Effect of spacer sequences between $bla_{CTX-M}$ and ISEcp1 on $bla_{CTX-M}$ expression

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Spacer sequences of 42 and 127 bp, respectively, between the ISEcp1 insertion sequence and $bla_{CTX-M}$ have been observed in Klebsiella pneumoniae. However, it is not known whether different spacers upstream of $bla_{CTX-M}$ and a promoter within the 127 bp spacer influence cephalosporin resistance. Three recombinant plasmids with different spacers and with or without ISEcp1 were constructed to compare $bla_{CTX-M-3}$ expression and susceptibility to cephalosporins. Our experiment revealed enhanced $bla_{CTX-M-3}$ expression and a relatively high level resistance tocefotaxime and cefepime in recombinant plasmid IS42CTX-M-3, which contained ISEcp1 and the 42 bp spacer. A minor difference in $bla_{CTX-M-3}$ expression was observed in recombinants IS127CTX-M-3 and 127CTX-M-3, which contained a 127 bp spacer with or without ISEcp1, respectively. In conclusion, the promoter within ISEcp1 and a shorter spacer (42 bp but not 127 bp) between ISEcp1 and $bla_{CTX-M}$ are necessary for high-level $bla_{CTX-M-3}$ expression.

INTRODUCTION

The CTX-M-type $\beta$-lactamase has become the most prevalent extended-spectrum $\beta$-lactamase (ESBL) (Bonnet, 2004; Cantón & Coque, 2006). Correlation between $bla_{CTX-M}$ genes and the ISEcp1 insertion sequence has verified the influence of ISEcp1 not only on expression, but also in mobilization and acquisition of $bla_{CTX-M}$ (Karim et al., 2001; Lartigue et al., 2004; Messai et al., 2008; Poirel et al., 2003, 2005; Rodriguez et al., 2004; Tamang et al., 2011; Woodford et al., 2004). ISEcp1B or ISEcp1 harbours promoter sequences for the high-level expression of $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-17}$ and $bla_{CTX-M-19}$ (Bonnet, 2004; Cao et al., 2002; Poirel et al., 2003; Tamang et al., 2011). ISEcp1 also plays an important role upstream of various resistance genes including ampC, rmtC, qnrB-like and $bla_{CMY-2}$-like genes (Cattoir et al., 2008; Haldorsen et al., 2008; Verdet et al., 2009; Wachino et al., 2006). Four differently sized spacer sequences ($bla_{CTX-M-14}$, 42 bp; $bla_{CTX-M-15}$, 48 bp; $bla_{CTX-M-17}$, 79 bp; and $bla_{CTX-M-19}$, 127 bp) separating the inverted repeat right (IRR) of ISEcp1 from the ATG site of the $bla_{CTX-M}$ genes have been reported (Eckert et al., 2006; Lartigue et al., 2004; Messai et al., 2008; Saladin et al., 2002). Among the six subgroups of CTX-M enzymes (Rossolini et al., 2008), the predominant CTX-M-type ESBLs in Taiwan are CTX-M-3 and CTX-M-14, which belong to the $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$ subgroup, respectively (Yu et al., 2006). Our previous study on 235 ESBL-producing Klebsiella pneumoniae isolates in Taiwan revealed that all CTX-M-type ESBLs, including the $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$ subgroups, possess ISEcp1 upstream of $bla_{CTX-M}$ (Ma et al., 2009). Either a 42 bp or 127 bp spacer sequence is present between ISEcp1 and the ATG start codon of $bla_{CTX-M}$ in these clinical isolates. For all $bla_{CTX-M-3}$ isolates, the spacer sequence is 127 bp upstream of $bla_{CTX-M-3}$, but most $bla_{CTX-M-14}$-harbouring isolates have a spacer of 42 bp. One putative promoter sequence has been predicted in the 127 bp spacer sequence (GenBank accession no. AF550415) (Eckert et al., 2006; Golebiewski et al., 2007). No promoter has been observed within the 42 bp spacer sequence. However, no experiment has proven the function of this predicted promoter in the 127 bp spacer sequence. Furthermore, the influence of spacer sequence size on the strength of $\beta$-lactamase expression has not been addressed.

In this study, we aimed to determine whether the existence of an additional promoter within the 127 bp spacer sequence upstream of $bla_{CTX-M-3}$ could influence the expression of $bla_{CTX-M-3}$. We compared the expression of $bla_{CTX-M-3}$ with three combinations of spacer sequences and ISEcp1 upstream of $bla_{CTX-M-3}$.

METHODS

Conjugation of $bla_{CTX-M}$ and susceptibility testing. Transfer of resistance was carried out by conjugation. One clinical K. pneumoniae
isolate producing CTX-M-3 and one producing CTX-M-14 with a 127
and 42 bp spacer sequence, respectively, between IS\text{Ecp1} and bla\text{CTX-M-3} were selected as template DNA source for plasmid reconstructions. A rifampicin-resistant strain of \textit{Escherichia coli} (strain JP-995) was used as the recipient (Siu et al., 1997). Recipients and donors were separately inoculated into brain heart infusion broth (Oxoid) and incubated at 37 °C for 4 h. They were then mixed at a ratio of 1:10 (v/v) for overnight incubation at 37 °C. A 0.1 ml volume of the overnight broth mixture was spread onto MacConkey agar containing rifampicin (100 µg ml⁻¹) and cefotaxime (2 µg ml⁻¹). Transconjugants were selected. T-42-CTX-M-14 was E. coli JP-995 with IS\text{Ecp1}-42 bp spacer–CTX-M-14. T-127-CTX-M-3 was \textit{E. coli} JP-995 with IS\text{Ecp1}-127 bp spacer–CTX-M-3. Antibiotic susceptibility was determined by the broth microdilution method (CLSI, 2006), according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2007). We rechecked the MIC with Etest (bioMe´rieux) when the MIC was above

**Construction of three plasmids with different spacer sequences with or without IS\text{Ecp1} upstream of bla\text{CTX-M-3}**

The sequence between bla\text{CTX-M-3} and IS\text{Ecp1} was 42 bp and that between bla\text{CTX-M-3} and IS\text{Ecp1} was 127 bp in our screened isolates. To compare the influence of the promoter, bla\text{CTX-M-3} was selected as a reporter gene. Three recombinant plasmids containing the entire bla\text{CTX-M-3} gene, with or without the IS\text{Ecp1}-borne promoter sequences, were constructed. Transconjugants with IS\text{Ecp1} and 42 or 127 bp spacer sequences were used as a template to construct the three recombinant plasmids (Fig. 1) by insertion of blunt-end PCR products into the EcoRV site of plasmid pACYC184. Recombinant plasmids pAC127 and pACISEcp127 were constructed using PCR-amplified DNA fragments with the primers listed in Table 1. Recombinant plasmid PAC127 was constructed using primers 127-F and 127-R and had the 127 bp sequence separating the IRR of IS\text{Ecp1} from the ATG start codon of bla\text{CTX-M-3}. Recombinant plasmid pACISEcp127 was constructed using primers IS127-F and IS127-R and had the 127 bp sequence separating the IRR of IS\text{Ecp1}-127 bp spacer–CTX-M-3. The forward primer was the same as IS127-F. The reverse primer, IS127-R. The plasmid included IS\text{cp127} was constructed by overlapping PCR. Fragment 1 included IS\text{Ecp1} and the 42 bp sequence separating it from bla\text{CTX-M-3}. The forward primer was the same as IS127-F. The reverse primer, 42-R, had an overlapping sequence with the start ofbla\text{CTX-M-3}. Fragment 2 included the whole sequence of bla\text{CTX-M-3}. The primer

**set for fragment 2 was CTX-M-3-F and CTX-M-3-R.** Three recombinant plasmids were transformed into \textit{E. coli} reference strain DH5\text{x} and the transformant strains were designated 127CTX-M-3, IS127CTX-M-3 and IS42CTX-M-3, respectively (Fig. 1).

**RNA extraction and primer extension analysis.** Overnight cultures were diluted 1:100 in LB medium and grown to mid-exponential phase (OD₆₀₀=0.8) at 37 °C with shaking. Aliquots (3 ml) were pelleted by centrifugation at 12 000 g for 15 min and lysed with 1 ml TRizol reagent (Invitrogen). Homogenized samples were incubated for 5 min at room temperature with vigorous shaking in 0.1 ml 1-bromo-3-chloropropane, incubated at room temperature for 15 min, and centrifuged for 15 min at 12 000 g. The RNA that distributed into the aqueous phase was precipitated with 0.5 ml ice-cold isopropyl alcohol. The RNA pellet was washed with 1 ml 70% ethanol, air-dried for 10 min, and dissolved in 30 µl diethylpyrocarbonate water at 55 °C. The total RNA concentration was measured using a BioPhotometer apparatus (Eppendorf). A 1 µg portion of total RNA was mixed with 1 U Baseline-ZERO DNase (Epicenter) in 2 µl 10× Baseline-ZERO DNase reaction buffer for 30 min at 37 °C. The DNase was inactivated with 2 µl 10× Baseline-ZERO DNase stop followed by 10 min at 65 °C. Primer extension was performed with the SuperScript First-Strand synthesis system for reverse transcriptase (RT)-PCR (Invitrogen). cDNA was generated from the CTX-M-3 REV primer (5’-CCATCGCATGACGCGCGCGCA-3’), which had been previously 5’-end-labelled with a fluorescent dye (6-FAM). Sample analyses were performed by the nucleic acid analysis service at Mission Biotech, Taiwan.

**RT-PCR.** The transcriptional expression of bla\text{CTX-M} of pACISEcp42, pAC127 and pACISEcp127 was analysed by RT-PCR. cDNA was synthesized using the SuperScript First-Strand synthesis system (Invitrogen). Differences in gene expression were estimated by PCR using the target-specific primers CTX-MA and CTX-MB (Bonnet et al., 2001). Amplifications were performed with an initial step of 5 min at 95 °C followed by 25 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C. Constitutive expression of the 16S rRNA gene assessed in the same cDNA preparation was used as a control, using primers 16S rRNA-F and 16S rRNA-R (Table 1) (Ping et al., 2007). The level of gene expression was estimated by comparison of

![Fig. 1. Structure of the three recombinant plasmids used in this study. Recombinant plasmid pAC127 possesses only its own promoter. Recombinant plasmid pACISEcp127 possesses both an IS\text{Ecp1}-provided promoter and its own promoter. Recombinant plasmid pACISEcp42 has only the IS\text{Ecp1}-provided promoter.](image-url)
the band intensities on a 1% agarose gel containing ethidium bromide.

**β-Lactamase activity.** Hydrolysis of 25 μM cefotaxime (Sigma-Aldrich) was monitored by UV spectrophotometry at 254 nm and 37 °C in 10 mM phosphate buffer (pH 7.0). Activity was standardized relative to protein concentration, which was based on the Bradford method (Bradford, 1976). BSA was used as the standard. β-Lactamase activity was assayed by growing bacterial cultures overnight in nutrient broth, diluting 10-fold into fresh broth, and incubating for 4 h. The cells were harvested into 2 ml vols 10 mM phosphate buffer (pH 7.0), disrupted by three cycles of freezing and thawing, and sonicated. Debris was removed by centrifugation at 6000 g for 30 min, and the supernatants were retained at −20 °C and assayed against cefotaxime.

### RESULTS

The antimicrobial susceptibility testing results of two transconjugants and three plasmid recombinants are presented in Table 2. The three recombinants, 127CTX-M-3, IS127CTX-M-3 and IS42CTX-M-3, were resistant to cefotaxime based on their MICs of ≥64 μg ml⁻¹. IS42CTX-M-3 displayed a trend to higher MICs of aztreonam, ceftazidime and cefepime than did IS127CTX-M-3 and 127CTX-M-3. The MIC of cefotaxime for IS42CTX-M-3 was ≥512 μg ml⁻¹ by Etest. IS42CTX-M-3 had more than fourfold increased MICs of cefotaxime, cefepime and aztreonam than did IS127CTX-M-3 and 127CTX-M-3. The two transconjugants, T-42-CTX-M-14 with the IS

### Table 1. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Location or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>127-F</td>
<td>GTGTGAGAAGCAGTCTAAAT</td>
<td>Right part of ISEcpI lacking −35 and −10 sequences, forward primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>127-R</td>
<td>CGTCTAAGGCGATAAAC</td>
<td>Right part near 3’-end of blaCTX-M-3 gene, reverse primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>IS127-F</td>
<td>GTGTTGCTCTGTGATAAC</td>
<td>Right part of ISEcpI including −35 and −10 sequences, forward primer of construct plasmid pACISEcp127</td>
</tr>
<tr>
<td>IS127-R</td>
<td>GAGCTTTAGGCTTGGTAT</td>
<td>Right part near 3’-end of blaCTX-M-3 gene, reverse primer of construct plasmid pACISEcp127</td>
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<tr>
<td>42-R</td>
<td>GCAGCGATTTTTTACTACATC</td>
<td>End of 42 bp sequence and beginning of blaCTX-M-3 gene, forward primer of construct plasmid pACISEcp42</td>
</tr>
<tr>
<td>CTX-M-3-F</td>
<td>ATGGTTAAAAAATCCTGG</td>
<td>5’-end of blaCTX-M-3 gene, forward primer</td>
</tr>
<tr>
<td>CTX-M-3-R</td>
<td>TGCAAAACGCTGGTGAC</td>
<td>3’-end of blaCTX-M-3 gene, reverse primer</td>
</tr>
<tr>
<td>CTX-M-3-REV</td>
<td>CCATCAGCTGAACTGGCCCA</td>
<td>5’-end of blaCTX-M-3 gene, FAM-labelled reverse primer for primer extension experiments</td>
</tr>
<tr>
<td>CTX-MA</td>
<td>CGCTTTGCATGTGCGAC</td>
<td>blaCTX-M-3 gene, forward primer, Bonnet et al. (2001)</td>
</tr>
<tr>
<td>CTX-MB</td>
<td>AGGCCTAAACATGCAAGT</td>
<td>blaCTX-M-3 gene, reverse primer</td>
</tr>
<tr>
<td>16S rRNA-F</td>
<td>AGGCCCTAACATGCAAGTC</td>
<td>16S rRNA gene, forward primer, Ping et al. (2007)</td>
</tr>
<tr>
<td>16S rRNA-R</td>
<td>TGCAATATTCGCCACTGCTG</td>
<td>16S rRNA gene, reverse primer</td>
</tr>
</tbody>
</table>

### Table 2. Antimicrobial susceptibility testing (MICs μg ml⁻¹) results of two transconjugants and three plasmid recombinants

<table>
<thead>
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<tbody>
<tr>
<td>Ampicillin</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>AMX/CLA</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Piperclillin</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>PIP/TAZ</td>
<td>4</td>
<td>8</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>4</td>
<td>4</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>≥256</td>
<td>≥256</td>
<td>≥256</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Cefazidime</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefepine</td>
<td>4</td>
<td>16</td>
<td>≥256</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

*AMX/CLA, amoxicillin/clavulanate; PIP/TAZ, pipercillin/tazobactam.
RNA secondary structure prediction with the Vienna RNA Secondary Structure Prediction website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) revealed the free energy of formation in the double promoter region can readily form RNA secondary structure in the double promoter region for IS127CTX-M-3 (Fig. 4).

**DISCUSSION**

In the GenBank database, the upstream region (127 bp) of blaCTX-M-1 subgroup enzymes (including CTX-M-1, CTX-M-3, CTX-M-15, CTX-M-37, CTX-M-42, CTX-M-54, CTX-M-57, etc.) is different from the region (42 bp) in the blaCTX-M-9 subgroup (including CTX-M-9, CTX-M-14/18, CTX-M-16, CTX-M-17, CTX-M-19, etc.). This may be related to fact that the blaCTX-M-3 and blaCTX-M-9 subgroups originated from different *Kluyvera* species (Bonnet *et al.*, 2001; Rodriguez *et al.*, 2004; Rossolini *et al.*, 2008). *ISEcp1B*, which differs from ISEcp1 by three nucleotide substitutions and an amino acid change in transposase, has a promoter function for the high-level expression of β-lactamase genes (Poirel *et al.*, 2003). The promoter sequences −35 (TTGAAA) and −10 (TACAAT) were identified in ISEcp1B near its IRR in blaCTX-M-17 andblaCTX-M-19 (Cao *et al.*, 2002; Poirel *et al.*, 2003). The 127 bp spacer sequence upstream of blaCTX-M-3 in our study was the same as the publishedblaCTX-M-1 subgroup upstream sequences of GenBank accession numbers AF550415 (CTX-M-3) and DQ061159 (CTX-M-42) and *Kluyvera ascorbata* (Eckert *et al.*, 2006; Rodriguez *et al.*, 2004). In contrast to the plasmid-located blaCTX-M-3 where ISEcp1 and a 127 bp spacer were present upstream, the chromosome-locatedblaCTX-M-3 was preceded only by a 127 bp spacer without ISEcp1 in *K. ascorbata* (Rodriguez *et al.*, 2004). Our study identified the transcription start site as being located 16 bp upstream of the ATG start codon of blaCTX-M-3 for the 127CTX-M-3 recombinant. For the promoter sequences within the 127 bp spacer sequence, the −35 sequence is similar to the consensus sequence TgTAgGAgGCaAgGCGCAAGGAAGGaTTGAAAGTGGTTGTAA

**Fig. 2.** Primer extension analysis of CTX-M-3. The transcription initiation site is indicated as P1. The promoter sequences within ISEcp1 are in bold. The possible promoter sequences within the 127 bp spacer between ISEcp1 and blaCTX-M-3 are in bold, underlined, and marked with −35 and −10.

**Fig. 3.** RT-PCR gel to evaluate 16S rRNA and CTX-M-3 mRNA levels. The IS42CTX-M-3 strain had enhanced expression of blaCTX-M-3, in contrast to IS127CTX-M-3 and 127CTX-M-3 strains.
et al., 2004). *K. ascorbata* does not have ISEcp1 before the 127 bp spacer and the bla$_{CTX-M}$ gene. For *K. ascorbata*, the 127 bp spacer sequence may function as a promoter, as found in our constructed 127CTX-M-3 recombinant.

RT-PCR and β-lactamase activity assays showed that the transcript levels and hydrolytic activity for cefotaxime of the three constructed plasmids were as follows: IS42CTX-M-3 > 127CTX-M-3 > IS127CTX-M-3. The difference in the expression of IS42CTX-M-3 and 127CTX-M-3 revealed that the ISEcp1-provided promoter is stronger than the promoter in the 127 bp spacer sequence. This is similar to the case of ISAba1 in *Acinetobacter baumannii*, where only those isolates with ISAba1 upstream and adjacent to the bla$_{OXA-51-like}$ gene display imipenem and/or meropenem resistance (Turton et al., 2006). Our work on bla$_{CTX-M-3}$ confirmed the previous findings that the adjacent ISEcp1 acts as a strong promoter for bla$_{CTX-M}$.

Comparison of the cefotaximase expression of IS42CTX-M-3 and IS127CTX-M-3 strains revealed that, although IS127CTX-M-3 possesses two upstream promoters, it has lower cefotaximase expression and hydrolytic activity than IS42CTX-M-3. The difference in distance of ISEcp1 to the start of bla$_{CTX-M}$ may contribute to the difference in promoter strength between IS42CTX-M-3 and IS127CTX-M-3. A similar situation was that the deletion of a seven-nucleotide portion in the spacer sequence between the qnrA gene and its promoter made the promoter closer to the qnrA gene and increased the MIC of ciprofloxacin (Xu et al., 2007). In our study, when ISEcp1 was closer to bla$_{CTX-M-3}$ (42 bp), enhanced β-lactamase activity was expressed.

IS127CTX-M-3 and 127CTX-M-3 displayed no significant difference in MIC level. IS127CTX-M-3 has two promoters, but displayed lower cefotaximase expression and activity than 127CTX-M-3. Although formation of RNA secondary structure in the double promoter region in IS127CTX-M-3 is predicted, it remains to be assessed whether RNA secondary structure formation, which may weaken the promoter action, does change its promoter activity.

The MIC values for the transconjugants and the plasmid recombinants were not the same. For example, the MIC of cefepime was low (4 μg ml$^{-1}$) for T-42-CTX-M-14 compared to that for IS42-CTX-M-3 (≥256 μg ml$^{-1}$). Although both had ISEcp1 and the 42 bp spacer sequence, this could be due to the difference in the recipient strains of the transconjugant and the plasmid recombinant. Also, CTX-M-3 and CTX-M-14 are of different subgroups of CTX-M and confer different cephalosporin MIC profiles, as demonstrated in Table 2, which is a limitation of the study. Though we observed that the promoter region functions better in the transconjugant than in the recombinant, the actual reasons were not identified.

In conclusion, even though a promoter for CTX-M is present in the 127 bp sequence upstream of the bla$_{CTX-M}$ gene in the CTX-M-1 subgroup, our results demonstrate that the promoter within ISEcp1 is more important. Both the promoter within ISEcp1 and its shorter distance (42 bp) to bla$_{CTX-M}$ are necessary to result in high-level bla$_{CTX-M}$ expression.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


