCR analysis was performed to evaluate the transcriptional level of *ramA* in NRKP. Results showed that NRKP had >100-fold increased expression of *ramA,* compared with the NIT-susceptible strain ATCC 13883 (Fig. 1A). Overexpression of *ramA* is often caused by the mutation of repressor gene, *ramR*. Sequence alignment analysis of *ramR* was conducted and revealed a R108stop mutation in *ramR* of NRKP. Complementation of NRKP with wild-type *ramR* significantly decreased the expression of *ramA* (Fig. 1A) and increased the susceptibility of NIT (from 128 to 32 mg/L) (Table 2), demonstrating that the elevation of *ramA* expression in NRKP was caused by *ramR* mutation. To further confirm that *ramA* was involved in NIT resistance in NRKP, *ramA* deletion mutant (NRKPΔ*ramA*) and complemented strain (NRKPΔ*ramA*/pHSG396*ramA*) were constructed. Deletion of *ramA* led to a four-fold decrease in the MIC of NIT, and complementation of *ramA* restored the MIC value (Table 2). In addition, the susceptibility of multiple other antibiotics was also affected by *ramA* (Table 2).

3.3. Efflux pumps AcrAB and OqxAB played important roles in NIT resistance

AcrAB and OqxAB were common efflux pumps contributing to antibiotic resistance in *K. pneumoniae*, and AcrAB was known under the regulation of RamA [15]. Thus, sequence alignment analysis was conducted to detect the presence of *oqxAB* and *acrAB* in NRKP. Results showed that NRKP harboured a single copy of the entire *rarA-oqxABR* locus and the *ramA-acrABR* locus on chromosomes (data not shown). qRT-PCR showed that NRKP had high expression of *oqxB* (Fig. 1D). Interestingly, the expression levels of *acrB* and *rarA* were not high (Fig. 1B,C). Deletion of *ramA* (NRKPΔ*ramA*) decreased the expression levels of *acrB* and *oqxB* by four- to seven-fold (Fig. 1B,D). Complementation of *ramA* restored the transcription levels of *acrB* and *oqxB* (Fig. 1B,D). These results suggested that the expression of efflux pumps AcrAB and OqxAB were *ramA*-dependent, and they may contribute to NIT resistance in NRKP.

To further confirm the role of AcrAB and OqxAB, *acrB* and *oqxB* mutant strains were constructed (designated as NRKPΔ*acrB*, NRKPΔ*oqxB* and NRKPΔ*acrB* Δ*oqxB*). Deletion of *acrB* or *oqxB* alone led to a four-fold decrease in the MIC of NIT (from 128 to 32 mg/L), and deletion of *acrB* and *oqxB* simultaneously led to a 16-fold decrease in the MIC of NIT (from 128 to 8 mg/L) (Table 2). The MICs of other antibiotics also showed significant changes in *acrB* and *oqxB* mutant strains (Table 2).

4. Discussion

With increasing resistance to TMP-SMX and fluoroquinolones, NIT and fosfomycin have been recommended for first-line therapy by guidelines for the treatment of uncomplicated UTIs since 2011 [7,17]. A systematic review by Huttner et al. showed that NIT had

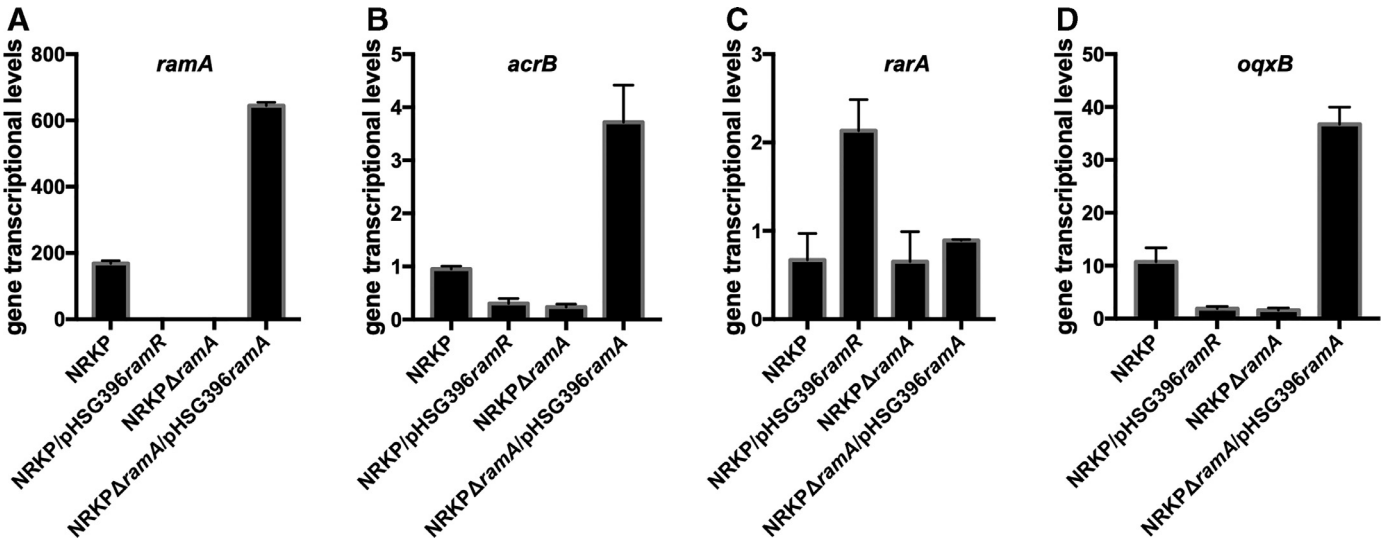

Fig. 1. Quantitative reverse transcriptase polymerase chain reaction assessment of expression of (A) *ramA*, (B) *acrB*, (C) *rarA* and (D) *oqxB*. Fold changes are compared with the reference strain ATCC 13883. *rrsE* was selected as the endogenous reference gene. NRKP, nitrofurantoin-resistant *Klebsiella pneumoniae*.

good clinical and microbiological efficacy against UTIs when given in the short term, and toxicity was mild and predominantly restricted to gastrointestinal issues [18]. Therefore, NIT has become an important and preferred treatment for UTIs.

However, resistance to NIT has emerged along with its clinical use in UTIs. Vigilance should be paid to the resistance mechanisms of common uropathogens, especially for *E. coli* and *K. pneumoniae*. As *E. coli* was the most frequently identified urological pathogen, mechanisms of NIT resistance have mainly been investigated in *E. coli*. The main NIT resistance mechanisms in *E. coli* were mutations in chromosomal nitroreductase genes *nfsA and nfsB* [6]. In addition, overexpression of the efflux pump OqxAB is a complementary mechanism that is adding up to *nfsA, nfsB* and *ribE* mutations to yield high-level NIT resistance [8,19]. In the present study, no mutations were observed in *nfsA, nfsB* and *ribE* of NRKP. When efflux pumps AcrAB and OqxAB were knocked out, the NIT susceptibility test of *acrB* or *oqxB* deletion strain (NRKP△*acrB* and NRKP△*oqxB*) showed that deletion of *acrB*/*oqxB* alone led to an increase in the sensitivity of NRKP to NIT, and deletion of *acrB* and *oqxB* simultaneously led to a 16-fold decrease in the MIC of NIT (from 128 to 8 mg/L), demonstrating that two efflux pumps, OqxAB and AcrAB, played a dominant role in NIT resistance. What is more, the present results also showed that AcrAB and OqxAB could mediate other antibiotic resistances, all of which were the common substrates of the two efflux pumps AcrAB and OqxAB.

It is generally acknowledged that efflux pump *acrAB* is under the control of transcriptional activator RamA, and *oqxAB* is controlled by another transcriptional activator RarA [15]. In this study, NRKP had increased expression of *oqxAB* without RarA overexpression, suggesting the existence of other factors involved in *oqxAB* regulation. Furthermore, deletion of *ramA* resulted in decreased *oqxB* transcription, and this phenotype was restored by the complementation of *ramA*, implying that the expression of *oqxAB* could be under the control of *ramA*. In addition, a previous study showed that expression of *acrAB* could be regulated by *rarA*, suggesting that these two efflux pump systems might 'cross-talk' with each other [20].

In conclusion, this study demonstrated that efflux pumps AcrAB and OqxAB mediate NIT resistance in NRKP, a *K. pneumoniae* clinical isolate. Attention should be paid when NIT is prescribed in the clinical setting for treating UTIs caused by *K. pneumoniae*. One limitation of this study is that data were obtained from a single *K. pneumoniae* isolate. Further epidemiological investigations are needed to evaluate the role of efflux pumps in NIT resistance among *K. pneumoniae* clinical isolates.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.06.004.

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