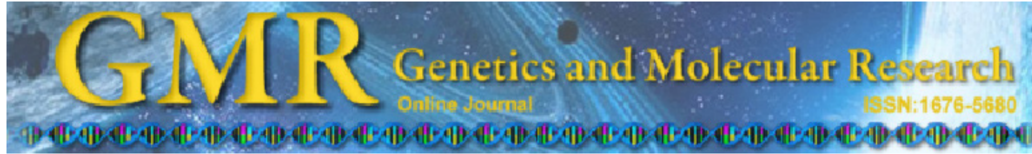

Genetic variations in the active efflux pump genes *acrA*/*B* and *tolC* in different drug-induced strains of *Escherichia coli* CVCC 1547

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ABSTRACT. This study aimed to investigate the properties of mutations of the active efflux pump genes *acrA/B* and *tolC* in *Escherichia coli* CVCC 1547 when induced by different drugs. The mutations were isolated *in vitro* by exposing *E. coli* CVCC 1547 to stepwise increases in the concentration of ceftriaxone (CRO), amikacin (AMK), or ciprofloxacin. The results showed that the minimum inhibitory concentrations for the corresponding drugs increased, as did the minimum inhibitory concentrations for other fluoroquinolones and β-lactam drugs that were not inducers. DNA sequence analyses of the *acrA*/*B* and *tolC* genes of the mutants and comparison with the parent strain revealed that genetic variations had occurred. Three point mutations resulted in amino acid changes in the proteins expressed. Specifically, strain CRO_{10} had a mutation in *acrA*, A309G, that resulted in a Thr-103 to Ala substitution, and a mutation in *tolC*, G735A, that changed Ala-245 to Thr; strain AMK_{20} (and AMK_{30}) had a Val-447 to Ile amino acid change in *acrB*. In addition to the missense mutations in these strains, we detected 7, 20, and 15 nonsense mutations in *acrA*, *acrB*, and *tolC*, respectively. To sum up, multiple genetic sequence variations and

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some changes in amino acid sequences were detected when *E. coli* CVCC 1547 was challenged *in vitro* with CRO, AMK, or ciprofloxacin. These changes may have given rise to multidrug-resistant strains.

Key words: *acrA*/*B-tolC*; MDR; Active efflux pump; Genetic variations

INTRODUCTION

Escherichia coli is a well-studied bacterium, often used in research at the forefront of advancing technology. Currently, all sequenced pathogenic *E. coli* strains have come from human hosts (Perna et al., 2001; Welch et al., 2002; Brzuszkiewicz et al., 2006; Chen et al., 2006). This bias has left a gap in our knowledge as many *E. coli* strains cause important and widespread diseases in animals, including those consumed by humans. We, therefore, wanted to investigate gene mutations in an *E. coli* strain representative of those from animal sources and chose the standard *E. coli* O_{73} CVCC 1547 strain (duck origin) as the object of study. Avian pathogenic *E. coli* strains cause respiratory disease and septicemia in poultry and are economically important worldwide, causing significant mortality. This is an increasing threat to public health owing to the emergence of multidrug-resistant (MDR) *E. coli* strains.

Efflux pump genes exist both in sensitive and drug-resistant bacteria. Some are induced by their substrates so that a sensitive strain could overexpress a pump and become resistant (Piddock, 2006). *E. coli* has been found to possess a variety of active efflux systems such as AcrAB-TolC, AcrEF-TolC (Jellen-Ritter and Kern, 2001), AcrAD-TolC (Rosenberg et al., 2000; Poole, 2004), OqxAB (Hansen et al., 2004), MdfA (Poole, 2004), FloR (Schwarz et al., 2004), and MacAB-TolC (Kobayashi et al., 2001). The first 4 belong to the RND pump family, and the last 3 to the MF, MF, and ABC pump families, respectively. In all of the above pumps, AcrAB-TolC provides the "overriding" phenotype of intrinsic drug resistance to compounds unrelated in structure. Other pumps may have overlapping substrate repertoires but are generally silent and under tight control by regulatory genes. The substrate range for AcrAB-TolC is the most extensive, including drugs such as β-lactams, fluoroquinolones, glycylcyclines, macrolides, and oxazolidinones.

In recent years, many studies have been performed to elucidate the structure and function of AcrAB-TolC, mainly in *E. coli*, but mutations and amino acid changes have not been reported in the active efflux genes *acrA*/*B* or *tolC* in drug-induced strains. It has already been demonstrated that knockout of *acrA*/*B* or the *tolC* gene of *E. coli* increases the sensitivity to drugs (Jellen-Ritter and Kern, 2001) and that increased expression of *acrA/B* or *tolC* can greatly increase the drug resistance level (Bailey et al., 2006). However, no report has investigated the association between point mutations in the above genes induced by sub-MIC drug concentration and MDR.

To better understand the mechanisms of MDR induced by sub-minimum inhibitory concentrations (MIC) in *E. coli* of animal origin, data need to be collected from sub-MIC-induced strains to assess the risks of increasing fluoroquinolone and β-lactam resistance. In this study, the standard strain, *E. coli* O_{73} CVCC 1547 (duck origin), was induced *in vitro* with ceftriaxone (CRO), a third-generation cephalosporin, amikacin (AMK), an aminoglycoside antibiotic, and ciprofloxacin (CIP), a synthetic fluoroquinolone drug. We compared the MIC values for the parent strain with those of strains having point mutations in *acrA*/*B* or *tolC*. Thus, we investigated the roles played by amino acid mutations in these *E. coli* genes in MDR to clarify the potential dangers of the misuse of low antibiotic doses in the clinic.

MATERIAL AND METHODS

Bacterial strain

E. coli CVCC 1547 and ATCC 25922 were purchased from the China Institute for Veterinary Drug Control and the Beijing Standard Microbiology Strain Store Center, respectively.

Chemicals and antibacterial agents

CRO, CIP, and AMK were purchased from the National Institute for the Control of Pharmaceutical and Biological Products; all were dissolved in accordance with manufacturer recommendations.

Multicycle induction *in vitro* and antimicrobial susceptibility tests

The *E. coli* CVCC 1547 parent strain is susceptible to CRO, CIP, and AMK, according to the Clinical and Laboratory Standards Institute broth micro-diffusion test method (CLSI, 2009), with MICs of 0.5, 2, and 1 µg/mL, respectively. It was used for *in vitro* induction for up to 30 generations. Drug-induced strains were sampled after 10, 20, and 30 generations for testing. These were identified as CRO_{10}, CRO_{20}, and CRO_{30}; AMK_{10}, AMK_{20}, and AMK_{30}; and CIP_{10}, CIP_{20}, and CIP_{30} for CRO, AMK, and CIP, respectively. The MIC for each agent was determined for all samples. *E. coli* ATCC 25922 was used as a quality control strain. All MICs were determined on at least three independent occasions.

Cross-resistance test

MIC values were determined for agents that were not used in the inductions. We compared the MICs of two other fluoroquinolone agents with CIP and of three other β-lactam agents with CRO.

PCR amplification and DNA sequence analysis of the *acrA*/*B* and *tolC* genes

E. coli genomic DNA was prepared using a TIANamp Micro DNA Purification Kit (Tiangen), according to the manufacturer protocol. DNA fragments containing the a*crA*/*B* and *tolC* genes were amplified by PCR using the primers shown in Table 1. All PCR amplicons were verified by gel electrophoresis on a 1.0% agarose gel (w/v) stained with ethidium bromide. The above amplicons were purified, ligated into the pMD-18T vector (TaKaRa) and sequenced. Sequence analyses were performed online using BLAST (www.ncbi.nlm.nih.gov/blast/).

Table 1. Primers used for PCR and sequencing of *acrA*, *acrB*, and *tolC* genes.

Primer*	Sequence (5'→3')	Location
acrA-F	CTCAAGTTAGCGGGATTATC	208-227
acrA-R	TCTGATCAACGGTAACGTC	789-807
acrB-F	GAAAGGCCAACAGCTTAAC	674-692
acrB-R	GAGCTGGAGTCAGGATCAAC	1454-1473
tolC-F	TGCTCCCCATTCTTATCGGC	49-68
tolC-R	GCTCTTGCTTGGCGTTGTAC	1241-1260

*GenBank accession No. CP001846.

RESULTS

Induction of resistant mutants of *E. coli*

Under drug-selective laboratory conditions, point mutations of an active efflux pump can broaden its drug substrate range (Mao et al., 2002). Moreover, the "natural pools" of isolates studied thus far generally have undefined regulation-based adaptations that alter the expression levels of homologous pump systems (Wang et al., 2001; Chen et al., 2007; Poole, 2007).

In order to examine the mechanisms of fluoroquinolone and β-lactam resistance in *E. coli*, a series of mutants originating from *E. coli* CVCC 1547 were isolated. MICs were measured for mutants isolated after antibiotic induction for different times. After 10 generations of growth with induction, the results showed that sensitivity to the inducing drug dropped remarkably, with MIC values increasing to 1024 (CRO), 64 (AMK), and 16 (CIP) times that of the parent strain, CVCC 1547. After 20 generations, the MICs increased to >1024, 256, and 64 times the MIC of the parent strain. From the 21st to the 30th generation, MICs did not further increase except for the CIP_{30} mutants, where the MIC was twice that of the CIP_{20} isolates. The results are presented in Table 2.

Table 2. Minimum inhibitory concentrations (MICs) of antibiotics (μg/mL) to induced mutants and parent strain.

Strains	MIC (μg/mL)							
	CRO	AMK	CIP	FEP	CEF	CTX	GAT	LVX
CVCC 1547	0.5	2	1	1	0.5	2	1	2
AMK_{10}	8	128	8	8	2	32	8	32
AMK_{20}	256	512	8	16	128	64	8	16
AMK_{30}	>512	512	32	128	512	512	16	32
CIP_{10}	32	128	16	8	8	16	8	32
CIP_{20}	256	256	64	16	128	64	8	32
CIP_{30}	512	512	128	32	128	128	16	64
CRO_{10}	512	128	8	16	32	128	16	8
CRO_{20}	>512	64	64	64	128	512	16	16
CRO_{30}	>512	128	128	256	512	512	32	16

CRO = ceftriaxone; AMK = amikacin; CIP = ciprofloxacin; FEP = sodium cefepime; CEF = ceftiofur; CTX = cefotaxime; GAT = gatifloxacin; LVX = levofloxacin.

The parent strain was sensitive to CRO, CIP, and AMK. For the first 10 generations of induction, the resistance level increased rapidly. In contrast, the resistance increased slowly from the 10th generation onwards, and, by the 30th generation, all strains had high resistance to the inducing drug. Clearly, the sensitive parent strain had become highly resistant under the pressure of antibiotic selection.

Cross-resistance results

The induced strains resistant to CRO were also completely resistant to the other β-lactam agents, ceftiofur and cefotaxime, and 57% (4/7) were resistant to another β-lactam, sodium cefepime. There was 100% cross-resistance to the three fluoroquinolones CIP, gatifloxacin, and levofloxacin (Table 2). A previous report had suggested that even silent muta-

tions could alter the structure and hence change the substrate specificity of the multidrug efflux pump, AcrAB-TolC (Kimchi-Sarfaty et al., 2007). We speculated that mutations in the *acrA*/*B* and *tolC* genes contributed to the MDR of the strains induced. In the following study, we sequenced these genes and analyzed the relationship between them.

DNA sequence analysis of the a*crA/B* and *tolC* genes from resistant mutants

After amplification, purification, and ligation of the a*crA*/*B* and *tolC* genes from the above induced and parental strains, sequence analysis was conducted. No deletions were found of the active efflux genes a*crA/B* and *tolC*.

CIP_{10} had the same mutations as strains CIP_{20}, CIP_{30}, CRO_{20}, and CRO_{30}. Similarly, AMK_{20} had the same mutations as AMK_{30} (Table 3). The *acrA/B* and *tolC* gene sequences of the drug-induced strains in this study were compared with the GenBank sequences (accession No. CP001846). We identified base changes that, in some of the strains, would give rise to amino acid changes in the encoded proteins.

Table 3. Gene and amino acid mutations of the a*crA*/*B* and *tolC* genes.

Strains	Gene/amino acid mutation					
	acrA		*acrB*		*tolC*	
	Gene	Amino acid	Gene	Amino acid	Gene	Amino acid
CVCC 1547	None	None	None	None	None	None
AMK_{10}	None	None	None	None	None	None
AMK_{20} (AMK_{30})	None	None	G764A, C1202T, C1209T, T1247G, G1256T, C1289T, G1341A*, G1391T, A1394T, T1397C	V447I	G395A, G488A, C557A, G677A, G686A, T845G, C902T, T932C, T956G	None
CIP_{10}(CIP_{20}/CIP_{30}/ CRO_{20}/CRO_{30})	G734A	None	A779G, T791C, G794C, G977A, T1226C, G1256T, C1289T, G1391T, A1394T, T1397C	None	C309T, G488A, G581A, G686T	None
CRO_{10}	A309G*, C356A, G419A, G503A, C527T, G569A, G692A	T103A	A779G, G800A, A890G, C980T, G989A, C1130G, G1211A, T1226C, G1256T, C1289T	None	A377T, G410A, C560T, G581A, G677A, G735A*, A782G	A245T

*Amino acid changes resulted from changes of the base.

Specifically, for *acrA*, none of the AMK-induced strains changed compared with the parent strain, CVCC 1547. CIP_{10}, CIP_{20}, CIP_{30}, CRO_{20}, and CRO_{30} had a single, silent, point mutation, that is, G734A. Interestingly, CRO_{10} had 7 point mutations, one of which, A309G, was a missense mutation, resulting in a Thr-103 to Ala change. For *acrB*, AMK_{20} and AMK_{30} had 10 point mutations, with one, G1341A, being a missense mutation changing Val-447 to Ile. CIP_{10}, CIP_{20}, CIP_{30}, CRO_{20}, and CRO_{30} also had 10 point mutations, all of which were silent and 50% (5/10) of which were the same as those of AMK_{20}. CRO_{10} had 10 point mutations, 20% (2/10) of which were the same as those of AMK_{20}, 40% (4/10) were the same as CIP_{10}, and all of which were also silent mutations. For *tolC*, AMK_{20} and AMK_{30} had 9 point mutations; CIP_{10}, CIP_{20}, CIP_{30}, CRO_{20}, and CRO_{30} had 4 point mutations, all of which were silent; and 50% (2/4) were the same as those of AMK_{20}. CRO_{10} had 7 point mutations, with one the same as that of AMK_{20}, one the same as CIP_{10}, and with a missense mutation, G735A, changing Ala-245 to Thr.

DISCUSSION

Relationship of drug-induced fluoroquinolone resistance with mutations in *acrA* and *acrB* in *E. coli*

Mechanisms of fluoroquinolone resistance fall into two principle categories: alterations in drug targets (such as DNA gyrase or topoisomerase IV) or decreased cellular accumulation of quinolones, involving the major multidrug efflux pump. The latter generally exhibit decreased expression of the OmpF porin and overexpression of the AcrAB efflux pump (Hooper, 2001). The expression of *acrA*/*B* was affected by the regulatory genes *marA* or *soxS* (Bratu et al., 2009). In previous studies, increased expression of *marA* was observed in CRO_{30}, CIP_{10}, CIP_{20}, CIP_{30}, and AMK_{20} strains. *soxS* was overexpressed in CRO_{20}, CRO_{30}, CIP_{20}, CIP_{30}, and AMK_{20} strains (data not shown). On the other hand, *acrB* was identified as a mutation of *gyrB*, a double-point mutation altering two consecutive amino acids (S759R/R760C) in the COOH-terminal region of the gyrase B subunit. The *acrB* mutation also significantly reduced the DNA binding of gyrase (Funatsuki et al., 1997). Mutations at the *acrA* locus also occurred in response to the deletion of *topA* encoding topoisomerase I. These mutations help the survival of Δ*topA* strains under specific growth conditions. It has been shown that the *acrB* gene is in the same cistron as *gyrB* on the genetic map of the *E. coli* chromosome. Asp-426 and Lys-447 were considered as sites for mutation to quinolone resistance (Funatsuki et al., 1997). Our experiments show that a Thr-103 to Ala change in *acr*A and a Val-447 to Ile change in *acrB* could be evidence of the relationship between fluoroquinolone resistance and active efflux pump mutations.

Alterations in *acrA/B* and *tolC* and β-lactam resistance in *E. coli*

The *acrA/B* and *tolC* genes of the CRO_{10}-induced strains had the most base sequence changes compared with the parent strain. The number of point mutations was 7, 10, and 7, whereas the numbers for CRO_{20} and CRO_{30} were reduced to 1, 10, and 4. Moreover, in the CRO_{10} strains, there was a single amino acid mutation in each of the *acrA* and *tolC* genes. Back mutations occurred in the progression to CRO_{20}/CRO_{30}. A possible reason is that non-specific mechanisms play a major role in resistance at the beginning, but with the increased induction times, a number of specific mechanisms (such as production of ESBLs) play a major role; therefore, there is less variation in mutation sites, and no amino acids are changed. In our previous study, we found that, from the 15th generation, strains induced with the above three antimicrobial agents could produce TEM-1 and OXA-1 (data not shown). This may be a possible explanation of the above phenomenon.

Role of aminoglycoside drugs in the induction of *acrB*

To our knowledge, the AcrAB-TolC transporters have a broad substrate range, including cationic dyes, tetracycline, fluoroquinolone, chloromycetin, erythromycin, β-lactams, and rifampicin, but not hydrophilic aminoglycosides. The substrates of the AcrAD-TolC system are mainly aminoglycosides, bile, and a few amphoteric drugs, but it has no significant affinity for lipophilic drugs or most amphoteric molecules (Rosenberg et al., 2000). In this study,

in order to detect whether aminoglycoside drugs can lead to mutations in *acrB*, we used an aminoglycoside, AMK, and the results show that AMK induction led to 10 point mutations and one missense mutation in the *acrB* gene (Table 3).

In summary, although antibiotics used at concentrations below their MICs cannot kill bacteria, they can modify their physicochemical characteristics and MDR phenotype. In this study, we examined drug-induced *E. coli* strains, and our results demonstrate that sub-MIC antibiotics can cause alterations in *acrA* (Thr-103 to Ala), *acrB* (Val-447 to Ile), and *tolC* (Ala-245 to Thr) and that the alterations in the *acrA* and *acrB* genes may contribute to fluoroquinolone resistance in *E. coli*.

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