Effect of spacer sequences between \( bla_{CTX-M} \) and ISEcp1 on \( bla_{CTX-M} \) expression

Ling Ma,¹ Leung Kris Siu¹ and Po-Liang Lu²,³

¹Division of Infectious Disease, National Health Research Institutes, Miaoli County, Taiwan, ROC
²Department of Internal Medicine and Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Taiwan, ROC
³School of Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Taiwan, ROC

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 to cefotaxime 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 \( bla_{CTX-M} \) 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., 2003, 2005; Rodriguez et al., 2004; Tamang et al., 2011; Woodford et al., 2004). ISEcp1 harbours promoter sequences for the high-level expression of \( bla_{CTX-M-1}, \) \( bla_{CTX-M-15}, \) \( bla_{CTX-M-17} \) 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; \( bla_{CTX-M-10}, 48 \) bp; \( bla_{CTX-M-17}, 79 \) bp and \( bla_{CTX-M-3}, 127 \) 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 \( bla_{CTX-M-1}, \) \( bla_{CTX-M-9}, \) and \( bla_{CTX-M-14} \) 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 \( bla_{CTX-M-3} \)-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; Golebiowski 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 $\textit{ISEcp1}$ 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 with $\textit{ISEcp1}$ and 42 bp sequences were selected. T-42-CTX-M-14 was produced to reference strain strain JP-995 with $\textit{ISEcp1}$–42 bp spacer–CTX-M-14. T-127-CTX-M-3 was $\textit{E. coli}$ JP-995 with $\textit{ISEcp1}$–127 bp spacer–CTX-M-3. Antibiotic susceptibility was determined by the broth microdilution method (CLSI, 2006), according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2007). We rechecked the MIC with Etest (bioMe´rieux) when the MIC was above 2 μg ml$^{-1}$ by the broth microdilution method.

Construction of three plasmids with different spacer sequences with or without $\textit{ISEcp1}$ upstream of $\textit{bla}_{\text{CTX-M-3}}$.

The sequence between $\textit{bla}_{\text{CTX-M-14}}$ and $\textit{ISEcp1}$ was 42 bp and that between $\textit{bla}_{\text{CTX-M-3}}$ and $\textit{ISEcp1}$ was 127 bp in our screened isolates. To compare the influence of the promoter, $\textit{bla}_{\text{CTX-M-3}}$ was selected as a reporter gene. Three recombinant plasmids containing the entire $\textit{bla}_{\text{CTX-M-3}}$ gene, with or without the $\textit{ISEcp1}$-borne promoter sequences, were constructed. Transconjugants with $\textit{ISEcp1}$ 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 $\textit{ISEcp1}$–127 bp spacer–CTX-M-3. Recombinant plasmid pACISEcp127 was constructed with primers IS127-F and 127-R and had the 127 bp sequence separating the IRR of $\textit{ISEcp1}$–127 bp spacer–CTX-M-3. The forward primer was the same as IS127-F. The reverse primer, 42-R, had an overlapping sequence with the start of $\textit{bla}_{\text{CTX-M-3}}$. Fragment 2 included the whole sequence of $\textit{bla}_{\text{CTX-M-3}}$. The primer

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 $\textit{ISEcp1}$-provided promoter and its own promoter. Recombinant plasmid pACISEcp42 has only the $\textit{ISEcp1}$-provided promoter.

RNA extraction and primer extension analysis.

Overnight cultures were diluted 1:100 in LB medium and grown to mid-exponential phase (OD$_{600}$=0.8) at 37 °C with shaking. Aliquots (3 ml) were pelleted by centrifugation at 12 000 g for 15 min and resuspended 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 isopropl 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′-CCATCAGCGTGAACTGGCGCA-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 transcripational expression of $\textit{bla}_{\text{CTX-M}}$ 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
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 and was determined by the Bio-Rad protein assay, 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 ISεcp1 lacking −35 and −10 sequences, forward primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>127-R</td>
<td>CGTCTAAGGCGATAAACA</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>GTGTTGCTCTGTGAAAC</td>
<td>Right part of ISεcp1 including −35 and −10 sequences, forward primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>IS127-R</td>
<td>GAGCTTATGGCCTGTGAT</td>
<td>Right part near 3’-end of blaCTX-M-3 gene, reverse primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>42-R</td>
<td>GCAGCTGATATTATTTAACCCTCATAAGGCGATGAATTCGAC</td>
<td>End of 42 bp sequence and beginning of blaCTX-M-3 gene, forward primer of construct plasmid pAC127</td>
</tr>
<tr>
<td>CTX-M-3-F</td>
<td>ATGCTGTTAAAATCTGTTAC</td>
<td>5’-end of blaCTX-M-3 gene, forward primer</td>
</tr>
<tr>
<td>CTX-M-3-R</td>
<td>TAAAACGCTGTTACGCTAC</td>
<td>3’-end of blaCTX-M-3 gene, reverse primer</td>
</tr>
<tr>
<td>CTX-M-3-REV</td>
<td>CATCAAGGCGATGAATTCGAC</td>
<td>5’-end of blaCTX-M-3 gene, FAM-labelledd reverse primer for primer extension experiments</td>
</tr>
<tr>
<td>CTX-MA</td>
<td>CGCTTTGCGATGTCGCA</td>
<td>blaCTX-M-3 gene, forward primer, Bonnet et al. (2001)</td>
</tr>
<tr>
<td>CTX-MB</td>
<td>ACGGCTATATCGTGGTGC</td>
<td>blaCTX-M-3 gene, reverse primer</td>
</tr>
<tr>
<td>16S rRNA-F</td>
<td>AGGCCTAACATGCAATAGT</td>
<td>16S rRNA gene, forward primer, Ping et al. (2007)</td>
</tr>
<tr>
<td>16S rRNA-R</td>
<td>TGCAATATTCACGCTGTC</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>
<tr>
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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>PIP/TAZ</td>
<td>4</td>
<td>8</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</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>≥256</td>
<td>≥256</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>≥256</td>
<td>≥256</td>
</tr>
<tr>
<td>Cefadizime</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefepime</td>
<td>4</td>
<td>16</td>
<td>4</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, piperacillin/tazobactam.
that indicated enhanced expression of \textit{bla}\textsubscript{CTX-M-14} containing plasmid and T-127-CTX-M-3 with the ISE\textit{Ecp1}–127 bp spacer–\textit{bla}\textsubscript{CTX-M-3}–containing plasmid, also had high cefotaxime MICs. The MIC of cefotaxime for T-42-CTX-M-14 was fourfold higher than that for T-127-CTX-M-3. The MIC of cefotaxime for recombinant IS42CTX-M-3 was \( \geq 256 \, \mu \text{g} \, \text{ml}^{-1} \), which is higher than the MIC of cefotaxime (16 \, \mu \text{g} \, \text{ml}^{-1}) for transconjugant T-127-CTX-M-3.

Primer extension experiments indicated that the transcription start site was located 16 bp upstream of the ATG start codon of \textit{bla}\textsubscript{CTX-M-3} for the 127CTX-M-3 recombinant. For the promoter sequences IS\textsubscript{127}CTX-M-3 in comparison to IS\textsubscript{127}CTX-M-3 and IS\textsubscript{127}CTX-M-3, in contrast to IS\textsubscript{127}CTX-M-3 and \textit{bla}\textsubscript{CTX-M-3} in \textit{Klebsiella ascorbata} (Rodrı´guez et al., 2004). Our study identified the transcription start site as being located 16 bp upstream of the ATG start codon of \textit{bla}\textsubscript{CTX-M-3} for the 127CTX-M-3 recombinant. For the promoter sequences within the 127 bp spacer, the −35 region is closely related to the consensus sequence T82T84G78A65C54A45 while within the 127 bp spacer between IS\textsubscript{Ecp1} and \textit{bla}\textsubscript{CTX-M-3} are in bold, underlined, and marked with −35 and −10.

**DISCUSSION**

In the GenBank database, the upstream region (127 bp) of \textit{bla}\textsubscript{CTX-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 \textit{bla}\textsubscript{CTX-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 \textit{bla}\textsubscript{CTX-M-1} and \textit{bla}\textsubscript{CTX-M-9} subgroups originated from different \textit{Kluvyera} species (Bonnet et al., 2001; Rodrı´guez et al., 2004; Rossolini et al., 2008). ISE\textit{Ecp1}B, which differs from ISE\textit{Ecp1} by three nucleotide substitutions and an amino acid change in transposase, has a promoter function for the high-level expression of \( \beta \)-lactamase genes (Poirel et al., 2003). The promoter sequences −35 (TTGAAA) and −10 (TACAAT) were identified in ISE\textit{Ecp1}B near its IRR in \textit{bla}\textsubscript{CTX-M-17} and \textit{bla}\textsubscript{CTX-M-19} (Cao et al., 2002; Poirel et al., 2003). The 127 bp spacer sequence upstream of \textit{bla}\textsubscript{CTX-M-3} in our study was the same as the published \textit{bla}\textsubscript{CTX-M-1} subgroup upstream sequences of GenBank accession numbers AF550415 (CTX-M-3) and DQ061159 (CTX-M-42) and \textit{Kluvyera ascorbata} (Eckert et al., 2006; Rodrı´guez et al., 2004). In contrast to the plasmid-located \textit{bla}\textsubscript{CTX-M-3} where ISE\textit{Ecp1} and a 127 bp spacer were present upstream, the chromosome-located \textit{bla}\textsubscript{CTX-M-3} was preceded only by a 127 bp spacer without ISE\textit{Ecp1} in \textit{K. ascorbata} (Rodrı´guez et al., 2004). Our study identified the transcription start site as being located 16 bp upstream of the ATG start codon of \textit{bla}\textsubscript{CTX-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 T\(_{82}\)T\(_{84}\)G\(_{78}\)A\(_{65}\)C\(_{54}\)A\(_{45}\) while the −10 sequence lacks similarity to the consensus sequence T\(_{80}\)A\(_{95}\)T\(_{45}\)A\(_{60}\)A\(_{50}\)T\(_{96}\) (Lewin, 2006). Such a −10 sequence has also been described in \textit{Geobacter sulfurreducens} (Núñez...
et al., 2004). *K. ascorbata* does not have ISEcp1 before the 127 bp spacer and the *bla*\textsubscript{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 \(\beta\)-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*\textsubscript{OXA-51-like} gene display imipenem and/or meropenem resistance (Turton et al., 2006). Our work on *bla*\textsubscript{CTX-M-3} confirmed the previous findings that the adjacent ISEcp1 acts as a strong promoter for *bla*\textsubscript{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*\textsubscript{CTX-M-3} 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*\textsubscript{CTX-M-3} (42 bp), enhanced \(\beta\)-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 \(\mu\)g ml\(^{-1}\)) for T-42-CTX-M-14 compared to that for IS42-CTX-M-3 (\(\geq 256 \mu\)g 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 cephalosporine 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*\textsubscript{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*\textsubscript{CTX-M} are necessary to result in high-level *bla*\textsubscript{CTX-M} expression.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


