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Horizontal Transfer of *bla*$_{CMY}$-Bearing Plasmids among Clinical *Escherichia coli* and *Klebsiella pneumoniae* Isolates and Emergence of Cefepime-Hydrolyzing CMY-19

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Nine *Escherichia coli* and 5 *Klebsiella pneumoniae* clinical isolates resistant to various cephalosporins and cephamycins were identified in a Japanese general hospital between 1995 and 1997. All nine *E. coli* isolates and one *K. pneumoniae* isolate carried *bla*$_{CMY-9}$, while the other four *K. pneumoniae* isolates harbored a variant of *bla*$_{CMY-9}$, namely, *bla*$_{CMY-19}$. The pulsed-field gel electrophoresis patterns of the nine CMY-9-producing *E. coli* isolates were almost identical, suggesting their clonal relatedness, while those of the five *K. pneumoniae* isolates were divergent. Plasmid profiles, Southern hybridization, and conjugation assays revealed that the genes for the CMY-9 and the CMY-19 β-lactamases were located on very similar conjugative plasmids in *E. coli* and *K. pneumoniae*. The genetic environment of *bla*$_{CMY-19}$ was identical to that of *bla*$_{CMY-9}$. A single amino acid substitution, I292S, adjacent to the H-10 helix region was observed between CMY-9 and CMY-19. This substitution was suggested to be responsible for the expansion of the hydrolyzing activity against several broad-spectrum cephalosporins, and this finding was consistent with the kinetic parameters determined with purified enzymes. These findings suggest that the *bla*$_{CMY-19}$ genes found in the four *K. pneumoniae* isolates might have originated from *bla*$_{CMY-9}$ gene following a point mutation and dispersed among genetically different *K. pneumoniae* isolates via a large transferable plasmid.

Resistance to β-lactam antibiotics in gram-negative bacilli is mainly mediated by the production of β-lactamases, which are divided into four major molecular classes, classes A, B, C, and D (1, 10). Genes for AmpC (class C) β-lactamases are generally encoded on the chromosomes in many gram-negative microbes, including *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Pseudomonas aeruginosa* (27). Chromosomal AmpC enzymes are usually inducible and are often responsible for resistance to cephalosporins (27) as well as to penicillins. Plasmid-mediated class C β-lactamases have mainly been described in *Klebsiella* spp., *Escherichia coli*, and *Salmonella* spp. throughout the world (25). A cephamycin-resistant *Klebsiella pneumoniae* strain producing a plasmid-mediated class C β-lactamase, CMY-1, was first reported in 1989 in Korea (7, 8). Plasmid-mediated class C enzymes are currently divided into at least five clusters (25) on the basis of amino acid sequence similarities, together with their putative progenitor chromosomal AmpC enzymes. In Japan, MOX-1 (16), CMY-8 (unpublished data), CMY-9 (12), CMY-2 (unpublished data), CFE-1 (23), and DHA-1 (unpublished data) have so far been found as plasmid-mediated AmpC β-lactamases, mainly in nosocomial isolates of the family *Enterobacteriaceae*.

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Between 1995 and 1997, eight additional *E. coli* isolates and five *K. pneumoniae* isolates resistant to both oximino-cephalosporins and cephamycins were isolated in the same hospital where the first CMY-9-producing *E. coli* strain (strain HKYM68) was isolated in 1995 (12). In the present study, the molecular and biochemical mechanisms underlying the multiple-cephalosporin resistance among these 14 isolates as well as their genetic relatedness were elucidated.

MATERIALS AND METHODS

Bacterial strains. Nine *E. coli* isolates and five *K. pneumoniae* isolates displaying a high level of resistance to cephalosporins and cephamycins were isolated between 1995 and 1997 in a general hospital in Yamaguchi Prefecture, Japan, and stored in our laboratory. Among these isolates, *E. coli* strain HKYM68 was previously found to produce CMY-9 (12). Phenotypic identification of each isolate was performed by using a commercial identification system (API 20E system; bioMerieux, Marcy 1′Etoile, France), according to the instructions of the manufacturer.

Phenotypic test for β-lactamase types. A simple initial screening test for the presumptive identification of the β-lactamase types in clinical isolates was performed by use of the double-disk synergy test with Kirby-Bauer disks. Two disks which contained ceftazidime (30 μg per disk) or cefotaxime (30 μg per disk) were used in combination with three different disks containing either amoxicillin-clavulanate (20 μg per disk/10 μg per disk), sodium mercaptoacetic acid (3 mg per disk), or 3-aminophenyl boronic acid (APB) (300 μg per disk), which are specific inhibitors of class A, class B, and class C β-lactamases, respectively (2, 32).

Identification of β-lactamase genes by PCR and sequencing analyses. The samples were screened by PCR with 12 sets of primers for the detection of TEM- and SHV-derived extended-spectrum β-lactamases; GES-type, CTX-M-2-type, CTX-M-3-type, and CTX-M-9-type class A β-lactamases; CMY-1-, CMY-2-, and DHA-1-type class C β-lactamases; and IMP-1-, IMP-2-, and VIM-2-type class B β-lactamases. The sets of PCR primers and the amplification conditions used to detect various plasmid-mediated β-lactamase genes found thus far in Japan have been reported previously (28, 31). The PCR amplicons were electrophoresed on

a 2% agarose gel and purified with a MinElute gel extraction kit (QIAGEN K. K., Tokyo, Japan), and both strands were sequenced.

Transfer of β-lactam resistance. A conjugation experiment was performed by the broth mating method with *E. coli* strain CSH-2 (*metB* F^- Rif^r Nal^r) as the recipient. The donor-to-recipient ratio was 1:4, and the mating time was 3 h. Transconjugants were selected on Luria-Bertani (LB) agar plates supplemented with both rifampin (100 μg/ml) and nalidixic acid (50 μg/ml), together with cefotaxime (10 μg/ml) or ceftazidime (10 μg/ml).

Antibiotic susceptibility tests. Susceptibilities to antibiotics were tested by the agar dilution method according to the procedure recommended by the CLSI (formerly the National Committee for Clinical Laboratory Standards) document M7-A5 (24). *E. coli* ATCC 25922 was used as the control strain for the antimicrobial susceptibility testing.

Isoelectric focusing of β-lactamases. Bacterial cells were grown in 10 ml of LB broth supplemented with cephalothin (50 μg/ml) and were harvested by centrifugation (4,000 × *g* for 15 min) The cell pellet was resuspended in 1 ml of 50 mM sodium phosphate buffer. The pI of β-lactamase was determined as described previously (31).

Pulsed-field gel electrophoresis (PFGE). Total DNA preparations containing both chromosomal and plasmid DNAs were extracted from each isolate and digested overnight with XbaI (New England Biolabs, Beverly, MA) in agarose gel plugs. The digested DNAs were subjected to electrophoresis with a CHEF-DRII drive module (Bio-Rad Laboratory, Hercules, CA), with pulses ranging from 12.5 to 40 s at 6 V/cm for 24 h at 16°C.

Plasmid analysis and Southern hybridization. Large plasmids mediating bla_{CMY} genes were prepared from clinical isolates and their transconjugants according to the procedure described by Kado and Liu (17) and electrophoresed on a 0.8% agarose gel. The plasmid DNAs of the transconjugants were also prepared by using a QIAGEN midi-prep kit (QIAGEN K. K.), digested with SacI, and then transferred to a nylon membrane (Bio-Rad Laboratories). The 999-bp digoxigenin (DIG)-labeled DNA probes were prepared by using a PCR DIG Probe Synthesis kit (Roche Diagnostics, Tokyo, Japan); and the DNA template was prepared from a bla_{MOX}-positive *E. coli* strain HKYM68 (12), together with two PCR primers, primers MOX-F (5′-AAC AAC GAC AAT CCA TCC-3′) and MOX-R (5′-TGT TGA AGA GCA CCT GGC-3′).

PCR and sequencing analyses of flanking regions of bla_{CMY}. To determine the genetic environments of the bla_{CMY} genes, standard PCR amplification experiments and sequencing analyses were performed with an Expand High-Fidelity PCR system (Roche) and several sets of primers, which were designed on the basis of the nucleotide sequences deposited in the EMBL/GenBank/DDBJ databases under accession number AB061794. The resultant PCR products were purified by using a MinElute gel extraction kit (QIAGEN) and were subsequently sequenced with the appropriate primers.

Cloning of bla_{CMY-9} and bla_{CMY-19} for purification of enzymes. To amplify bla_{CMY-9} and bla_{CMY-19}, conjugative plasmids pK209 and pK466 were used as the template DNA, respectively. A highly reliable PCR amplification was performed with primers CMY-S1 (5′-CAG GGC GTG AGG ATA AAG-3′) and CMY-S2 (5′-GGG ACG AGA TAG AGA AAT-3′) by using the Expand High-Fidelity PCR system (Roche). Each amplicon was ligated to the pGEM-T vector (Promega, Madison, WI) and subjected to confirmatory sequencing. Selected plasmids with no amplification error, pGEM-CMY-9 and pGEM-CMY-19, which carry bla_{CMY-9} and bla_{CMY-19}, respectively, were digested with XhoI and EcoRI. The resultant fragments were ligated to pBCSK+ (Stratagene, La Jolla, Calif.) restricted with the same enzymes; and competent cells of *E. coli* strain DH5α [*supE44* Δ*lacU169* (ϕ*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1 acrAB*$^+$], purchased from TOYOBO, Co., Ltd, Tokyo, Japan, were transformed by electroporation with the mixture of the constructed plasmids.

Purification of CMY-9 and CMY-19 β-lactamases. *E. coli* strain DH5α, which harbored pBC-CMY-9 carrying the bla_{CMY-9} gene or pBC-CMY-19 carrying the bla_{CMY-19} gene, was separately cultured overnight in 2 liters of LB broth containing cephalothin (50 μg/ml) and chloramphenicol (30 μg/ml). The cells were harvested by centrifugation and washed in 50 mM sodium phosphate buffer (pH 7.0). The pellets were resuspended with 10 ml of 20 mM Tris-HCl buffer (pH 7.5) and destroyed with a French press. After low-speed centrifugation (3,300 × *g* for 15 min) to remove the cellular debris and unbroken cells, the supernatant was again centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant containing β-lactamase was chromatographed through a HiTrap Q HP column (Amersham Biosciences) that had been preequilibrated with 20 mM Tris-HCl buffer (pH 7.5). β-Lactamase activity was detected in the flowthrough fraction, which was then dialyzed against 50 mM sodium phosphate buffer (pH 6.0). This partially purified fraction was again applied to a HiTrap SP HP column (Amersham Biosciences) that had been preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The enzymes were eluted with a linear gradient of NaCl in the same buffer. Fractions with β-lactamase activity were dialyzed against 50 mM sodium phosphate buffer (pH 7.0) and condensed by use of an Ultrafree-15 centrifuge filter device (Millipore Corporation, Bedford, MA). The production of CMY-19 was not enough in the *E. coli* transformant, so the following method was used. The bla_{CMY-19} gene was amplified with primers CMY-F2 (5′-CAT ATG CAA CAA CGA CAA TCC ATC C-3′), which has an NdeI linker (underlined), and CMY-R2 (5′-GAA TTC TCA ACC GGC CAA CTG CGC CA-3′), which has an EcoRI linker (underlined), and the Expand High-Fidelity PCR system (Roche). The amplicon was ligated with a pGEM-T vector (Promega), subjected to confirmatory sequencing, and then excised by digestion with NdeI and EcoRI and subcloned into the expression vector pET29a(+) (Novagen, Madison, WI), which was cleaved with the same enzymes. The constructed expression vector, named pET-CMY-19, was introduced into *E. coli* BL21(DE3)pLysS [F^- *ompT hsdS*B ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS], which was obtained from Novagen through TAKARA BIO Inc., Kyoto, Japan. The transformant was cultured in 1 liter of LB broth containing kanamycin (50 μg/ml) and chloramphenicol (30 mg/ml) at 37°C. Isopropyl-β-D-thiogalactopyranoside was added when the culture reached an optical density at 600 nm of 0.55, and the culture was incubated for an additional 6 h at 25°C. CMY-19 was purified by the same methods used for the purification of CMY-9. The purity of the β-lactamases was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue (CBB) staining. The purified CMY-9 and CMY-19 β-lactamases were also subjected to isoelectric focusing analysis with an Ampholine PAG plate (Amersham Biosciences) and stained with CBB.

Assay of kinetic parameters. The kinetic parameters of CMY-9 and CMY-19 against various β-lactam substrates were assayed at 30°C in 50 mM sodium 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; piperacillin, 232 nm; cephalothin, 262 nm; cephaloridine, 297 nm; ceftizoxime, 257 nm; ceftazidime, 274 nm; cefotaxime, 264 nm; cefpirome, 267 nm; cefepime, 275 nm; cefoxitin, 270 nm; cefmetazole, 259 nm; moxalactam, 274 nm; imipenem, 298 nm. K_m and k_{cat} values were obtained by a Michaelis-Menten plot of the initial steady-state velocities at different substrate concentrations. K_i was determined by the procedure described in our previous study (13), with cephalothin used as a reporter substrate.

Nucleotide sequence accession number. The open reading frame of bla_{CMY-19} was deposited in the EMBL/GenBank databases through DDBJ and assigned accession number AB194410.

RESULTS

Properties of nine *E. coli* and five *K. pneumoniae* clinical isolates. The MICs of six β-lactams for the 14 clinical isolates are shown in Table 1. The *K. pneumoniae* and *E. coli* clinical isolates exhibited resistance to oximino-cephalosporins and cephamycins but were susceptible to carbapenems, although *E. coli* HKYM68 also showed resistance to imipenem. In a double-disk synergy test, no synergistic effect of clavulanic acid on the activities of ceftazidime and cefotaxime was detectable in any of the 14 isolates. A lack of metallo-β-lactamase production was also suggested by the results of the sodium mercaptoacetic acid disk tests. An apparent expansion of the growth inhibitory zone was observed with the 14 clinical isolates only between a disk containing 300 μg of 3-aminophenyl-boronic acid and a disk containing ceftazidime or cefotaxime, suggesting the production of a class C β-lactamase. These findings indicate that the property of resistance to oxyimino-cephalosporins and cephamycins was likely due to the production of a class C β-lactamase.

PCR detection of various β-lactamase genes and sequencing revealed that a *K. pneumoniae* isolate (HKY209) carried bla_{CMY-9}, while the other four *K. pneumoniae* isolates carried bla_{CMY-19}, a variant gene of bla_{CMY-9} (Table 1). A single nucleotide mutation at position 944 was found between bla_{CMY-9} and the newly identified bla_{CMY-19} gene, and this point mutation resulted in the I292S substitution near the H-10 helix domain in CMY-19, as shown in Fig. 1. All nine *E. coli* clinical isolates carried both the bla_{CMY-9} and the bla_{TEM} genes (Table 1).

TABLE 1. MICs for parent strains and their transconjugants

Strain	Date of isolation (mo and yr)	Patient	Source	β-Lactamase	MIC (μg/ml)[a]							Transconjugant (*E. coli* CSH-2)	β-Lactamase	MIC (μg/ml)[a]						
					PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM			PIP	CAZ	CAZ + APB[b]	CTX	FEP	CMZ	IPM
K. pneumoniae												*E. coli* CSH-2		1	0.13	0.13	≤0.06	≤0.06	0.5	0.25
HKY209	Jul. 95	A	Sputum	CMY-9	32	>128	1	>128	0.25	>128	0.25	*E. coli*(pK209)	CMY-9	4	64	0.25	128	≤0.06	128	0.25
HKY327	Apr. 95	B	Sputum	CMY-19	128	>128	32	128	4	64	0.25	*E. coli*(pK327)	CMY-19	64	>128	8	128	4	32	0.5
HKY363	Jun. 96	C	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK363)	CMY-19	32	>128	8	64	2	16	0.25
HKY466	Oct. 96	D	Sputum	CMY-19	128	>128	16	64	4	64	0.25	*E. coli*(pK466)	CMY-19	32	>128	8	64	2	16	0.25
HKY474	Jan. 97	E	Sputum	CMY-19	64	>128	16	64	4	64	0.13	*E. coli*(pK474)	CMY-19	32	>128	4	64	4	16	0.25
E. coli																				
HKY154	Mar. 95	F	Sputum	CMY-9 and TEM-1-like[c]	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE154)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY191	Jun. 95	G	Pus	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.13	*E. coli*(pE191)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY200	Jun. 95	H	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE200)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY215	Jul. 95	H	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE215)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY224	Aug. 95	I	Stool	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE224)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKYM68	Nov. 95	J	Sputum	CMY-9 and TEM-1-like	32	>128	2	>128	2	>128	32[d]	*E. coli*(pEM68)	CMY-9	8	64	0.5	128	≤0.06	128	0.25
HKY297	Mar. 96	K	Sputum	CMY-9 and TEM-1-like	32	>128	1	>128	0.25	>128	0.13	*E. coli*(pE297)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY315	Apr. 96	L	Throat swab	CMY-9 and TEM-1-like	32	>128	1	>128	0.5	>128	0.25	*E. coli*(pE315)	CMY-9	4	64	0.5	128	≤0.06	128	0.25
HKY334	Apr. 96	K	Sputum	CMY-9 and TEM-1-like	64	>128	1	>128	0.5	>128	0.13	*E. coli*(pE334)	CMY-9	4	64	0.5	128	≤0.06	128	0.25

[a] Abbreviations: PIP, piperacillin; CAZ, ceftazidime; APB, 3-aminophenyl-boronic acid; CTX, cefotaxime; FEP, cefepime; CMZ, cefmetazole; IPM, imipenem.
[b] APB was used at a concentration of 300 μg/ml.
[c] The nucleotide sequence of the PCR amplicon derived from the *bla* gene was identical to that of the bla_{TEM-1} gene, although the total nucleotide sequence of the *bla* gene was not determined.
[d] Imipenem resistance may be due to alteration in bacterial membrane as reported previously (3, 9, 29).

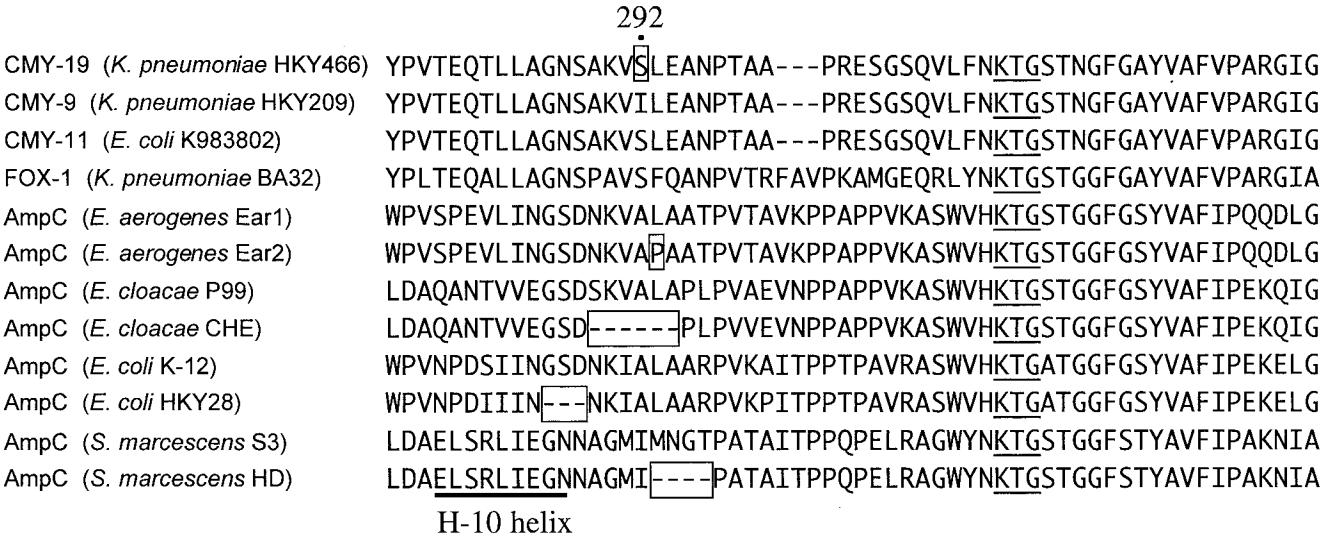

FIG. 1. Alignments of amino acid residues near the H-10 helix. A partial amino acid sequence alignment of CMY-9 (12), CMY-19 (this study), CMY-11 (21), FOX-1 (15), AmpC of *E. cloacae* Ear1 and Ear2 (5), AmpC of *E. cloacae* P99 and HD (6), AmpC of *E. coli* K-12 and HKY28 (13), and AmpC of *S. marcescens* S3 and HD (22) is shown. Square boxes show the amino acid substitutions or deletions that are predicted to affect the hydrolyzing activity of cefepime. The conserved motif KTG is underlined. Dashes indicate deletions of amino acid residues. CMY-11- and FOX-type enzymes have a serine residue at amino acid position 292, but no observation about their property against cefepime was described in the articles. The numbering of the amino acid residues is in reference to that of the mature CMY-1 reported by Bauernfeind et al. (7).

The mechanism of imipenem resistance of HKYM68 was not characterized in this work.

Transferability of β-lactam resistance. The oximino-cephalosporin and cephamycin resistance trait of the five *K. pneumoniae* was transferred to a recipient *E. coli* strain (strain CSH-2) at a frequency of 10^{-4} to 10^{-5} cells per recipient cell by broth mating. Conjugal transfer of the resistance trait from the nine *E. coli* isolates was also observed at a frequency of about 10^{-3} to 10^{-4} cells per recipient cell.

PCR analyses confirmed the presence of bla_{CMY-9} or bla_{CMY-19} in each transconjugant, indicating that these genes are located on transferable plasmids. PCR analysis of the transconjugants also revealed no cotransmission of the bla_{TEM} gene to the *E. coli* transconjugants that harbored the bla_{CMY-9} gene.

PFGE analysis. The PFGE patterns of the five *K. pneumoniae* isolates after XbaI digestion were highly variable (Fig. 2A), which

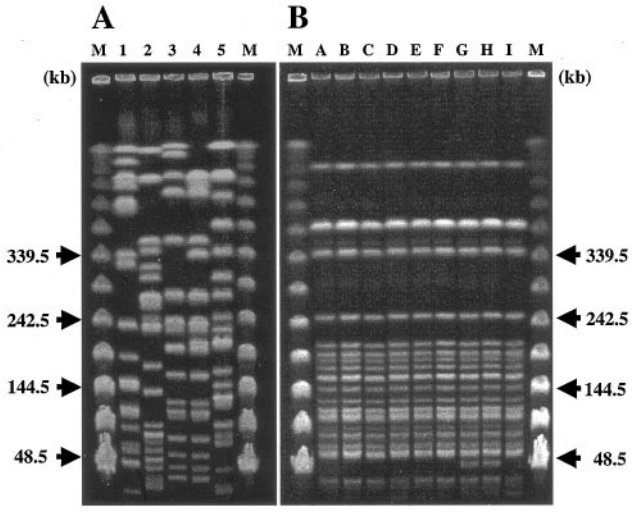

FIG. 2. PFGE analysis of *K. pneumoniae* and *E. coli* isolates. (A) Lanes: M, PFGE marker; 1, *K. pneumoniae* HKY209; 2, *K. pneumoniae* HKY327; 3, *K. pneumoniae* HKY363; 4, *K. pneumoniae* HKY466; 5, *K. pneumoniae* HKY474. (B) Lanes: M, PFGE marker; A, *E. coli* HKY154; B, *E. coli* HKY191; C, *E. coli* HKY200; D, *E. coli* HKY215; E, *E. coli* HKY224; F, *E. coli* HKY297; G, *E. coli* HKY315; H, *E. coli* HKY334; and I, *E. coli* HKYM68.

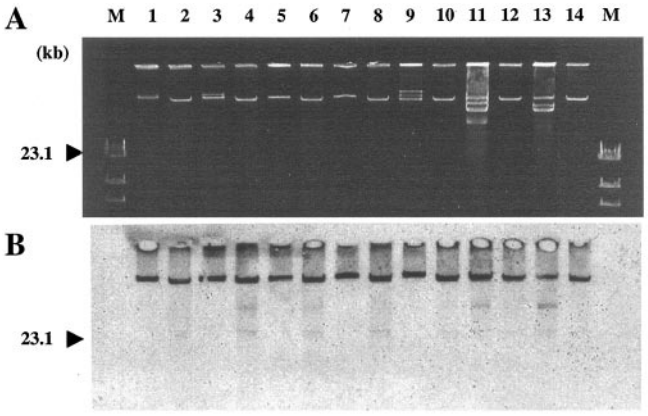

FIG. 3. Plasmid profiles and Southern hybridization. (A) Plasmid profiles of clinical isolates and their tranconjugants; (B) hybridization with the probe specific for the CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *K. pneumoniae* HKY209; 2, *E. coli* CSH-2/pK209; 3, *K. pneumoniae* HKY327; 4, *E. coli* CSH-2/pK327; 5, *K. pneumoniae* HKY363; 6, *E. coli* CSH-2/pK363; 7, *K. pneumoniae* HKY466; 8, *E. coli* CSH-2/pK466; 9, *K. pneumoniae* HKY474; 10, *E. coli* CSH-2/pK474; 11, *E. coli* HKY154; 12, *E. coli* CSH-2/pE154; 13, *E. coli* HKYM68; and 14, *E. coli* CSH-2/pEM68.

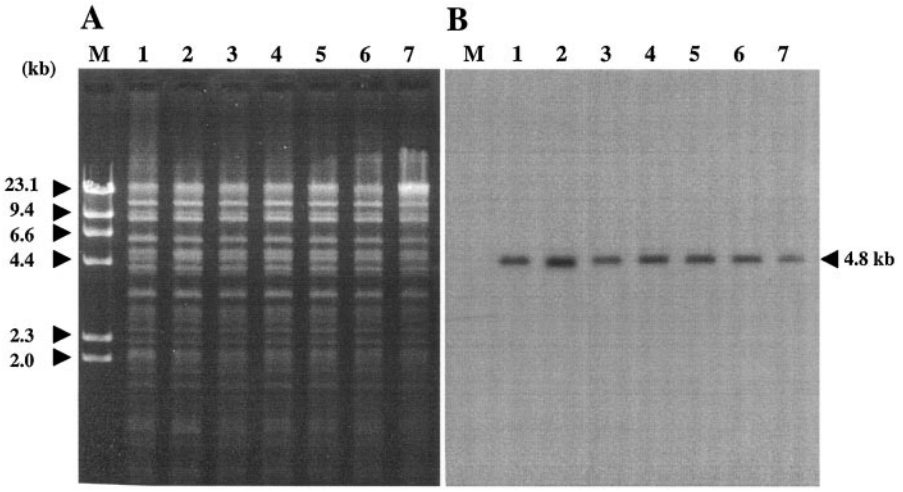

FIG. 4. Plasmid patterns after restriction enzyme digestion and Southern hybridization. (A) SacI-digested plasmid DNAs prepared from the representative transconjugants; (B) hybridization patterns with the probe specific for CMY-1- and MOX-1-type β-lactamase gene. Lanes: M, HindIII-digested DNA marker; 1, *E. coli* CSH-2/pK209; 2, *E. coli* CSH-2/pK327; 3, *E. coli* CSH-2/pK363; 4, *E. coli* CSH-2/pK466; 5, *E. coli* CSH-2/pK474; 6, *E. coli* CSH-2/pE154; and 7, *E. coli* CSH-2/pEM68.

revealed their clonal diversity. In contrast, the PFGE patterns of the *E. coli* isolates were very similar to one another (Fig. 2B), which revealed their clonal relatedness.

Plasmid analyses and Southern hybridization. The plasmid DNA profiles prepared from the five *K. pneumoniae* isolates and their transconjugants and from two representative *E. coli* isolates (isolates HKY154 and HKYM68) and their transconjugants are shown in Fig. 3A. One to three large plasmids were apparently present in the five *K. pneumoniae* isolates, and one large plasmid was transferred to *E. coli* from each strain in the conjugation experiment. *E. coli* isolate HKY154 had four plasmids, and identical plasmid profiles were found in seven other *E. coli* isolates (isolates HKY191, HKY200, HKY215, HKY224, HKY297, HKY315, and HKY334) (data not shown). *E. coli* HKYM68 harbored three plasmids that were similar to three of the four plasmids found in HKY154. All the *E. coli* transconjugants carried a single plasmid apparently identical to that transferred from the *K. pneumoniae* isolates (Fig. 3A and data not shown).

A plasmid of similar size hybridized with the DNA probe specific for the CMY-1-type β-lactamase genes in all the *K. pneumoniae* and *E. coli* isolates and their transconjugants (Fig. 3B). The SacI restriction profiles of the plasmid DNAs from representative transconjugants were very similar to each other (Fig. 4A), and the DNA probe specific for $bla_{\text{CMY-1}}$-group genes hybridized with a band of about 4.8 kb in size in all cases (Fig. 4B).

Isoelectric focusing of β-lactamases. A β-lactamase band with a pI of >8.45 was detected in all *K. pneumoniae* and *E. coli* clinical isolates and their transconjugants but not in *E. coli* CSH-2 (data not shown). This band likely corresponded to the CMY-9 or CMY-19 β-lactamase. No band with an acidic pI value was detected in the nine *E. coli* clinical isolates carrying the bla_{TEM} gene, likely because of a low level of expression of that gene.

Genetic environments of $bla_{\text{CMY-9}}$ and $bla_{\text{CMY-19}}$ genes. The structure of the flanking regions of the $bla_{\text{CMY-9}}$ gene in *E. coli* HKYM68 was already reported in a previous study (12). The structure surrounding the bla_{CMY} genes in the other 13 isolates was identical to that found in HKYM68. Both $bla_{\text{CMY-9}}$ and $bla_{\text{CMY-19}}$ were located at the 3′ end of a putative transposase gene, *orf513*. A *sul1*-type class 1 integron structure consisting of *intI1* (an integrase gene), a fused *aacA1-orfG* gene cassette (responsible for aminoglycoside resistance), *qacEΔ1*, and *sul1* (responsible for trimethoprim-sulfamethoxazole resistance) were found at the 5′ end of *orf513* (Fig. 5).

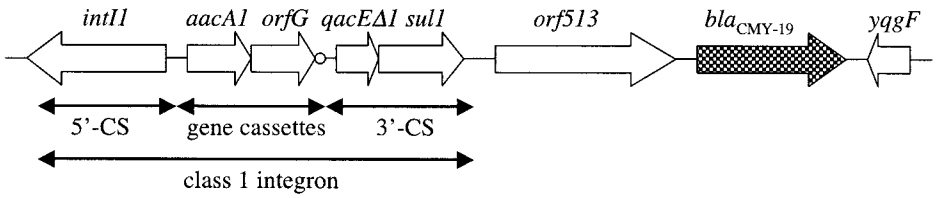

FIG. 5. Gene organization around bla_{CMY} genes. The bla_{CMY} gene on the conjugative plasmid found in the *K. pneumoniae* and *E. coli* clinical isolates located just downstream of *orf513* is shown as it was found in our previous study on a CMY-9 producing *E. coli* HKHM68 (12). Open circle, position of the 59-base element; CS, conserved segment of a class 1 integron. *orf513* is speculated to encode a putative transposase, and various antimicrobial resistance genes tend to be integrated just downstream the *orf513*. The product from the *yqgF* gene encodes a hypothetical protein very similar to the YqgF identified in *Aeromonas hydrophila* (EMBL accession no. AJ276030), but the function is unknown.

TABLE 2. MICs of β-lactams for CMY-9-producing and CMY-19-producing *E. coli* transformants

β-Lactam	MIC (μg/ml)		
	Transformant		Recipient, *E. coli* DH5α (pBCSK+)
	E. coli DH5α (pBC-CMY-9) CMY-9	*E. coli* DH5α (pBC-CMY-19) CMY-19	
Ampicillin	64	>128	2
Piperacillin	8	64	0.5
Piperacillin + TAZ[a]	4	32	0.5
Cephalothin	>128	>128	2
Cephaloridine	64	128	2
Ceftizoxime	64	16	≤0.06
Ceftazidime	64	>128	≤0.06
Ceftazidime + APB[b]	0.5	8	≤0.06
Cefotaxime	>128	128	≤0.06
Cefotaxime + APB[b]	2	1	≤0.06
Cefpirome	8	16	≤0.06
Cefepime	0.13	4	≤0.06
Cefoxitin	>128	128	2
Cefmetazole	128	32	0.5
Cefminox	128	32	0.5
Moxalactam	8	8	≤0.06
Aztreonam	4	16	≤0.06
Imipenem	0.25	0.25	0.13
Meropenem	≤0.06	≤0.06	≤0.06

[a] TAZ, tazobactam, which was used at a concentration of 4 μg/ml.
[b] APB, 3-Aminophenyl boronic acid, which was used at a concentration of 300 μg/ml.

MICs for CMY-9- or CMY-19-producing *E. coli* transformants. The MICs of various β-lactams for CMY-9- or CMY-19-producing *E. coli* transformants are shown in Table 2. Some notable differences were observed between the MICs of the two strains. The MICs of ampicillin and piperacillin for the CMY-19 producer were higher than those for the CMY-9 producer. Concerning ceftizoxime and cefotaxime, the MICs for the CMY-9 producer were higher than those for the CMY-19 producer, but in the case of ceftazidime, the level of resistance was reversed. The CMY-19 producer showed higher levels of resistance to cefpirome and cefepime than the CMY-9 producer. The MICs of cephamycins, such as cefoxitin, cefmetazole, and cefminox, were higher for the CMY-9 producer than for the CMY-19 producer. A remarkable reduction in the MICs by the addition of a class C β-lactamase specific inhibitor, 3-aminophenyl boronic acid, was observed with both the CMY-9 and the CMY-19 producers.

Kinetic parameters. To purify the CMY-9 and the CMY-19 β-lactamases, initially, *E. coli* DH5α(pBC-CMY-9) and *E. coli* DH5α(pBC-CMY-19) were cultured in 2 liters of LB broth. However, the yield of purified CMY-19 β-lactamase was insufficient for the assay of kinetic parameters. Therefore, a pET29a(+) expression vector and an *E. coli* BL21(DE3) pLysS strain were used for overproduction and purification of that enzyme. The purified enzymes gave a single band on SDS-PAGE with CBB staining that suggested >95% purity (data not shown).

The kinetic parameters of CMY-9 and CMY-19 against selected β-lactams are shown in Table 3. The hydrolyzing activity (k_{cat}/K_m) of CMY-19 for penicillins, including ampicillin and piperacillin, were higher than those of CMY-9. Although CMY-9 and CMY-19 had similar k_{cat} values for cefotaxime, CMY-19 had a 100-fold-higher K_m than CMY-9, resulting in a lower catalytic efficiency for this substrate. Ceftazidime, cefpirome, and cefepime behaved as poor substrates for CMY-9 due to the high K_m values for these agents, while CMY-19 showed different behaviors against these compounds. CMY-19 had a 140-fold-lower K_m against ceftazidime than CMY-9. The k_{cat} value of CMY-9 for cefepime could not be determined, but CMY-19 measurably hydrolyzed this compound. The hydrolyzing efficiencies (k_{cat}/K_m values) of CMY-19 against cephamycins such as cefoxitin and cefmetazole were lower than those of CMY-9. Although CMY-19 had a lower K_m against cephamycins than CMY-9, it showed a much lower k_{cat} against these compounds.

DISCUSSION

A plasmid-mediated class C β-lactamase (CMY-1) was first reported in 1989 in a *K. pneumoniae* isolated in South Korea (8). Subsequently, several variants of that enzyme, such as MOX-1 (16), CMY-8 (33), CMY-9 (12), CMY-10 (20), and CMY-11 (21), have been identified, mainly in East Asian countries, including Taiwan and Japan. The dissemination of CMY-

TABLE 3. Kinetic parameters of CMY-9 and CMY-19

Substrate	CMY-9			CMY-19		
	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Ampicillin	91 ± 28	1.0 ± 0.1	1.1×10^4	16 ± 1	0.35 ± 0.01	2.2×10^4
Piperacillin	97 ± 21	0.14 ± 0.01	1.4×10^3	8.9 ± 0.5	0.031 ± 0.001	3.5×10^3
Cephalothin	120 ± 10	630 ± 10	5.3×10^6	230 ± 10	380 ± 10	1.7×10^6
Cephaloridine	1200 ± 100	99 ± 2	8.3×10^4	1500 ± 100	240 ± 10	1.6×10^5
Ceftizoxime	5.5 ± 0.2	1.3 ± 0.1	2.4×10^5	11 ± 1	0.71 ± 0.03	6.5×10^4
Ceftazidime	560 ± 110	1.8 ± 0.3	3.2×10^3	3.7 ± 0.1	0.085 ± 0.002	2.3×10^4
Cefotaxime	0.28 ± 0.01	0.27 ± 0.01	9.6×10^5	31 ± 2	0.33 ± 0.01	1.1×10^4
Cefpirome	390 ± 50	3.6 ± 0.3	9.2×10^3	25 ± 2	0.58 ± 0.02	2.3×10^4
Cefepime	950 ± 50	NH[a]	ND[b]	630 ± 170	1.8 ± 0.4	2.9×10^3
Cefoxitin	60 ± 2	50 ± 1	8.3×10^5	0.90 ± 0.03	0.12 ± 0.01	1.3×10^5
Cefmetazole	5.1 ± 0.2	1.7 ± 0.1	3.3×10^5	0.26 ± 0.01	0.045 ± 0.001	1.7×10^5
Moxalactam	0.22 ± 0.01	NH	ND	0.40 ± 0.03	NH	ND
Imipenem	4.6 ± 0.3	NH	ND	4.3 ± 0.1	NH	ND

[a] NH, not hydrolyzed.
[b] ND, not determined.

10- and CMY-11-producing isolates of the family *Enterobacteriaceae* was also reported in Korea (18, 19). In the present study, CMY-type β-lactamase-producing *K. pneumoniae* and *E. coli* clinical isolates from a Japanese general hospital were investigated. Through PFGE analysis, it was found that the five *K. pneumoniae* isolates had little genetic relatedness to each other, while the nine *E. coli* isolates belonged to the same clonal lineage. Interestingly, plasmid analysis showed that all 14 isolates harbored a very similar conjugal plasmid that encodes a CMY-type β-lactamase which was either CMY-9 or CMY-19, a variant that differs from CMY-9 by a single amino acid substitution (I292S). Since the flanking structures of the bla_{CMY} genes were identical in all plasmids, it is probable that one conjugal plasmid carrying bla_{CMY-9} was horizontally transferred to *E. coli*, *K. pneumoniae*, and then an *E. coli* clone and various *K. pneumoniae* strains harboring the bla_{CMY} gene-bearing plasmids might have spread in the hospital.

β-Lactamases can modify their substrate specificity through a single amino acid substitution (30). CMY-19 had a single amino acid substitution, I292S, near the H-10 helix domain, compared with the sequence of CMY-9 (Fig. 1). Indeed, a serine residue at the same amino acid position was found in all the FOX-type enzymes (15, 26), including CAV-1 (14), and also in CMY-11 (21), as shown Fig. 1; but no peculiar behavior against cefepime was documented with those enzymes. Through the I292S substitution, CMY-19 would have developed extended substrate specificity against cefepime and cefpirome, as well as ampicillin, piperacillin, cephaloridine, and ceftazidime, compared with that of CMY-9, although the hydrolyzing activities against ceftizoxime, cefotaxime, and cephamycins were impaired. The expansion of hydrolyzing activity against cefepime found in CMY-19 was a most remarkable property because cefepime is generally stable against AmpC β-lactamases (11). Similar developments of extended hydrolyzing activity against cefepime through amino acid substitutions or deletions adjacent to the H-10 helix have been observed in several chromosomally encoded AmpC β-lactamases, such as the AmpC of *Serratia marcescens* that lacks four amino acids at positions 293 to 296 (22), an AmpC of *E. coli* that lacks three amino acids at positions 286 to 288 (13), an AmpC of *Enterobacter cloacae* that lacks six amino acids at positions 289 to 294 (6), and an AmpC of *Enteroacter aerogenes* that acquired an L293P substitution (5). Furthermore, Barlow and Hall reported on the in vitro selection of CMY-2 β-lactamase variants with several amino acid substitutions, including replacements at positions 292, 293, 294, 296, and 298, which demonstrated increased resistance to cefepime (4). From our previous molecular modeling analyses (13), it was found that the expansion of an open space in the vicinity of the R-2 side chain of ceftazidime or cefepime through the deletion of tripeptides in the H-10 helix of *E. coli* AmpC (Fig. 1) played a crucial role in the acquisition of the greater hydrolyzing activity against those agents possessing a bulky R side chain. Although the actual mechanism for the higher cefepime-hydrolyzing activity in CMY-19 compared to that in CMY-9 has not been elucidated, a similar molecular distortion at the active center of the enzyme might well have occurred in CMY-19. This speculation would be substantiated by molecular modeling and X-ray crystallographic analyses.

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