Characterization of a Novel Plasmid-Mediated Cephalosporinase (CMY-9) and Its Genetic Environment in an Escherichia coli Clinical Isolate

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To date in Japan, clinical isolates belonging to the family Enterobacteriaceae usually demonstrate high degrees of susceptibility to oxyimino-cephalosporins, cephamicins, and carbapenems. While bacteria producing extended-spectrum \( \beta \)-lactamases have been spreading globally since the 1980s (7, 20), these \( \beta \)-lactamases in general hardly hydrolyze cephamicins or carbapenems. Meanwhile, the rate of resistance to cephamicins as well as broad-spectrum cephalosporins has arisen among members of the family Enterobacteriaceae, mainly Escherichia coli and Klebsiella pneumoniae, in recent years (7). Production of plasmid-mediated cephalosporinases is a major cause of resistance to these agents (6, 8). CMY-1 from South Korea was the first of these enzymes to be reported in East Asia (2), but no sequence information was reported. MOX-1 was then reported from Japan as an enzyme capable of efficiently hydrolyzing moxalactam (18, 19), and the two enzymes were later found to be closely related (4). In addition, CMY-8 was recently described from Taiwan and was found to be a variant of MOX-1 (39).

Meanwhile, the genetic environment of these plasmid-mediated cephalosporinase genes has remained unclear. Recently, \( \textit{Morganella morganii} \) was shown to be associated with an integron on a transmissible plasmid of a Salmonella enterica serovar Enteritidis strain (34). An integron is a genetic element that contains a site-specific recombination system that recognizes and captures mobile gene cassettes (14, 15). Many of the gene cassettes found to date include antibiotic resistance genes (16). The novel \( \text{sul}^{\text{I}} \)-type integron associated with \( \text{bla}_{\text{DHA-1}} \) was similar to integrons In6 and In7 (31) in that it contained a 2.1-kb common region including \( \text{orf341} \) and ended with a truncated \( \text{CS} \) conserved segment. The following 2.1 kb was almost identical to the common region of integrons In6 and In7 and the integrase of pSAL-1, except that \( \text{orf513} \) encoding a putative transposase was identified instead of \( \text{orf341} \) due to addition of a single nucleotide. \( \text{bla}_{\text{CMY-9}} \) was closely located downstream of the end of the common region. These observations are indicative of the exogenous derivation of \( \text{bla}_{\text{CMY-9}} \) from some environmental microorganisms such as aeromonads.

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Meanwhile, the genetic environment of these plasmid-mediated cephalosporinase genes has remained unclear. Recently, \( \text{bla}_{\text{DHA-1}} \), a cephalosporinase gene that presumably originated from \textit{Morganella morganii}, was shown to be associated with an integron on a transmissible plasmid of a Salmonella enterica serovar Enteritidis strain (34). An integron is a genetic element that contains a site-specific recombination system that recognizes and captures mobile gene cassettes (14, 15). Many of the gene cassettes found to date include antibiotic resistance genes (16). The novel \( \text{sul}^{\text{I}} \)-type integron associated with \( \text{bla}_{\text{DHA-1}} \) was similar to integrons In6 and In7 (31) in that it contained a 2.1-kb common region including \( \text{orf341} \) of unknown function and a partial duplication of the \( \text{CS} \) conserved segment (\( \text{CS} \)-CS). It was speculated that \( \text{bla}_{\text{DHA-1}} \) was mobilized by site-specific recombination (34).

We studied the genetic and biochemical properties of a novel plasmid-mediated cephalosporinase, CMY-9, cloned from an \textit{E. coli} clinical isolate in Japan. We also determined the nucleotide sequences of the neighboring regions of \( \text{bla}_{\text{CMY-9}} \) and demonstrated that a \( \text{sul}^{\text{I}} \)-type integron is present upstream of \( \text{bla}_{\text{CMY-9}} \) along with a putative transposase gene, which may have played a role in the acquisition of \( \text{bla}_{\text{CMY-9}} \) by the plasmid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains and plasmids used in this study are listed in Table 1. E. coli HKYM68, which displays high levels of resistance to various broad-spectrum \( \beta \)-lactams including cephamicins, was isolated from a sputum specimen of an inpatient in Yamaguchi Prefecture, Japan, in November 1995. Bacteria were grown in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, unless specified otherwise.

Antibiotics and susceptibility testing. Antibiotics were obtained from the following sources: ampicillin and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; ceftazidime and cefpirome, Aventis Pharma, Ltd., Tokyo, Japan; cefotaxime, GlaxoSmithKline K. K., Tokyo, Japan; cepepine, Bristol Pharmaceuticals K. K., Tokyo, Japan; aztreonam, Eisai Co., Ltd., Tokyo, Japan; cefotaxin and imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; and rifampin, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. MICs were determined by the agar dilution method by the protocol recommended by the National Committee for Clinical Laboratory Standards in document M7-A4 (23).

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HKYM68 was prepared and digested with various enzymes; the resultant fragments, TEM- and SHV-derived extended-spectrum β-lactamases, as described elsewhere (30, 39).

Transfer of resistance. Conjugation analysis was performed with E. coli CSH2 (F* metB, with resistance to both nalidixic acid and rifampin; provided by T. Sawai, Chiba University School of Medicine, Chiba, Japan) as the recipient by pBX682 A cloning vector; CHL r Stratagene (La Jolla, Calif.). Electroporant E. coli HB101 was transformed with this plasmid in one of the putative promoter regions for blaCMY-9, we constructed plasmid pCSK* (Stratagene, La Jolla, Calif.). Electrocompetent E. coli HB101 harboring pCMYP8B9 or pCMY8 was used for subsequent cloning of the β-lactamase genes. Basic recombinant DNA techniques were carried out as described by Sambrook et al. (27). The plasmid DNA of E. coli HKYM68 was prepared and digested with various enzymes; the resultant fragments were ligated in plasmid vector pCSK* (Stratagene, La Jolla, Calif.). Electrocompetent E. coli HB101 was transformed with these recombinant plasmids. Transformants were selected according to their resistance to the appropriate antibiotics.

DNA sequencing. The DNA sequences of both strands were always determined as described by Sanger et al. (28) with BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). The sequences of the cloned fragments were determined with custom primer sequences. The enzymes used for gene manipulation were purchased from Nippon Gene Co. Ltd. (Tokyo, Japan) or New England Biolabs, Inc. (Beverly, Mass.).

Affinity expression cassette-PCR cloning. Since plasmid pCMY9 had deletions in one of the putative promoter regions for blaCMY-9, we constructed plasmid pCMYP8B9 by fusing the promoter region of blaCMY-9, and the coding region of blaCMY-9 of E. coli HKYM68. The plasmid also conferred resistance to kanamycin, but not gentamicin, streptomycin, chloramphenicol, tetracycline, or co-trimoxazole (trimethoprim-sulfamethoxazole) buffer (pH 5.5). The cells were frozen at −80°C and then thawed and ultracentrifuged at 100,000 × g for 3 h at 4°C. The supernatant was used for subsequent chromatographic purification. Size-exclusion chromatography was performed on a HiLoad 16/60 Superdex 200 pregrade column (Pharmacia Biotech, Uppsala, Sweden) preequilibrated with 50 mM MES buffer (pH 5.5). Fractions containing β-lactamase activity were collected. Then, cation-exchange chromatography was performed on a Hitrap SP HP column (Pharmacia Biotech) preequilibrated with MES buffer by using a high-performance liquid chromatography system (Pharmacia Biotech). Proteins were eluted with a linear gradient of 0 to 0.7 M NaCl in MES buffer. Fractions with activity were pooled and concentrated with an Ultrafree-15 centrifugal filter device (Millipore Corporation, Bedford, Mass.). The purified enzymes were used for subsequent β-lactamase assays. The purities of the enzymes were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Enzyme assays. CMY-9 and CMY-8 were assayed against various β-lactam substrates at 30°C in 50 mM phosphate buffer (pH 7.0) by using an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The absorption maxima of the substrates used were as follows: ampicillin, 235 nm; aztreonam, 315 nm; cefminox, 275 nm; cefotaxime, 264 nm; ceftazidime, 272 nm; cephaloridine, 295 nm; imipenem, 297 nm; and moxalactam, 274 nm. The molar extinction coefficients were calculated by the method of Seeberg et al. (29). Km and kcat values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. Inhibition studies were done at 30°C in 50 mM phosphate buffer (pH 7.0) with ceftazidime as the substrate and clavulanate and tazobactam as the inhibitors. To determine the isoelectric points, 10 µl of enzyme solution was loaded onto an Immobiline DryStrip (pH 6 to 11; Pharmacia Biotech), and electrophoresis was carried out with an IPGphor electrophoresis system (Pharmacia Biotech).

Nucleotide sequence accession number. The entire nucleotide sequence determined in this study has been submitted to the EMBL/GenBank/DDBJ databases and assigned accession no. AB061794.

RESULTS

Transfer and PCR analyses of β-lactamase genes. The PCR analyses revealed the presence of blaTEM-1-like and blaMOX-1-like β-lactamase genes in parental strain E. coli HKYM68. The ceftazidime resistance determinant of E. coli HKYM68 was successfully conjugated into recipient strain E. coli CSH2, and the blaMOX-1-like gene was detected by PCR analysis, indicating that the blaMOX-1-like gene was located on the transferable plasmid of E. coli HKYM68. The plasmid also conferred resistance to kanamycin, but not gentamicin, streptomycin, chloramphenicol, tetracycline, or co-trimoxazole (trimethoprim-sulfamethoxazole) buffer.
famethoxazole), as determined by standard disk diffusion test. The \( \text{bla}_{\text{TEM-1}} \)-like gene was not detected by PCR in the exconjugant strain.

**Cloning and sequencing of \( \beta \)-lactamase genes.** A 1.6-kb EcoRI-XhoI fragment containing the \( \beta \)-lactamase gene of \( E. \text{coli} \) HKYM68 was cloned into plasmid vector pBCS\(^{+}\), and the resultant plasmid, termed pCMY9, conferred ceftazidime resistance to \( E. \text{coli} \) HB101 (MIC, 64 \( \mu \)g/ml). Plasmid pCMY8 carrying \( \text{bla}_{\text{CMY-8}} \) of \( K. \text{pneumoniae} \) KPW142 on a 1.6-kb EcoRI-XhoI fragment was likewise obtained.

The nucleotide sequence of the \( \beta \)-lactamase gene of pCMY9 revealed a cephalosporinase gene variant. It had a point mutation of G to T at nucleotide position 255 compared with the sequence of \( \text{bla}_{\text{CMY-8}} \). The mutation resulted in a single amino acid substitution at amino acid residue 85 from highly conserved glutamic acid (E) to aspartic acid (D) (32). This novel \( \beta \)-lactamase was designated CMY-9. The deduced amino acid sequence of CMY-9 had 78 and 74% identities with those of CepH and CepS, respectively; CepH and CepS are chromosomal cephalosporinases of \( A. \text{hydrophila} \) and \( A. \text{sobria} \), respectively (1, 36). A phylogenetic tree of plasmid-mediated cephalosporinases and representative chromosomal cephalosporinases was constructed by using the CLUSTAL W program with the sequences in the DDBJ database (http://www.ddbj.nig.ac.jp/E-mail/homology.html) and is shown in Fig. 1.

The nucleotide sequence of TEM-type \( \beta \)-lactamase gene of \( E. \text{coli} \) HKYM68 revealed \( \text{bla}_{\text{TEM-1}} \). Therefore, its presence did not appear to be responsible for the high levels of resistance of \( E. \text{coli} \) HKYM68 to various \( \beta \)-lactams.

**Susceptibility to \( \beta \)-lactams.** The MICs of representative \( \beta \)-lactams for parental strain \( E. \text{coli} \) HKYM68 and the clones harboring either plasmid pCMY9 or pCMY8 are shown in Table 2. The expression of CMY-9 or CMY-8 in \( E. \text{coli} \) HB101

![FIG. 1. Dendrogram for plasmid-mediated cephalosporinases and the chromosomal cephalosporinases from which they may have originated. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges.](http://aac.asm.org/Downloadedfromhttp://aac.asm.org/ September 7, 2017 by guest)
significantly increased the MICs of cefminox, cefoxitin, and cephaloridine. The MICs of ceftazidime, moxalactam, and aztreonam were increased for the clones but were not as high as those for *E. coli* HKYM68. Of note, the MICs of cepirome were significantly elevated for the clones, which is an uncommon finding for plasmid-mediated cephalosporinases (3, 4, 10, 13). The presence of tazobactam reduced the MICs of piperacillin for the clones by 8- to 32-fold, implicating this agent as an effective inhibitor of these enzymes. No inhibitory effect was detected with clavulanic acid.

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**Purification of β-lactamase and isoelectric focusing.** CMY-9 and CMY-8 were purified to give a single band on SDS-PAGE (data not shown). The pI's were estimated to be 9.0 for both enzymes.

**Kinetic parameters.** \( k_{cat} \) was calculated with 1 µg of the purified enzymes per ml. The kinetic parameters (\( K_m \) and \( k_{cat} \)) and hydrolytic efficiency (\( k_{cat}/K_m \)) of CMY-9 and CMY-8 against various β-lactams are given in Table 3. Their hydrolytic efficiencies for cephaloridine and cefminox were much greater than those for ampicillin, characteristic of cephalosporinases. They shared similar profiles for ampicillin and cefoxatime. CMY-9 showed a higher hydrolytic efficiency for ceftazidime than CMY-8 did due to the greater \( k_{cat} \). CMY-8, on the other hand, hydrolyzed cephaloridine and cefminox more efficiently than CMY-9 did. No detectable hydrolysis was observed for aztreonam or imipenem with either β-lactamase as the substrate under the experimental conditions used in the present study.

Inhibition of enzyme activity was not detected when clavulanic acid was used as the inhibitor and ceftazidime was used as the substrate, a common observation for cephalosporinases. On the other hand, tazobactam inhibited the hydrolysis of ceftazidime by both enzymes, although it did so much more moderately than it inhibited the hydrolysis of ceftazidime by the penicillinases (\( K_i \), 20 ± 6 µM for CMY-9). These findings were consistent with the susceptibility testing results, in which tazobactam but not clavulanic acid showed an inhibitory effect against CMY-9 and CMY-8.

**Cloning and sequencing of the neighboring regions of blaCMY-9.** In characterizing the genetic environment of *blaCMY-9*, recombinant plasmids with overlapping fragments were obtained, and together they covered an 8-kb region containing *blaCMY-9* (Table 1).

The nucleotide sequence of the whole region revealed an upstream of *blaCMY-9* a *sul1*-type class 1 integron consisting of intI1 (which encodes integrase), a fused *aacA1-orfG* gene cassette, *qacEΔ1* (which encodes the quaternary ammonium compound resistance protein), and *sul1* (which encodes dihydropteroate synthase) (Fig. 2). *aacA1* encodes aminoglycoside 6'-N-acetyltransferase, which confers resistance to kanamycin.

**Table 2. Results of antibiotic susceptibility testing**

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th><em>E. coli</em> HKYM68 MIC (µg/ml)</th>
<th><em>E. coli</em> HB101(pCMY9) MIC (µg/ml)</th>
<th><em>E. coli</em> HB101(pCMY8) MIC (µg/ml)</th>
<th><em>E. coli</em> HB101 MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>&gt;128</td>
<td>128</td>
<td>&gt;128</td>
<td>8</td>
</tr>
<tr>
<td>Amoxicillin-clavulanate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;128</td>
<td>128</td>
<td>&gt;128</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;128</td>
<td>128</td>
<td>&gt;128</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin-tazobactam&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;128</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>&gt;128</td>
<td>64</td>
<td>&gt;128</td>
<td>4</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>0.0625</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>0.0625</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>&gt;128</td>
<td>32</td>
<td>64</td>
<td>0.0625</td>
</tr>
<tr>
<td>Cefepime</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0625</td>
</tr>
<tr>
<td>Cefminox</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>0.5</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>4</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>&gt;128</td>
<td>32</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>64</td>
<td>8</td>
<td>4</td>
<td>0.0625</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fixed concentration of clavulanate, 4 µg/ml.

<sup>b</sup> Fixed concentration of tazobactam, 4 µg/ml.

**Table 3. Substrate profiles for CMY-9 and CMY-8**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CMY-9</th>
<th>CMY-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_m ) (µM)</td>
<td>( k_{cat} ) (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>1,200 ± 300</td>
<td>130 ± 20</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>69 ± 22</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>3.4 ± 2.0</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>120 ± 30</td>
<td>0.53 ± 0.14</td>
</tr>
<tr>
<td>Cefminox</td>
<td>11 ± 2</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>2.4 ± 1.6</td>
<td>0.017 ± 0.003</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>NH*</td>
<td>NH*</td>
</tr>
<tr>
<td>Imipenem</td>
<td>NH*</td>
<td>NH*</td>
</tr>
</tbody>
</table>

<sup>a</sup> NH, no measurable hydrolysis was detected after 2 h of incubation.
This fused \textit{aacA1-orfG} gene cassette has been reported in integron In21 (GenBank accession no. AF047479) and has also been reported in integron In53 (22). The deduced amino acid sequence of AACA1 reported in this study was identical to that of In21 and shared 99% identity with that of In53, which had been designated AACA1b (22). The product of \textit{orfG} has no homology to any known amino acid sequences, and its function has not been characterized.

The 3'-CS of the integron was truncated 24 bases downstream of the stop codon for \textit{sul1}, an observation made with integron In6 of pSa, integron In7 of pDGO100, and the integron of pSAL-1 (17, 31, 33, 34). The sequence past the end of the truncated 3'-CS was almost identical to the 2.1-kb common regions for In6, In7, and the integron of pSAL-1 (31, 34). However, \textit{orf341} was replaced by \textit{orf513}. An insertion of a guanine residue at nucleotide position 960 of \textit{orf341} resulted in a transcriptional frameshift, extending the putative protein product to 513 amino acids. \textit{orf513} has been reported to be a putative structural gene constituting In60, which carries the \beta-lactamase gene for CTX-M-9 (GenBank accession no. AF373104). The deduced amino acid sequence of \textit{orf513} shares 65% identity with that of \textit{orfA}, which encodes a putative transposase of the transferable plasmid from \textit{E. coli} BN10660 (9), and shares lower degrees of identity with those of putative IS801-related transposases (GenBank accession nos. AF261825 and AB020531) (26), indicating that \textit{orf513} encodes a complete putative transposase. Also, a deletion of 9 bases was noted in the noncoding sequence of our integron immediately prior to the 3' end of the common region compared with the sequences of the other integrons (Fig. 3). This deletion was not observed for the corresponding sequences upstream of \textit{bluCMY-9}, \textit{bluCMY-1}, or \textit{bluCMY-8}. A putative promoter region of \textit{bluCMY-9} started only 2 bases from the point of divergence.

Two open reading frames were identified downstream from \textit{bluCMY-9}. The first encoded a putative 140-amino-acid protein, which shared 89% amino acid identity with the amino acid sequence of YqgF of \textit{A. hydrophila} (1). The function of YqgF is unknown. For the second open reading frame, only a deduced sequence of 76 amino acids was available. It shared 54% amino acid identity with the sequence of YqgE of \textit{E. coli}, the function of which is also unknown (5). Unlike In6, In7, and the integron of pSAL-1, no duplication of the 3'-CS was observed within the sequenced region.

**DISCUSSION**

Plasmid-mediated cephalosporinases conferring resistance to oximino-cephalosporins and cephamycins as well as to \beta-lactamase resistance inhibitors have been described from 1989 to date (8). Many of them have deduced amino acid sequences close to those of chromosome-encoded and inducible AmpC enzymes of members of the family \textit{Enterobacteriaceae}. As shown in Fig. 1, CMY-2, CMY-3, CMY-4, CMY-5, LAT-1, LAT-2, LAT-3, LAT-4, and BIL-1 are very similar to the AmpC enzymes of \textit{Citrobacter freundii} (11, 12, 35, 37), MIR-1 and ACT-1 are very similar to the enzymes of \textit{Enterobacter cloacae} (21), DHA-1 and DHA-2 are very similar to the enzymes of \textit{M. morganii} (10), and ACC-1 is very similar to the enzymes of \textit{Hafnia alvei} (13). The phylogenies of CMY-1, CMY-8, MOX-1, FOX-1, FOX-2, FOX-3, FOX-4, and FOX-5 have remained unclear, although they are related to each other...
and are similar to CepH or CepS, chromosomal cephalosporinases of *A. hydrophila* and *A. sobria*, respectively (24). Of those, CMY-1, MOX-1, and CMY-8 are reported from East Asia, and all have been identified from *K. pneumoniae*. CMY-9 was named after its sequence similarity to the sequence of CMY-8. This is the first time that a plasmid-mediated cephalosporinase has been isolated from an *E. coli* strain in this East Asian cluster. A comparison of the nucleotide sequences upstream of *bla*<sub>CMY-9</sub> and the other related cephalosporinase genes is depicted in Fig. 3. CMY-10 and CMY-11 have recently been reported to be variants of CMY-1 from South Korea (GenBank accession nos. AF381615 and AF381619, respectively). These nucleotide sequences share up to 64% identity with the corresponding region of *bla*<sub>CMX</sub> (1). The homology starts at the end of the region common with integrons In6, In7, and In60 and the integron of pSAL-1 and continues into the cephalosporinase-coding regions, enforcing the idea that these genes originated from chromosomal cephalosporinase genes such as *bla*<sub>CepH</sub>.

The kinetic studies of CMY-9 and CMY-8 revealed notable differences in hydrolytic efficiencies of the two enzymes, although they shared characteristics as broad-spectrum cephalosporinases. They both hydrolyzed cephaloridine and cefmoxoxeficiently, but CMY-8 hydrolyzed them even more efficiently. On the other hand, CMY-9 showed greater hydrolytic efficiency than CMY-8 against ceftazidime. Since the two enzymes differ by only one amino acid, the results may be attributed to the E85D substitution, considering that it is located within the recognition site for the R1 side chain of β-lactams and constitutes a surface residue (34). The R side chain of aspartic acid (—OCH<sub>2</sub>COO) is shorter than that of glutamic acid (—OCH<sub>2</sub>COO). Curtailment of the side chain at amino acid position 85 by the E85D substitution may have modified the recognition site of the enzyme for bulky R1 side chains of some β-lactams, altering its substrate specificity.

Genes responsible for the carbapenem resistance of *E. coli* HKYM68 were not identified. The hydrolytic activity of the strain against imipenem was investigated with a crude enzyme preparation, but no apparent hydrolysis was detected after overnight incubation. Isoelectric focusing with the crude extract and nitrocefin revealed two bands corresponding to CMY-9 and TEM-1. Molecular mechanisms other than the production of β-lactamases, such as the impermeability of the outer membrane or an augmented active efflux system, are possible explanations for the carbapenem resistance.

It was previously suggested that parts of the upstream sequences of *bla*<sub>MOX-1</sub> and *dfrA10* carried on integrase In7 were
similar (19). The present study demonstrated that the \textit{blaCMY-9} gene is surrounded by a genetic structure similar to those of integrons \textit{In6}, \textit{In7}, and \textit{In60} and the integron of \textit{pSAL-1} (Fig. 2), all of which include the 2.1-kb so-called common regions, except that \textit{orfS13}, which encodes a putative transposase, instead of \textit{orfS41} was identified. In fact, the nucleotide sequences of \textit{In6} and \textit{In7} were recently updated to contain \textit{orfS13}, not \textit{orfS41} (GenBank accession nos. L06418 and L06822, respectively) (Fig. 2). The common regions are always located downstream of the truncated 3’-CS of the integrons and end at the same site approximately 0.2 kb downstream of the 3’ end of \textit{orfS13}. The fact that the motif is highly conserved among these plasmids points to the possibility that the putative transposase is functional.

The genetic environment surrounding the 3’ end of the common region revealed that, compared with the upstream sequence of \textit{blaCMY-1}, the upstream sequence of \textit{blaCMY-9} had an additional 15 bases, whereas the last 18 bases of the common region were duplicated in the sequence of \textit{blaCMY-11} (Fig. 3). On the other hand, the upstream sequences of \textit{blaCMY-9}, \textit{blaCMY-9}, and \textit{blaMION-1} lacked 50 bases compared with the upstream sequence of \textit{blaCMY-1}. This variation is also indicative of past transposition events.

These findings suggest that the 2.1-kb common regions represent one end of an undefined transposon which comprises these unique \textit{sul1}-type integrons with resistance gene cassettes. Moreover, a variety of genes conferring resistance to antibiotics such as broad-spectrum \textit{\beta}-lactams, chloramphenicol, and trimethoprim are located immediately downstream of this putative transposon (Fig. 2). It can therefore be speculated that these gram-negative bacteria have accumulated an array of antibiotic resistance genes through transposition of the putative transposon, which carries integrons with gene cassettes conferring resistance to aminoglycosides, into the vicinity of other classes of resistance genes, effectively acquiring phenotypes of multidrug resistance.

Finally, this report highlights the genetic and phenotypic diversities of the plasmid-mediated cephalosporinases distributed across East Asia. Caution must be taken in clinical settings to prevent further dissemination of these emerging cephamycin-resistant gram-negative bacteria that produce various cephamycin-hydrolyzing \textit{CMY}-type \textit{\beta}-lactamases.

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