

Contribution of Insertion Within *acrR* to *acrAB* Expression and Ciprofloxacin Resistance in a Veterinary *Escherichia coli* Isolate

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Abstract: Mutation in *acrR* would result in enhanced expression of *acrAB*, increasing fluoroquinolone resistance and partially contributing to the increase in resistance levels to several unrelated antibiotics. An insertion of 777 bp fragment within *acrR* gene was detected in a clinical *E. coli* isolates from a diseased duck. In order to determine whether the insertion of *acrR* was responsible for the elevated expression of *acrAB*, complementation with wild-type *acrR* and expression levels of efflux pump and regulator genes by qPCR were carried out. Complementation led to increased sensitivity to ciprofloxacin and to ethidium bromide and decreased expression of *acrA* and *acrB* and increased expression of global regulators *marA* and *SoxR* compared with that of the parental strains suggesting that the insertion of *acrR* playing important role in increased *acrAB* expression and ciprofloxacin resistance.

Key words: Mutation, *acrR*, expression, *E. coli*, antibiotics, fragment, China

INTRODUCTION

Resistance to fluoroquinolones in *Escherichia coli* usually occurs due to mutations of the Quinolone Resistance Determining Region (QRDR) in chromosomal genes (*gyrA*, *gyrB*, *parC* and *parE*) encoding the target proteins and/or increased efflux via the *acrAB*-TolC efflux system with or without decreased expression of outer membrane porins (Martinez *et al.*, 2009; Piddock, 2006). *AcrAB*-TolC can expel a wide variety of unrelated antibiotics and over-expression leads to multiple-antibiotic resistance. Expression of *acrAB* can be modulated either by the local repressor *acrR* or by global transcriptional activators like *MarA*, *SoxS* or *Rob* (Martinez *et al.*, 2009). Mutations affecting expression of these regulator genes can confer resistance via increased expression of *acrAB*. Previous studies suggested that mutation in *acrR* resulted in enhanced expression of *acrAB*, increasing sensitivity to ciprofloxacin and to ethidium bromide and contributed to ciprofloxacin resistance (Webber and Piddock, 2001). Mutations in the *acrR* repressor gene are more likely to be involved in *acrB* derepression than mutations which lead to *marA* or *SoxS* over-expression (Webber *et al.*, 2005). Researchers

investigated a veterinary isolate of *E. coli* from a diseased duck which had an insertion of 777 bp in the *acrR* gene. The aim of this study was to determine whether the insertion of *acrR* was responsible for the elevated expression of *acrAB* and antibiotic resistance.

MATERIALS AND METHODS

Strains and susceptibility testing: *E. coli* strain ED28 was isolated from a cloacal swab of a diseased duck from a Veterinary Teaching Hospital in Guangdong Province, China. Susceptibility to ampicillin, cefotaxime, ceftiofur, kanamycin, chloramphenicol, tetracycline, nalidixic, ciprofloxacin, enrofloxacin and ethidium bromide was measured with the Agar Dilution Method according to the guidelines described by the Clinical and Laboratory Standards Institute. *E. coli* ATCC25922 was used as the control.

Efflux activity detection: For evaluation of active efflux of the ED28 strain, the MICs for nalidixic, ciprofloxacin and enrofloxacin were determined by the Agar Dilution Method in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 100 μ M) and

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efflux pump inhibitor L-Phenylalanyl-arginyl- β -Naphthylamide (PA β N, 80 mg L⁻¹) (Everett *et al.*, 1996; O'Regan *et al.*, 2009). ED28 strain was also tested for tolerance to the organic solvents hexane and cyclohexane described previously (Ricci and Piddock, 2009).

PCR detection and DNA sequencing of chromosomal and plasmid mediated resistance genes: Mutations within the QRDR in the *gyrA*, *gyrB*, *parC* and *parE* genes were identified by PCR with specific primers (Ricci and Piddock, 2009) followed by DNA sequencing. The presence of Plasmid Mediated Quinolone Resistance (PMQR) genes (*qnr*, *aac(6')-Ib-cr* and *qepA*) and β -lactamases encoding genes (*bla_{CTX-M}* and *bla_{CMV}*) was determined by PCR with primers specific for these genes described previously (Pitout *et al.*, 2008) and the allele or subtype of each target gene was determined by sequencing of both strands of each PCR products. The *aac(6')-Ib-cr* was further confirmed by digestion of the PCR product of *aac(6')-Ib* with BtsCI.

Complementation experiment: In order to determine the contribution of the insertion within *acrR* to the multidrug resistance phenotype of ED28, complementation experiment with a plasmid-borne wild type *acrR* gene was carried out as described previously (Webber *et al.*, 2005). Briefly, wild-type *acrR* was amplified from EC319, a wild type *E. coli* strain and after verification by sequencing cloned into pGEM-T Easy (Promega, USA) to create the *acrR*-complementing plasmid. The created recombinant plasmid was transformed by CaCl₂ heat shock into ED28.

Gene expression experiments: Quantitative real-time PCR (qPCR) was used to assess expression of *acrA*, *acrB*, *marA* and *soxS*. Protocols for qPCR were adapted from a previously described method (Vinson *et al.*, 2010). Briefly, overnight cultures were diluted 1/100 in LB medium and

grown to the mid-logarithmic phase (OD_{600nm} = 0.5) at 37°C with shaking. Total RNA was isolated and purified using RNAiso Plus according to the manufacturer's instructions. The synthesis of cDNA was performed using the PrimeScript RT reagent kit with gDNA Erase (TaKaRa, Japan) according to the manufacturer's instructions. 16SrRNA used as endogenous reference for a quantitative control. The qRT-PCR was performed using an iCycler IQ5 (Bio-Rad Laboratories) with the following cycling conditions: an initial incubation of 95°C for 3 min followed by 40 cycles of 15 sec at 94°C, 15 sec at 58°C and 72°C for 30 sec. All test samples were run in duplicate and the 2^{- $\Delta\Delta$ CT} Method was used to analyze the relative changes in gene expression from real-time qPCR experiment.

RESULTS AND DISCUSSION

ED28 was resistant to all tested antibiotics and ethidium bromide (Table 1). The MICs of nalidixic acid, ciprofloxacin and enrofloxacin were >512, 32 and 64 μ g mL⁻¹, respectively. ED28 presented confluent growth when overlaid with hexane or cyclohexane suggesting increased efflux pump activity.

Mutations were detected within the QRDR of *gyrA* (Ser83Leu and Asp87Asn) and *parC* (Ser80Ile). No mutations were found within *gyrB* or *parE*. High level resistance to ciprofloxacin in ED28 was mainly due to double mutations in *gyrA* and *parC* rather than the increased intrinsic mechanism such as active efflux pump which only confers moderate resistance (Ruiz and Levy, 2010). ED28 carried a plasmid borne *aac(6')-Ib-cr* which is responsible for reduced susceptibility to ciprofloxacin by N-acetylation of a piperazinyl amine and co-produced *bla_{CTX-M-27}* which corresponded with the phenotype for resistance to the ED28 for expanded-spectrum cephalosporin cefotaxime. Complementation of mutated *acrR* with wild-type *acrR* restored susceptibility to

Table 1: Resistance mutations in QRDR of *gyrA* and *parC* along with MICs of antibiotics with and without EPI, organic solvent tolerance, expression levels of efflux pump and regulator genes in ED28 and Complemented ED28 (C-ED28)

Strains	MIC ^a (mg L ⁻¹)								
	NAL	+CCCP	+PA β N	CIP	+CCCP	+PA β N	ENR	+CCCP	+PA β N
ED28	>512	256	256	32.000	32.000	16.000	64.000	16.000	4.000
C-ED28	64	16	16	0.016	0.016	0.004	0.016	0.016	0.003
Strains	Resistance mutations in QRDR ^b				Fold change in gene expression ^c				
	GyrA	ParC	CYC	Plasmid borne genes	<i>acrA</i>	<i>acrB</i>	<i>marA</i>	<i>SoxS</i>	
ED28	Ser83 Leu (Asp87 Asn)	Ser80Ile	R	<i>aac(6')-Ib-cr (bla_{CTX-M-27})</i>	4.75 \pm 0.14	3.82 \pm 0.520	2.07 \pm 0.012	-0.83 \pm 2.00	
C-ED28	ND (Asp87Asn)	ND	S	ND (<i>bla_{CTX-M-27}</i>)	1.96 \pm 0.56	1.45 \pm 0.024	5.17 \pm 0.440	13.80 \pm 8.66	

^aMIC = Minimum Inhibitory Concentration; NAL = Nalidixic Acid; CIP = Ciprofloxacin; ENR = Enrofloxacin; CCCP = Carbonyl Cyanide m-Chlorophenylhydrazone; PA β N, L-Phenylalanyl-Arginyl- β -Naphthylamide; ^bQRDR = Quinolone Resistance Determining Region; no mutations detected in the known resistance regions of *gyrB* or *parE*; ND = Not Detected. CYC = Cyclohexane; S = Susceptible; T = Tolerant; ^c Fold change compared with that of ATCC25922

ciprofloxacin (MIC, 0.016 $\mu\text{g mL}^{-1}$) and enrofloxacin (MIC, 0.016 $\mu\text{g mL}^{-1}$) and significantly decreased the nalidixic acid MIC (64 $\mu\text{g mL}^{-1}$) in ED28. Besides, ED28 was turned to be hyper-susceptible to cefotaxime, ceftiofur, kanamycin and chloramphenicol wild-type *acrR*. The Complemented ED28 (C-ED28) was more susceptible to ethidium bromide and was unable to grow in the presence of cyclohexane. Only 1 mutation (Asp87Asn) in QRDR of *gyrA* was detected in C-ED28 and of 2 plasmid borne resistant genes, *aac(60)-Ib-cr* was lost. It was worth noting that C-ED28 restored susceptibility to cefotaxime and ceftiofur despite harboring *bla*_{CTX-M-27}.

In order to determine the influence of the insertion in *acrR* on the expression of *acrAB*, qPCR data were compared with that in *E. coli* ATCC25922. In ED28 there was a 4.75, a 3.82 and 2.07 fold more *acrA*, *acrB* and *marA*, respectively expressed. There was 0.83 fold less SoxS (Table 1). However, complementation with wild type *acrR* decreased the expression of *acrA* and *acrB* and increased the production of *marA* and SoxS, compared with that of the parental strain ED28. In *E. coli*, over-production of *acrB* and enhanced efflux can be due to a mutation in *marR* (O) or SoxR, leading to increased production of *marA* and SoxS. However, the findings disagreed with previous conclusion that enhanced efflux seemed to be closely related to derepression by *acrR* due to mutation in *acrR* rather than inevitably resulted from increasing expression of global regulators (Ricci and Piddock, 2009; Ruiz and Levy, 2010). The surprising high expression of SoxS in complementary ED28 presumably associated with DNA self-repairing in bacteria due to heat shock during a constructed plasmid transferred into cell (Krapp *et al.*, 2011).

CONCLUSION

The results suggest that insertion of 777 bp fragment within *acrR* contributes to resistance to ciprofloxacin in combination with substitutions in *GyrA* and *ParC*. Previous studies suggested that mutation at amino acid 45 is important as a contributor to antibiotic resistance (Webber and Piddock, 2001).

However, none amino acid mutations within *acrR* were found in the clinical *E. coli* isolates with high-level resistance to ciprofloxacin, diversity mutations within *acrR* were detected among *E. coli* isolates from patients in China (Wang *et al.*, 2001), suggesting various selective conditions favored diversity mutation within *acrR* (Webber *et al.*, 2005).

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