Plasmid-borne *armA* methylase gene, together with *bla*_{CTX-M-15} and *bla*_{TEM-1}, in a *Klebsiella oxytoca* isolate from China

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An *armA*-producing *Klebsiella oxytoca* isolate, strain 157, was detected after screening of 447 extended-spectrum β -lactamase-producing *Enterobacteriaceae* isolates in China. *K. oxytoca* 157 was resistant to aminoglycosides, ciprofloxacin and most β -lactams. Resistance to aminoglycosides and β -lactams could be transferred to recipient *Escherichia coli* by conjugation. *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes were detected in *K. oxytoca* 157 and transconjugant *E. coli* strain 600(pEC157). Mutation of aa 87 in GyrA was found in *K. oxytoca* 157. A plasmid of ~55 kb was extracted from *K. oxytoca* 157(pKO157) and *E. coli* 600(pEC157). Southern blot hybridization confirmed that the *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes were all located on this conjugative plasmid (pEC157). PCR mapping was also performed to investigate the genetic environment of *armA*. The *armA* gene was found to be flanked by the same putative transposable elements as reported previously in *E. coli*, *Klebsiella pneumoniae* and *Citrobacter freundii* isolates from different countries.

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INTRODUCTION

Aminoglycoside antibiotics are widely used in clinical settings, particularly for the treatment of life-threatening infections caused by Gram-negative bacteria. The 16S rRNA methylase confers high-level resistance to 4,6-substituted deoxystreptamides, including arbekacin, amikacin, kanamycin, tobramycin and gentamicin, by post-transcriptional methylation of 16S rRNA leading to loss of affinity for aminoglycosides (Yamane *et al.*, 2005). The *armA* gene was first isolated from an isolate of *Klebsiella pneumoniae* (Galimand *et al.*, 2003). It has been reported that the *armA* gene is flanked by putative transposable elements (González-Zorn *et al.*, 2005). In previous studies, most of the *armA*-positive isolates of *Escherichia coli* and *K. pneumoniae* have been found to have CTX-M-type enzymes (Yan *et al.*, 2004). *Klebsiella oxytoca* is an opportunistic pathogen involved in antibiotic-associated diarrhoea and nosocomial infections (Decré *et al.*, 2004). The chromosome of wild-type *K. oxytoca* carries the *bla*_{OXY} gene. This gene is constitutively expressed at low levels, which usually confers low-level resistance to amino- and carboxypenicillins but no significant resistance to other β -lactams (Livermore, 1995).

The aim of this study was to determine the prevalence of the 16S rRNA methylase gene in clinical extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* isolates and to characterize the genetic environment of these genes.

METHODS

Bacterial isolates. Clinical isolates of ESBL-producing *Enterobacteriaceae* isolates were collected from six provinces in China (Beijing, Zhejiang, Xinjiang, Henan, Jiangsu and Hubei) from September 1998 to November 2002 (Yu *et al.*, 2007a). These strains were identified using the API 20E system (bioMérieux). *E. coli* strain 600 (resistant to rifampicin) was used as the recipient for conjugation, *armA*-producing *Acinetobacter baumannii* and *rmtB*-producing *Pseudomonas aeruginosa* were used as positive controls for PCR, and *E. coli* ATCC 25922 was used as the control strain in susceptibility tests.

Screening for 16S rRNA methylase-producing isolates and detection of resistance genes and the genetic environment. 16S rRNA methylase genes (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA*) were detected by PCR using primers and conditions described previously (Yokoyama *et al.*, 2003; Doi *et al.*, 2004; Yamane *et al.*, 2005; Wachino *et al.*, 2007; Yu *et al.*, 2007b). For the 16S rRNA methylase-producing isolates, amplification and identification of ESBL-encoding genes were performed with previously described primers (Yu *et al.*, 2007a) for *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M-1-like}, *bla*_{CTX-M-2-like}, *bla*_{CTX-M-8-like} and *bla*_{CTX-M-9-like} genes. The *bla*_{OXY}, *parC*

Abbreviation: ESBL, extended-spectrum β -lactamase.

and *gyrA* genes were detected as reported previously (Maurin *et al.*, 2001; Decré *et al.*, 2004). PCR mapping experiments were performed to investigate the gene environment of the *armA* gene. Primers were designed according to the reported sequence of *K. pneumoniae* strain BM4536 (GenBank accession no. AY220558). All primers used are listed in Table 1. PCR products were sequenced on an ABI 3730 automatic sequencer.

Susceptibility testing of 16S rRNA methylase-producing isolates. The MICs of 20 antimicrobial agents for 16S rRNA methylase-producing isolates were determined using an Etest (AB Biodisk) according to the manufacturer's instructions. Results were interpreted according to published recommendations (CLSI, 2006). The break-point of netilmicin was used for isepamicin (Gomez-Flores *et al.*, 2004).

Conjugation and MIC determination of transconjugants. Conjugation experiments were performed as described previously (Yan *et al.*, 2001), with rifampicin-resistant *E. coli* 600 as the recipient. Mueller–Hinton agar plates supplemented with rifampicin (256 µg ml⁻¹) and amikacin (128 µg ml⁻¹) were used to select transconjugants. The MICs of 20 antimicrobial agents for transconjugants were determined by Etest according to the manufacturer's instructions.

Plasmid extraction and Southern blot hybridization analysis of the 16S rRNA methylase and β-lactamase genes. A Plasmid Midi kit (Qiagen) was used to extract plasmids from the donor isolate and the transconjugant. For Southern blot hybridization, plasmids were transferred from electrophoresis gels to nylon membranes (Bio-Rad) and then hybridized with *armA*, *bla*_{CTX-M-15} or *bla*_{TEM-1} gene fragments labelled with [α -³²P]dCTP (DuPont). After washing, the membrane was compressed with a storage phosphor screen (Kodak) for 48 h and scanned for positive hybridization.

RESULTS

Description of clinical isolates

Among the 447 *Enterobacteriaceae* isolates screened, 27 harboured *bla*_{SHV}-type ESBL, 341 isolates were positive for *bla*_{CTX-M-9} group ESBLs and 112 isolates were positive for *bla*_{CTX-M-1} group ESBLs (Yu *et al.*, 2007a). Twenty-one isolates were positive for *bla*_{CTX-M-14} and *bla*_{CTX-M-3}, two isolates were positive for *bla*_{CTX-M-3} and *bla*_{SHV-12}, one isolate was positive for *bla*_{CTX-M-9} and *bla*_{SHV-12}, four isolates were positive for *bla*_{CTX-M-14} and *bla*_{SHV-12}, one isolate was positive for *bla*_{CTX-M-24} and *bla*_{SHV-12}, one isolate was positive for *bla*_{CTX-M-3}, *bla*_{CTX-M-14} and *bla*_{SHV-12},

and one isolate was positive for *bla*_{CTX-M-3}, *bla*_{CTX-M-14} and *bla*_{SHV-43} (Yu *et al.*, 2007a).

Screening of 16S rRNA methylase genes

All 447 isolates were negative for *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* genes by PCR tests. Only one isolate was positive for *armA*. Sequencing analysis confirmed that the PCR product had 100 % nucleotide identity to the *armA* gene of *K. pneumoniae* strain BM4536 (GenBank accession no. AY220558). This isolate, named strain 157, was identified as *K. oxytoca*.

Characterization of resistance genes in *K. oxytoca* strain 157

K. oxytoca 157 was negative for *bla*_{SHV}, *bla*_{CTX-M-1} group, *bla*_{CTX-M-2} group and *bla*_{CTX-M-8} group ESBL genes, but positive for *bla*_{TEM} and *bla*_{CTX-M-9} group genes. Sequencing analysis confirmed the PCR products as *bla*_{TEM-1} and *bla*_{CTX-M-15}. The PCR products of *bla*_{OXY} showed 100 % nucleotide identity to *bla*_{OXY-2} (GenBank accession no. Z49084). One substitution of Asp⁸⁷→Asn in GyrA was identified in *K. oxytoca* 157.

Transfer of resistance

Resistance to aminoglycosides and most of the β-lactams could be transferred by conjugation. The conjugative transfer frequency was 1.5×10^{-6} transconjugants per recipient. The resistance characteristics of the transconjugant, named *E. coli* 600(pEC157), are shown in Table 2. PCR product analysis confirmed that the *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes were co-transferred by conjugation.

Susceptibility profile of *K. oxytoca* 157 and the transconjugate

K. oxytoca 157 showed an extraordinarily high level of resistance (MIC >1024 µg ml⁻¹) to the five tested aminoglycosides. It was also resistant to ciprofloxacin, most of the β-lactams except the carbapenems and ceftazidime/clavulanic acid. Tigecycline and polymyxin E had good activity against *K. oxytoca* 157, as shown in Table 2.

Table 1. PCR primers used in this study (designed according to the sequence of GenBank accession no. AY220558)

Gene	Primer	Sequence (5'→3')	Position of fragment
<i>orf513-tnpU</i>	P29	GTGTCGCTACATTAGTCGG	nt 15–1470
	P30	GAAGTGAAGCAATGGAAGA	
<i>tnpU-armA</i>	P31	GACGCAAGATAAAACAGGT	nt 1284–2170
	P32	GTCTTACTATTCTGCCATCCT	
<i>armA</i>	P33	AGGTTGTTTCCATTTCGAG	nt 2053–2645
	P34	GGAGAAGGGAATGGAAGAGA	
<i>armA-tnpD</i>	P35	AAGATGCTTCCTGTGCTAA	nt 2529–3944
	P36	ACCCAAGGACTGTAAGCC	

Table 2. Antimicrobial susceptibility patterns of *K. oxytoca* 157, *E. coli* 600(pEC157) and *E. coli* 600

Antimicrobial agent	MIC (mg l ⁻¹)		
	<i>K. oxytoca</i> 157	<i>E. coli</i> 600(pEC157)	<i>E. coli</i> 600
Gentamicin	>1024	>1024	0.5
Amikacin	>1024	>1024	0.5
Isepamicin	>1024	>1024	0.25
Netilmicin	>1024	>1024	0.5
Tobramycin	>1024	>1024	0.25
Cefepime	>256	>256	0.064
Ceftazidime	>256	>256	0.25
Ticarcillin/clavulanic acid	>256	>256	2
Aztreonam	>256	>256	0.25
Ceftriaxone	>256	>256	0.064
Cefotaxime	>256	>256	0.05
Ceftazidime/clavulanic acid	0.75	0.75	0.25
Imipenem	0.5	0.5	0.38
Meropenem	0.25	0.094	0.08
Ertapenem	0.75	0.38	0.08
Ciprofloxacin	6	0.015	0.015
Minocycline	6	1.0	0.5
Polymyxin E	0.5	0.25	0.25
Tigecycline	0.75	0.25	0.25
Rifampicin	128	>1024	>1024

Plasmid extraction and Southern blot analysis of the *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes

A plasmid of the same size (~55 kb) was extracted from both *K. oxytoca* 157 and *E. coli* 600(pEC157) (Fig. 1). The plasmid DNA showed a hybridization pattern represented by ~55 kb bands for both *K. oxytoca* 157 and transconjugate *E. coli* 600(pEC157) using a labelled *armA* gene fragment (Fig. 1). When labelled *bla*_{CTX-M-15} or *bla*_{TEM-1} gene fragments were used for hybridization, the plasmid DNA also showed a positive hybridization pattern (data not shown).

Exploration of the regions surrounding the *armA* gene

PCR mapping and sequencing experiments revealed that the sequences upstream and downstream of the *armA* gene in *K. oxytoca* 157 were identical to the genetic environment of *armA* genes in isolates of *K. pneumoniae* in France (GenBank accession no. AY220558), *E. coli* in Japan and Spain (GenBank accession nos AB117519 and AY522431, respectively) and *Citrobacter freundii* in Poland (GenBank accession no. AF550415).

DISCUSSION

In China, an *armA*-producing *A. baumannii* clone was found to be widely spread in different hospitals (Yu *et al.*, 2007b) and *rmtB*-producing *E. coli* and *Enterobacter cloacae* have been isolated from the commensal flora of healthy, food-producing animals (Chen *et al.*, 2007). Here, we have described for the first time the occurrence of plasmid-

borne 16S rRNA methylases among ESBL-producing *Enterobacteriaceae* isolates in China; this is the first report of an *ArmA*-producing *K. oxytoca* isolate.

Fig. 1. Southern blot analysis of the *armA* gene. Left three panels: plasmid DNA extracted from *K. oxytoca* 157(pKO157) and transconjugate *E. coli* 600(pEC157). Right panels: DNA was transferred to a membrane and hybridized with an *armA* probe. V517, plasmid size markers. The results showed that the *armA* gene is located on the ~55 kb plasmid.

Transfer of high-level aminoglycoside resistance to *E. coli* has been successful among *armA*-containing *E. coli*, *K. pneumoniae*, *C. freundii*, *Salmonella enterica* and *Shigella flexneri* isolates from Taiwan (Yan *et al.*, 2004) and France (Galimand *et al.*, 2005), and co-transfer with *bla*_{CTX-M-3} and *bla*_{TEM-1} genes has also been observed (Yan *et al.*, 2004). In this study, the *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes were readily co-transferred from *K. oxytoca* 157 to *E. coli* 600.

Descriptions of the genetic basis of *armA* in clinical and animal isolates have given insight into the spread of this methylase (Galimand *et al.*, 2005; González-Zorn *et al.*, 2005). Our results confirm that the *armA* genetic background of *K. oxytoca* 157 in China is the same as that of *Enterobacteriaceae* isolates of clinical and animal origin from other countries. This is an important indication that this gene may soon be detected in other species in other regions worldwide.

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