First detection of OKP-A β-lactamase in two Serratia marcescens isolates in China

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INTRODUCTION

The chromosomally encoded blaOKP enzymes are grouped in a single new family of Other Klebsiella pneumoniae β-lactamases, which belongs to molecular class A of Ambler (Hæggman et al., 2004; Mendonça et al., 2009). K. pneumoniae isolates may be naturally resistant to ampicillin and first-generation cephalosporins while being susceptible to cefotaxime, ceftiofur, ceftriaxone, imipenem and aztreonam. It was found that blaOKP had been identified first from the two S. marcescens strains, ch1 and ch2. The isolates were closely related as shown by pulsed-field gel electrophoresis (PFGE). The narrow-spectrum OKP-A β-lactamase gene blaOKP-A-13 was found to be chromosomally located in S. marcescens. The isolates produced a β-lactamase with a pI of approximately 8.2, which corresponds to the OKP-A family. Findings indicate that OKP enzymes are not Klebsiella pneumoniae-specific chromosomal β-lactamases, and the first isolation of S. marcescens producing OKP-A β-lactamase suggests that the blaOKP gene may be disseminated among different species.

KEY WORDS: Detection, OKP-A β-lactamase, Serratia marcescens, China

SUMMARY

Two strains of Enterobacteriaceae producing prodigiosin were isolated from meat in the Sichuan province of China in 2010. The strains were identified by Vitek system, 16S rDNA, rpoB, pfs and luxS genes. Minimum inhibitory concentrations were determined using the broth microdilution method. The two strains were screened for the presence of β-lactamase genes (blaTEM, blaSHV, blaOKP, and blaCTX-M genes). Based on PCR amplification and 16S rDNA sequencing the analysed strains were identified as Serratia marcescens. In addition, morphological and biochemical identification showed that the two strains were definitely S. marcescens. Antimicrobial susceptibility test showed that both strains were resistant to ampicillin and first-generation cephalosporins but not to extended-spectrum β-lactams because of a constitutively expressed chromosomal β-lactamase, including the blaSHV, blaLEN, and blaOKP (Hæggman et al., 2004; Mendonça et al., 2009). Studies have shown that the blaOKP gene diversified into many variants, which can be classified into two subgroups based on nucleotide sequences: the blaOKP-A and blaOKP-B β-lactamases. The blaOKP chromosomal β-lactamase gene has been used as a phylogenetic marker in K. pneumoniae in case of its low level of nucleotide divergence and evolved in parallel with the K. pneumoniae phylogenetic group KpII (Fevre et al., 2005; Hæggman et al., 2004).

To date, there have been no reports on the distribution of the blaOKP gene among Serratia marcescens strains. The spread of β-lactamases among members of the family Enterobacteriaceae has been increasingly observed throughout the world (Queenan et al., 2006). S. marcescens is an opportunistic pathogen associated with respira-
tory tract infections, urinary tract infections, septicemia, meningitis, and wound infections. Moreover, it has been shown to be resistant to a wide variety of antimicrobial agents (Begic et al., 2008; Chen et al., 2003; Thompson et al., 2007). The emergence of extended-spectrum β-lactamases (ESBL)-exhibiting *S. marcescens* causing nosocomial infection represents serious problem worldwide (Ivanova et al., 2008; Po-An et al., 2010). Moreover, *S. marcescens* could cause Cucurbit yellow vine disease (CYVD) in plants such as squash, pumpkin, watermelon, rice and cotton (Zhang et al., 2005). Besides, laboratory contamination was observed due to *S. marcescens* as a cause of a pseudo-outbreak reported by Dundar et al. (2009).

Here we describe the *bla*<sub>OKP-A</sub> gene found in *S. marcescens* strains, isolated from meat in Sichuan, China. The two strains producing narrow spectrum β-lactamase OKP-A-13 are resistant to ampicillin and cephalosporins.

**MATERIALS AND METHODS**

**Bacteria strains and identification**

During the study carried out from 2009 to 2010 to demonstrate the β-lactam-resistance phenotypes and genotypes of *Enterobacteriaceae* strains isolated from food samples, two *S. marcescens* stains were collected from meat in Sichuan, China. The two strains will be referred to as strain ch1 and ch2 throughout the paper. Identification of the two strains to the species level was performed by Vitek system (bioMerieux, Nurtingen, Germany).

Genomic DNA for PCR amplification was prepared with a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. They were also confirmed using 16S rDNA sequences (Singh et al., 2007; Zhu et al., 2007) and *rpoB* gene (Mollet et al., 1997) by PCR assay. In addition, the *S. marcescens* stains were examined for the presence of *pfs* and *luxS* genes (Hu et al., 2008). The primers used to amplify 16S rDNA, *rpoB*, *pfs* and *luxS* genes are listed in Table 1. Each 50 l PCR mixture consisted of 1 l of template, 5 l 10×PCR Buffer, 1.5 mM MgCl₂, 200 M dNTP, 0.4 M primers and 2.5U TaqDNA polymerases. PCR for 16S rDNA and *rpoB* was performed in a DNA thermal cycler (Bio-Rad, Hercules, CA) using the following program: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C or 51°C for 1 min and extension at 72°C for 1.5 min or 1 min, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 0.8% (w/v) agarose gels. PCR amplification conditions for *pfs* and *luxS* were as follows: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C or 51°C for 40 s and extension at 72°C for 25 s, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 2.0% (w/v) agarose gels.

The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa, Dalian, China), cloned into the pUCm-T Vector (Sangon, Shanghai, China) using TaKaRa DNA Ligation Kit Version 2.1 (TaKaRa, Dalian, China), and transformed into *E. coli*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence(5’-3’)</th>
<th>Tm</th>
<th>Extension time</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rDNA</td>
<td>AGATTTTGATCTTGGCTCAG</td>
<td>50°C</td>
<td>1.5 min</td>
<td>1507</td>
</tr>
<tr>
<td></td>
<td>TACGGCTACCTTGTAGCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoB</td>
<td>AACCAGTTCCGCGTGGCTGG</td>
<td>55°C</td>
<td>1 min</td>
<td>1090</td>
</tr>
<tr>
<td></td>
<td>CCTGAAACACGCGCGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>luxS</td>
<td>GCTGGAACACCTGTCGG</td>
<td>51°C</td>
<td>25s</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>ATGTGAAGAACCGGGCGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pfs</td>
<td>CCGGCATCGGGAAAGTCT</td>
<td>55°C</td>
<td>25s</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>ATCTGGCCCCGGCTGAGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The presence of *pfs* and *luxS* genes was confirmed by PCR amplification using primers listed in Table 1. The PCR products were analyzed on 0.8% (w/v) agarose gels. PCR amplification conditions for *pfs* and *luxS* were as follows: an initial denaturation step of 94°C for 5 min, 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C or 51°C for 40 s and extension at 72°C for 25 s, followed by a final elongation at 72°C for 10 min. Amplified PCR products were analyzed on 2.0% (w/v) agarose gels.

The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit Version 2.0 (TaKaRa, Dalian, China), cloned into the pUCm-T Vector (Sangon, Shanghai, China) using TaKaRa DNA Ligation Kit Version 2.1 (TaKaRa, Dalian, China), and transformed into *E. coli*.
DH5α. Subsequently, the amplicons were sequenced by Shanghai Sangon Bioengineering Co., Ltd. The nucleotide sequences were analyzed with software (BLAST) available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Confirmed isolates were stored in Trypticase soy broth containing 20% glycerol at -80°C until use.

**MICs determination**

The minimum inhibitory concentrations (MICs) of 19 antimicrobial agents alone or associated with β-lactamase inhibitors were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2009). Antimicrobials tested were ampicillin, ampicillin/sulbactam, ticarcillin, ticarcillin/ clavulanic acid, cefoxitin, cephalexin, cefotaxime, ceftiofur, ceftiraxone, imipenem, aztreonam, gentamicin, amikacin, kanamycin, netilmicin, streptomycin, norfloxacin, and ciprofloxacin. Escherichia coli ATCC25922 and E. coli ATCC35218 were used for quality control.

**Amplification of β-lactamase genes and sequencing**

Different primers were used to amplify blaTEM, blaSHV, blaOKP, and blaCTX-M β-lactamase genes. The 861-bp blaOKP gene was amplified by PCR using the primers OKP1F (TGGTTATGCGTTATATTCGCCTG) (positions 1 to 18 of the OKP-A-13 coding sequence; GenBank FJ534513) and OKP1R (GCTTAGCGTTGCCAGTGCT) (positions 844 to 861 of the OKP-A-13 coding sequence; GenBank FJ534513), and primers OKP2F (TCTTTACTCGCCTTATCGGCC) and OKP2R (TTAGCGTTGCCAGTGTGCTGCT) (Ribot et al., 2006). Presence of blaTEM, blaSHV, and blaCTX-M resistance genes were also checked by PCR using specific oligonucleotide primers as follows: TEM-F and TEM-R, specific for bla (Guo-Bao et al., 2007); SHV-F and SHV-R, specific for bla (Melano et al., 2006; Yagi et al., 2000); and CTX-M-F and CTX-M-R, specific for bla (Edelstein et al., 2003). Briefly, the blaOKP gene was amplified on a DNA thermal cycler (Bio-Rad, Hercules, CA) with a final volume of 50 l consisting of 5 l of template, 5 µl 10×PCR Buffer; 1.5 mM MgCl2, 200 M dNTP, 0.4 M each of the primers, and 2.5 U Taq DNA polymerase. The cycling conditions were as follows: an initial denaturation step of 94°C for 4 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 50s, followed by a final elongation at 72°C for 10 min. PCR products were ligated to pUCm-T Vector (Sangon, Shanghai, China) using TaKaRa DNA Ligation Kit Version 2.1 (TaKaRa, Dalian, China) and transformed into E. coli DH5α. Subsequently, the PCR products were sequenced by the Shanghai Sangon Bioengineering Co., Ltd. The nucleotide and deduced protein sequences were compared with those available in GenBank and at www.lahey. org/studies/webt. html.

**Plasmid analysis and transformation**

Plasmid DNA was extracted from the strains using the TIANpure Midi Plasmid Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The extracted plasmid DNA was digested with XbaI or HindIII. The plasmid DNA from S. marcescens ch1 and ch2 was electroporated into Escherichia coli DH10B according to the manufacturer's instructions (MicroPulser™, Bio-Rad) (Naas et al., 2003). Elimination of plasmid DNA from strain ch1 and ch2 was as previously described (El-Mansi et al., 2000). Plasmid DNA was also extracted from cured (plasmid eliminated) derivative isolates as above described. Electrophoresis was performed in a 0.8% agarose gel at 80 V for 2h, DL15,000 and λDNA/Hind III DNA (Tiangen, Beijing, China) were used as the molecular size marker.

**PFGE**

Pulsed-field gel electrophoresis (PFGE) was used to study the clonal relatedness of the strains. XbaI digested DNA fragments were analyzed by pulse-field gel electrophoresis (PFGE) using 1% agarose gels and a CHEF MAPPER electrophoresis system (Bio-Rad). The electrophoresis conditions were as previously described (Ribot et al., 2006). After migration, the gel was stained in a solution of 0.5 µg/ml ethidium bromide for 30 min. Relatedness of the isolates was interpreted using the criteria established by Tenover et al. (Tenover et al., 1995).

**Isoelectric focusing**

Crude β-lactamase extracts were prepared by ultrasonic treatment of bacterial cells. After re-
moval of whole cells and debris by centrifuga-
tion, the supernatant was used to determine the
isoelectric point (pI). Electrophoresis was per-
formed using precast polyacrylamide gels, pH 7
to 10 (Bio-Rad, Hercules, CA, USA). Enzyme ac-
tivity was visualized by gel staining with
Nitrocefin (Oxoid, Basingstoke, Hampshire, UK).
pIs were determined after comparison with the
know pI of β-lactamase SHV12 (pI of 8.2,
GenBank FJ685654).

**Nucleotide sequence accession number**
The nucleotide sequences of the 16s rDNA,
blaTEM-1, and blaOKP-A-13 genes reported in
this work are available in the GenBank nucleotide
database under the accession numbers
GU991997, HM348775 and HM348776, respec-
tively.

**RESULTS AND DISCUSSION**

Among all 536 strains recovered from meat in
2009 and 2010, two *S. marcescens* isolates pro-
ducing prodigiosin were identified by Vitek sys-
tem, 16S rDNA, *rpoB*, *pfs* and *luxS* genes (Figure
1). The identities of the PCR products also were
confirmed by commercial sequencing. Morpho-
logical and biochemical identification, PCR am-
plification and 16S rDNA sequencing showed that
these two stains were definitely *Serratia marces-
cens*. Antimicrobial susceptibility test showed
that both stains were resistant to ampicillin,
amoxicillin, cefoxitin, and cephalaxin while be-
ing susceptible to cefotaxime, ceftiofur, ceftriax-
one, imipenem, and aztreonam. The isolates were
also tested for antimicrobial susceptibility by
broth microdilution. The MICs of 19 antimicro-
bial agents determined by broth microdilution
are shown in Table 2. The transformants exhib-
ted lower MICs of ampicillin (ch1, 2.5 µg/ml; ch2,
5 µg/ml) and cefoxitin (ch1, 2.5 µg/ml; ch2, 5
µg/ml) compared to the parent strains.

Sequencing of the amplicons of both strains
showed 100% sequence identity with the *blaOKP-
A-13*. At present, this is the first report of *blaOKP-
gen* in a food-borne *S. marcescens* strain.

Furthermore, specific PCR and sequencing re-
sults revealed that both isolates harbored the
*blaTEM-1* gene. Primers specific for *blaSHV* and
*blaCTX-M* genes gave negative results. OKP en-
zymes have been found only in *K. pneumoniae*
isolates (Siebor *et al.*, 2005) prior to this study. To
date, there are only a few studies on OKP chro-

**FIGURE 1 A** - PCR amplification of 16rDNA genes. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes
1 and 2: *S. marcescens* strains ch1 and ch2, respectively.

**FIGURE 1 B** - PCR amplification of pfs and luxS genes. Lane M:
DL2,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: pfs detected in *S. marcescens* strains ch1 and ch2,
respectively. lanes 3 and 4: luxS detected in *S. marcescens* strains ch1 and ch2, respectively.
mosomal β-lactamase genes in *K. pneumonia*. Majority of OKP β-lactamase-producing isolates have been found *et al.*, in Europe and Canada, and focuses on human blood, and respiratory and urinary tracts (Fevre 2005; Häggman *et al.*, 2004; 10, Mendonça *et al.*, 2009; Siebor *et al.*, 2005). A previous study had discovered four new *bla*OKP genes encoding for OKP-A-13 (FJ534513), -14 (FJ534512), -15 (FJ755841), and -16 (FJ755840) in *K. pneumoniae* isolates in China in 2009, and the diversity of these OKP β-lactamase genes has been confirmed by phylogenetic analysis (Li-Kou *et al.*, 2011). Results show that ch1 and ch2 contained plasmids of approximately 18 kb (Figure 2). Digestion with *Hind* III endonuclease yielded seven bands of approximately 0.15 to 7.5 kb (Figure 3). The plasmids have identical banding patterns. PCR amplification of plasmid DNA extracts from both parent and transformant strains were *bla*TEM-positive and *bla*OKP-negative. Plasmid DNA from two *S. marcescens* cured (plasmid eliminated) derivative strains was extracted as described above and the presence or absence of plasmid DNA was tested using agarose gel electrophoresis. Clearly, as can be seen from Figure 4, the *S. marcescens* strains appeared to have completely lost their indigenous plasmid. Subsequently, PCR amplification of *bla*TEM and *bla*OKP genes in two *S. marcescens* cured derivative strains total DNA was *bla*TEM-negative and *bla*OKP-positive (Figure

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>S. marcescens ch 1/transformants</th>
<th>S. marcescens ch 2/transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antimicrobial agent(s)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≥32/2.5</td>
<td>≥32/5</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>≥32/2.5</td>
<td>≥32/5</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>≥32</td>
<td>≥32</td>
</tr>
<tr>
<td>Cefotaxine</td>
<td>≤1</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>≤1</td>
<td>≤1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>≤0.5</td>
<td>≤0.5</td>
</tr>
</tbody>
</table>

**FIGURE 2** - Plasmid of *S. marcescens* isolates. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: *S. marcescens* strains ch1 and ch2, respectively.
FIGURE 3 - Restriction patterns of HindIII-digested plasmid DNA fragments of transformants. Lane M: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2 transformants, respectively.

FIGURE 4 - Plasmid of S. marcescens and its cured derivative isolates. Lane M1: DNA marker III, Lane M2: DL15,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively. Lanes 3 and 4: S. marcescens strains ch1 and ch2 derivative isolates, respectively.

FIGURE 5 - PCR amplification of blaTEM and blaOKP genes in S. marcescens derivative isolates. Lane M: DL2,000 DNA marker (Tiangen, Beijing, China), lanes 1 and 2: blaTEM negative in S. marcescens strains ch1 and ch2 derivative isolates, respectively. Lanes 3 and 4: blaOKP positive in S. marcescens strain ch1 derivative isolates. Lanes 5 and 6: blaOKP positive in S. marcescens strain ch2 derivative isolates.

FIGURE 6 - PFGE patterns of S. marcescens isolates after digestion with XbaI. Lane M: CHEF DNA Size Marker (Saccharomyces cerevisiae) (Bio-Rad, Hercules, CA), lanes 1 and 2: S. marcescens strains ch1 and ch2, respectively.
5). However, as described above, it was blaTEM-positive and blaOKP-negative for PCR in both parent and transformant strains' plasmid DNA extracts. Elimination of plasmid DNA, specific PCR analysis of plasmids and transformant experiments failed to produce transformants containing OKP enzymes implies that the blaOKP gene encoding for these β-lactamases is present in the chromosome of S. marcescens and that the blaOKP antibiotic resistance gene may exist in other species. Likewise, SHV β-lactamase which had a narrow spectrum of activity was first described as a chromosomally encoded β-lactamase in K. pneumoniae isolates, then it evolved and was found in other Enterobacteriaceae species (Heritage et al., 1999). PFGE patterns of the two OKP β-lactamase-producing S. marcescens isolates are shown in Figure 6. Interestingly, results show that the two strains behave like one in PFGE, although they originated from meat grown in different geographic areas of Sichuan province approximately 150 km apart. pIs were determined after comparison with the known pI of β-lactamase SHV12 (pI of 8.2, GenBank FJ685654). Both strains showed a single band with pI of approximately 8.2. This value corresponds to the OKP-A family, consistent with the OKP positive PCR result (Fevre et al., 2005). In our study, the prevalence of OKP among enterobacteria was low: the blaOKP gene was detected in 4.10% (n=22) of all the isolates, among which two were from S. marcescens and 20 from K. pneumonia. OKP-producing Serratia species are rare, and this is the first report of two OKP-producing S. marcescens strains isolated from meat. The phenotypes of strains producing OKP enzymes were similar to those previously described for these types of β-lactamases (Fevre et al., 2005; Hæggman et al., 2004). The results of the present study clearly showed that OKP, previously found only in K. pneumoniae, has been detected in two S. marcescens related isolates from separate geographic locations. The study indicates that OKP enzymes are not K. pneumoniae-specific chromosomal β-lactamases and the blaOKP gene may be disseminated between different species.

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