Association of IS5 with divergent tandem bla\textsubscript{CMY-2} genes in clinical isolates of \textit{Escherichia coli}

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Objective: To characterize a unique tandem \textit{bla}_{CMY-2} gene arrangement found in two non-identical clinical strains of \textit{Escherichia coli}.

Methods: Both plasmid and chromosomal DNA were evaluated using PFGE, restriction digest analysis, plasmid profiling and Southern hybridization. \textit{bla}_{CMY-2} gene expression and gene copy number were evaluated by real-time PCR. Susceptibilities to selected \textit{\beta}-lactam antibiotics were determined by agar dilution.

Results: A tandem arrangement for \textit{bla}_{CMY-2} was identified in both isolates and was the only arrangement for \textit{bla}_{CMY-2} observed. These isolates had distinct PFGE and plasmid profiles. Each strain exhibited 2-fold higher \textit{bla}_{CMY-2} mRNA expression and up to 8-fold lower \textit{\beta}-lactam susceptibility compared with a strain with a single copy of \textit{bla}_{CMY-2}.

Conclusion: This is the first report of IS5 being associated with tandem \textit{bla}_{CMY-2}. IS5 has previously been associated with antibiotic resistance through tandem gene amplification. The unique tandem arrangement provides a mechanism for increased \textit{bla}_{CMY-2} expression.

Keywords: AmpC, insertion element, gene copy number

Introduction

\textit{CMY-2} is the most frequently encountered plasmid-encoded AmpC \textit{\beta}-lactamase in \textit{Escherichia coli} worldwide.\textsuperscript{1,2} The enzyme is encoded by the gene \textit{bla}_{CMY-2}, which was derived from the \textit{Citrobacter freundii} chromosomal \textit{ampC} gene.\textsuperscript{3} \textit{E. coli} that produce CMY-2 can be associated with resistance to almost all \textit{\beta}-lactam drugs, with resistance to carbopenems achievable when strains exhibit porin deficiencies.\textsuperscript{4} Organisms that produce CMY-2 represent a global antibiotic challenge and it is important to understand the mechanisms that contribute to CMY-2 production.

The genetic organization of resistance genes plays an important role in the ability of those genes to be expressed and cause a resistant phenotype. Some resistance genes are mobilized by integrons and the position within the integron determines its level of expression.\textsuperscript{5} In other cases, the mobilization of an insertion sequence upstream of a resistance gene can contribute to increases in gene expression by supplying a stronger promoter.\textsuperscript{6} \textit{\beta}-lactamase genes have been associated with plasmids, transposons, integrons and insertion sequences. To date, the gene encoding CMY-2 has been associated with plasmids and transposons, and in many cases with the insertion sequence \textit{ISEcp1}, typically as a single gene copy arrangement.

In this report we describe a novel tandem arrangement of \textit{bla}_{CMY-2} found in two unrelated strains of \textit{E. coli}. The orientation of the two \textit{bla}_{CMY-2} genes was divergent and separated by an IS5 insertion sequence. Strains with this arrangement exhibited 2-fold higher \textit{bla}_{CMY-2} expression compared with a single copy arrangement, and reduced \textit{\beta}-lactam susceptibility.

Materials and methods

\textbf{Strains and susceptibility testing}

\textit{E. coli} strains CUMC-201 and CUMC-243 were collected from different patients at different healthcare facilities as part of a community surveillance study in 2005.\textsuperscript{7} Strain CUMC-50 was an \textit{E. coli} strain obtained from a clinical urine specimen in 2003. In each of the strains, gene-specific PCR detected \textit{bla}_{CMY-2} but did not detect CTX-M, SHV or TEM \textit{\beta}-lactamase genes using methods described previously.\textsuperscript{8,9} MICs of select \textit{\beta}-lactams were determined for all strains by agar dilution according to CLSI guidelines.\textsuperscript{11}

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A divergent tandem bla\textsubscript{CMY-2} DNA sequence arrangement was initially identified in strains CUMC-201 and CUMC-243 during attempts to amplify the bla\textsubscript{CMY-2} upstream region for sequencing. A 1963 bp amplicon was generated using the single bla\textsubscript{CMY-2} internal reverse primer CMY7-PE1 (GTGGAGAAAGAGGCTGTCAG). To evaluate this amplicon, it was cloned into the pCR 2.1 vector and propagated in Top10 E. coli (Invitrogen, Carlsbad, CA, USA). The cloned amplicon was sequenced using M13 primers and internal IS5 primers, IS5-Int-F1 (CATGCTACGCATTCACTGC) and IS5-Int-R1 (GAACTGTCGCTTGATGATGC). Sequence for the entire bla\textsubscript{CMY-2} tandem arrangement was obtained by sequencing two PCR amplicons. The first amplicon was generated to sequence the bla\textsubscript{CMY-2} gene 5' to IS5 using primer IS5-Int-F2 (GTAAAGTGTAGATGACAGG) with primer ISEcp1-2-201-F (GATATGTAAT-CATGAAGTTGTCG). The second amplicon was generated to sequence the bla\textsubscript{CMY-2} gene 3' to IS5 using primer IS5-Int-F2 (CATCATCAAGCGA).
CAGTTCG) with Citro-ampC-3′ Flank. Internal portions of \(\text{bla}_{\text{CMY-2}}\) were sequenced for both amplicons using primers CMY-2-Int-F (GCACTTAGCACCTATACG) and CMY-2-Int-R (CTGGTCATTGCCTCTTCG).

PFGE was performed on XbaI (New England Biolabs, Ipswich, MA, USA) digested total cell DNA using CDC Pulse Net protocols. Whole cell DNA was also prepared in agarose plugs by the same PFGE protocol to limit DNA shearing, then digested with EcoRI and NruI (New England Biolabs) and separated by unidirectional electrophoresis. Plasmids were isolated using methods described by Sinnett and Montpetit and treated with Plasmid-Safe DNase (EPICENTRE Biotechnologies, Madison, WI, USA) for 1 h. Plasmids were separated through a 0.5% agarose gel in Tris/acetate/EDTA (TAE) at 5 V/cm and 4 °C for 12 h, and an EPICENTRE BacTracker supercoiled DNA ladder was used to estimate the size of the plasmids.

Southern hybridizations were performed on restriction endonuclease digestion products and plasmid isolations using a 172 bp \(\text{bla}_{\text{CMY-2}}\)-specific probe synthesized with a Roche PCR DIG Probe Synthesis Kit (Roche, Penzberg, Germany) using primers CMY-2 RT-F (CGTAAATGCACCATCACC) and CMY-2 RT-R (CTTCCTAcCAGTTCG).

**Table 1.** \(\beta\)-lactam susceptibilities, \(\text{bla}_{\text{CMY-2}}\) relative copy number and transcript level

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC mg/L</th>
<th>ATM</th>
<th>FEP</th>
<th>CAZ</th>
<th>IPM</th>
<th>MEM</th>
<th>TZP</th>
<th>Relative (\text{bla}_{\text{CMY-2}}) transcript level</th>
<th>SD</th>
<th>Relative (\text{bla}_{\text{CMY-2}}) gene copy number</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUMC-50</td>
<td>2</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.015</td>
<td>4</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>CUMC-201</td>
<td>8</td>
<td>0.5</td>
<td>64</td>
<td>0.12</td>
<td>0.03</td>
<td>16</td>
<td>1.80</td>
<td>0.76</td>
<td>2</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>CUMC-243</td>
<td>16</td>
<td>0.5</td>
<td>64</td>
<td>0.12</td>
<td>0.03</td>
<td>16</td>
<td>1.75</td>
<td>0.37</td>
<td>2</td>
<td>0.29</td>
<td></td>
</tr>
</tbody>
</table>

ATM, aztreonam; FEP, cefepime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; TZP, piperacillin/tazobactam. \(\text{bla}_{\text{CMY-2}}\) transcript level and gene copy number are relative to that of CUMC-50. SD is standard deviation of relative quantity values.

Figure 3. Southern blot analysis of digested genomic DNA isolated from CUMC-201 and CUMC-243 using a \(\text{bla}_{\text{CMY-2}}\)-specific probe. An Invitrogen 1 kb DNA ladder (left) was used to determine the size of restricted fragments separated by electrophoresis. Lanes 1-4 and lanes 5-8 represent DNA isolated from CUMC-201 and CUMC-243, respectively. Lanes 1 and 5, unrestricted DNA; lanes 2 and 6, EcoRI digest only; lanes 3 and 7 NruI digest only; lanes 4 and 8 EcoRI and NruI double digests.
Real-time PCR assays

bla\textsubscript{CMY-2} gene copy number and transcript levels were evaluated by real-time PCR. \textit{E. coli} strain CUMC-50, with a single copy of \textit{bla\textsubscript{CMY-2}} located on a 100 kb plasmid (GenBank accession number JF300163) was used as a comparator strain. Total RNA and DNA of each strain were obtained from 3 mL of log phase culture grown in Mueller–Hinton broth. RNA was extracted as described previously,\textsuperscript{14} and DNA was obtained using a QIAGEN DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). Real-time reactions were performed with a QIAGEN QuantiTect SYBR Green PCR Master Mix according to manufacturer instructions using 250 ng of total DNA or RNA template and 25 pmol of each primer. Primers CMY-2 RT-F and CMY-2 RT-R were used to measure the \textit{bla\subscript{CMY-2}} transcript level and copy number. Primers \textit{E.coli AmpC-F} (GAATCCTCAAGGACTTGC) and \textit{E.coli AmpC-R} (GGCCGGAACACCGATACTG) were used to measure chromosomal amp\textsubscript{C} transcript levels. The single copy gene amp\textsubscript{D} was used to normalize gene copy number assays in this study using primers \textit{E.coli AmpD-F3} (CCTCACCACATTACGATTGC) and \textit{E.coli AmpD-R3} (CTGCAGATCATAGTTCC). 23S RNA was measured to normalize real-time RT–PCR assays using primers EcoliRNA-F (CCCCGTACCAAAAAATGC) and EcoliRNA-R (CCCTGCTGTTACATCAGTGC). At least three independent DNA/RNA isolations were used to determine gene copy number/transcript levels for each gene. Relative quantities of gene copy number and transcript level were determined by the $2^{-\Delta\Delta Ct}$ method. For every gene analysed, only data with a collective cycle threshold value coefficient of variation less than 10% were used to calculate relative transcript level or gene copy number.

Results and discussion

Sequencing of the \textit{bla\subscript{CMY-2}} regions of strains CUMC-201 and CUMC-243 determined that both strains had divergent tandem \textit{bla\subscript{CMY-2}} genes separated by 1857 bp of DNA. The intragenic region featured a complete ISS element flanked by partial 3' end sequences of the insertion element IS\subscript{Ecp1} and identical 116 bp regions of non-coding DNA of \textit{C. freundii} origin (GenBank accession numbers HQ680722, and HQ680723) (Figure 1). The ISS CAR target consensus sequence\textsuperscript{15} was not found flanking the ISS element, nor was any other repeat. This suggests that the association of each \textit{bla\subscript{CMY-2}} gene with the ISS element occurred independently.

Southern hybridization of a \textit{bla\subscript{CMY-2}}-specific probe on isolated plasmids showed both strains had the tandem arrangement located on a plasmid ~160 kb in size as estimated with a supercoiled DNA ladder (Figure 2). However, both strains had different plasmid profiles (Figure 2) and differed by nine bands in their XbaI PFGE patterns, indicating they were not clonal. Additional Southern blotting of total DNA restricted with NruI yielded hybridization to a single fragment ~3200 bp (Figure 3). Blots of NruI, EcoRI double digests yielded hybridization only to fragments ~1164 and 2036 bp in size (Figure 3). These were the predicted target fragment sizes of the tandem arrangement digested by these endonucleases (Figure 1). These results indicated that the tandem arrangement was the only arrangement of \textit{bla\subscript{CMY-2}} in these strains. This conclusion was supported by real-time PCR assays that determined the \textit{bla\subscript{CMY-2}} copy number, and the transcript level of CUMC-201 and CUMC-243 was 2-fold higher than that of the single copy \textit{bla\subscript{CMY-2}} strain CUMC-50 (Table 1).

Agar dilution MICs of aztreonam, cefepime and piperacillin/tazobactam were 4- to 8-fold higher for CUMC-201 and CUMC-243 compared with CUMC-50, but were not considered resistant using the 2010 CLSI breakpoints (Table 1). When chromosomal amp\textsubscript{C} expression was measured by real-time RT–PCR, CUMC-201 and CUMC-243 had 20-fold higher amp\textsubscript{C} expression levels than CUMC-50, and this likely contributed to the susceptibility differences that were observed.

ISS has been previously associated with resistant phenotypes. Studies have demonstrated that the multidrug efflux pump gene acrAB and the sulphonamide resistance gene sur can be amplified in tandem arrangements during antibiotic stress when located between two ISS sequences.\textsuperscript{16,17} These tandem amplifications were thought to be due to homologous recombination events involving ISS, and they were associated with resistance to ampicillin and sulfathiazole, respectively, for the \textit{E. coli} strains in which they occurred.\textsuperscript{16,17}

This is the first report of a tandem plasmid-encoded \(\beta\)-lactamase gene associated with an ISS element. Although it seemed to have little influence on the resistance profile of the strains, this arrangement may be important for further ISS-mediated gene amplification of \textit{bla\subscript{CMY-2}}.

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Transparency declarations

None to declare.

References


