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Comparative Characterization of the Cephamycinase bla_{CMY-1} Gene and Its Relationship with Other β-Lactamase Genes

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A plasmidic β-lactamase which hydrolyzes cephamycins was first detected and reported in 1989. At that time its description was restricted to phenotypic characteristics. We analyzed the nucleotide sequence of its gene and explored its genetic relationship with other *bla* genes. The deduced amino acid sequence of the bla_{CMY-1} product was compared with those of other known plasmidic cephamycinases and of chromosomal AmpC β-lactamases. The results indicate that the relationship of CMY-1 is closest to MOX-1 among the plasmidic cephamycinases and to AmpC of *Pseudomonas aeruginosa* among the chromosomal cephalosporinases. We conclude that the plasmidic cephamycinases described up to now may be classified into three families, as follows: CMY-1, MOX-1, and FOX-1 with AmpC of *P. aeruginosa*; CMY-2, BIL-1, and LAT-1 with AmpC of *Citrobacter freundii*; and MIR-1 with AmpC of *Enterobacter cloacae*. Plasmidic cephamycinases are now recognized as clinically relevant class C β-lactamases.

bla genes encoding β-lactamases which slowly hydrolyze 7-α-methoxy-cephalosporins, e.g., cefoxitin, are generally localized on the bacterial chromosome. Many have been classified as class C β-lactamases (1, 16). However, in 1989 a cephamycinase encoded by a plasmidic gene was found (2). At that time the characterization of the enzyme was restricted mainly to its phenotype, e.g., antibiotic resistance profile and isoelectric point. The comparison of its characteristics with known β-lactamases resulted in postulation of the first plasmidic expanded-spectrum β-lactamase, including resistance to cephamycins (cephamycinase CMY-1). In this study we confirmed the phenotypic definition of CMY-1 by molecular analysis of its gene (bla_{CMY-1}). Furthermore, the positioning of the CMY-1 β-lactamase within the genetic β-lactamase classification system and its relationship with other cephamycinases described since then (4, 8, 10, 14, 21, 26) were analyzed.

Bacterial strains. *Klebsiella pneumoniae* CHO, the cefoxitin-resistant clinical isolate, was isolated from a wound infection of a patient in Seoul, South Korea, in 1989 (2). *Escherichia coli* C600 (nalidixic acid MIC, 1,024 mg/liter) was the recipient strain for transfer of the resistance factor from *K. pneumoniae* CHO. *E. coli* DH5α was the host for the cloning experiments.

Vector. Vector pBC (Stratagene, Heidelberg, Federal Republic of Germany [FRG]) carrying a chloramphenicol resistance marker was used for cloning of the bla_{CMY-1} gene.

Antibiotics. The following antibiotics were obtained from the respective manufacturers: cefoxitin and imipenem (Merck, Sharp & Dohme, Haar, FRG); clavulanate, temocillin, and cefepime (SmithKline Beecham Pharma, Munich, FRG); sulbactam (Pfizer, Karlsruhe, FRG); cefotetan and meropenem (Zeneca, Plankstadt, FRG); cefmetazole (Sankyo Europe, Düsseldorf, FRG); moxalactam (Eli Lilly, Bad Homburg, FRG); cefotaxime and cefpirome (Hoechst, Frankfurt am Main, FRG); ceftazidime (Cascan, Wiesbaden, FRG); aztreonam (Bristol-Myers Squibb, Munich, FRG); tazobactam (Lederle, Wolfratshausen, FRG); and flomoxef (Shionogi, Düsseldorf, FRG). Combinations of cefoxitin with β-lactamase inhibitors were used at proportions of 1:4 (clavulanate), 1:1 (sulbactam), or 1:7 (tazobactam).

MICs. MICs were determined by an agar dilution technique using Mueller-Hinton agar (Difco, Augsburg, FRG). An inoculum of 10^4 CFU per spot was deposited on the agar by a multipoint inoculator (Denley, Billinghurst, United Kingdom). MICs were read after 16 h of incubation at 35°C. *E. coli* ATCC 25922 was used as a quality reference strain.

Transfer of resistance determinants. The wild-type and recipient strains (10^9 CFU/ml per strain) were suspended in Mueller-Hinton broth (Difco) and incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Oxoid, Wesel, FRG) and supplemented with nalidixic acid (64 mg/liter) and cefoxitin (64 mg/liter) to inhibit the growth of the donor strain or the recipient strain, respectively.

TABLE 1. Antibiotic susceptibility of *K. pneumoniae* CHO, its transconjugant, its transformant, and the *E. coli* C600 recipient

Antibiotic	MIC (mg/liter) for:			
	K. pneumoniae CHO	*E. coli* C600 R^+	*E. coli* DH5α T^+ (pMVP-1-1a)	*E. coli* C600 R^-
Cefoxitin	512	256	128	4
+ Clavulanate	64	64	64	2
+ Sulbactam	64	32	16	2
+ Tazobactam	128	64	64	4
Cefotetan	256	256	128	0.13
Cefametazole	256	128	64	1
Moxalactam	8	8	4	0.13
Flomoxef	32	32	16	0.06
Cefotaxime	128	64	64	0.03
Ceftazidime	4	4	4	0.13
Cefpirome	2	2	1	0.03
Cefepime	0.5	0.25	0.25	0.03
Aztreonam	32	16	8	0.06
Piperacillin	256	128	64	1
Temocillin	8	8	8	8
Imipenem	0.25	0.25	0.25	0.25
Meropenem	0.06	0.06	0.03	0.06

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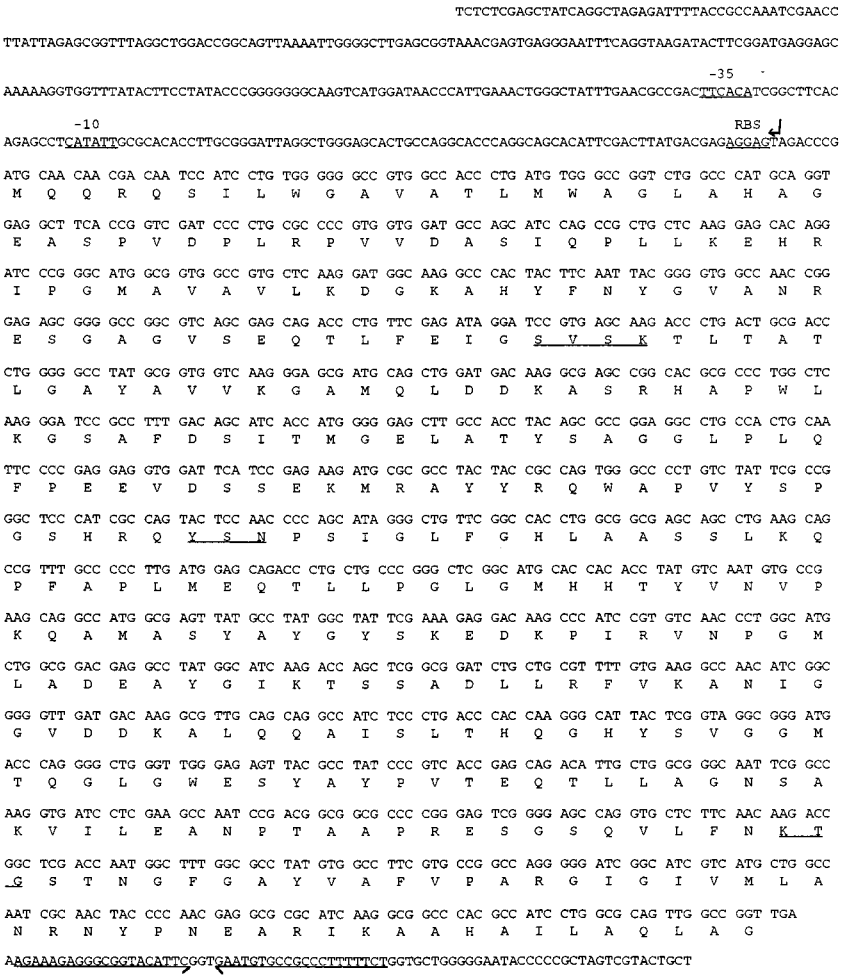

FIG. 1. Nucleotide sequence of the *bla*$_{CMY-1}$ gene (pMVP-1-1a; the part of the sequence taken from pMVP-1-1b is marked [↵]). The deduced amino acid sequence of CMY-1 is shown in the line below the nucleotide triplets. The β-lactamase active site S-V-S-K, the conserved triad K-T-G, and the class C typical motif Y-X-N are underlined. Possible promoter sequences (−35, −10) and a ribosome binding site (RBS) can be found upstream of the start codon. A terminator hairpin following the stop codon is marked.

Isoelectric focusing of β-lactamases. Crude homogenates of β-lactamases were prepared as described previously (3). For isoelectric focusing, the procedure of Matthew et al. (19) was modified (3).

Plasmid DNA preparation. Cells grown overnight in 150 ml of tryptic soy broth (Difco) were used to prepare R-factor plasmids or recombinant plasmids. The DNA preparation was performed by alkaline lysis (6). Plasmid DNA in the lysate was purified with an anion-exchange column (tip 100; Qiagen, Hilden, FRG) according to the recommendations of the manufacturer.

Cloning and sequencing of the *bla*$_{CMY-1}$ gene. Cloning experiments were performed by following standard procedures (23). All enzymes used were purchased from Boehringer Mannheim (Mannheim, FRG). The resistance plasmid carrying the *bla*$_{CMY-1}$ gene (pMVP-1) was isolated from the *E. coli* C600 transconjugant strain and partially digested with *Pst*I. Ligation to vector pBC, followed by transformation of $CaCl_2$-treated *E. coli* DH5α and selection on Mueller-Hinton agar supplemented with chloramphenicol (64 mg/liter) and cefoxitin (8 mg/liter), resulted in the detection of cefoxitin-resistant *E. coli* transformants harboring a recombinant plasmid with a 4.4-kb insert (pMVP-1-1). The complete 4.4-kb *Pst*I fragment could be excised by a double digest with *Eco*RI and *Xba*I. *Acc*I and *Dra*I digests of this 4.4-kb fragment resulted in two smaller subclones of 2.1 and 2.4 kb, respectively (pMVP-1-1a and pMVP-1-1b), which were sequenced. Sequencing was performed by the dideoxy chain termination procedure of Sanger et al. (25) by using consecutive primers for both strands with an automatic sequencer (373A; Applied Biosystems, Weiterstadt, FRG).

Sequence analysis. Related β-lactamases were identified by comparison with the EMBL and Swissprot databases (Fasta). Multiple alignment was calculated by Clustal V (11, 12).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL database under accession number X92508.

Phenotypic characterization of the CMY-1 β-lactamase. The isoelectric point of the CMY-1 β-lactamase from *K. pneumoniae* CHO and its *E. coli* transconjugant was 8.0 (2). High MICs of cephamycins, cefotaxime, aztreonam, and piperacillin were observed for the transconjugant strain and the transformant, but the MICs of ceftazidime were increased only 32-fold (Table 1). Reduction of cefoxitin MICs by β-lactamase inhibitors was only four- to eightfold; sulbactam was more active

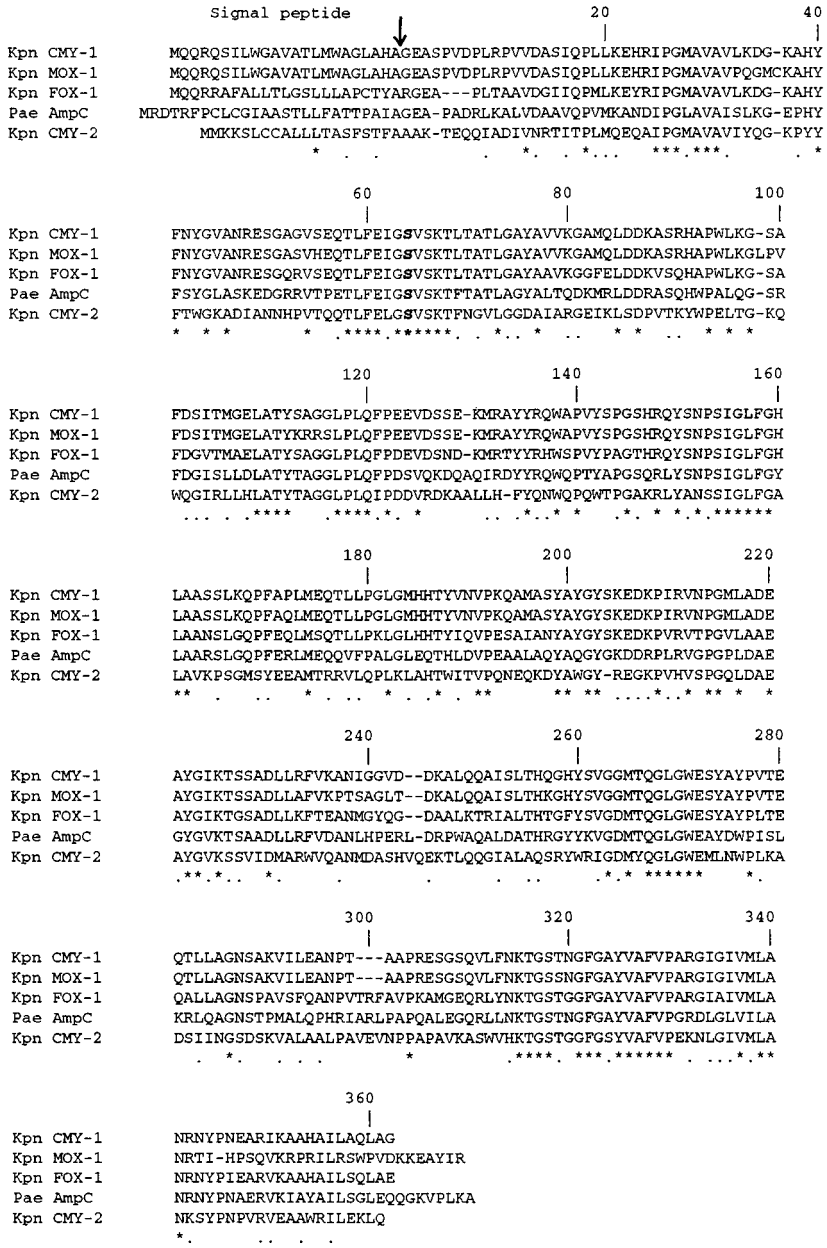

FIG. 2. Multiple sequence alignment of the amino acid sequences of CMY-1, MOX-1, FOX-1, AmpC of *P. aeruginosa*, and CMY-2. Identical amino acids are marked with an asterisk, and conservative exchanges are marked by a dot. Active-site serine (position 64) is marked by bold letters. The putative cleavage site of the signal peptidase is marked by an arrow.

than clavulanate or tazobactam. These phenotypic markers indicated a unique β-lactamase.

Analysis of the *bla*$_{\text{CMY-1}}$ gene. The nucleotide sequence of a 2.1-kb *Acc*I subclone (pMVP-1-1a) was determined. Only one large open reading frame was found. It consisted of 1,149 nucleotides, which corresponds to a putative protein of 382 amino acids (Fig. 1). Sequence comparison with other known β-lactamases (EMBL and Swissprot databases) revealed a relationship to other β-lactamases only of class C. The CMY-1 β-lactamase is classified as a class C β-lactamase because of the molecular mass of 38 kDa for the mature protein, the position of the active-site serine (amino acid 64 of the mature protein), and the class C-specific amino acid motif Y-X-N (Fig. 1). Multiple sequence alignment of the amino acid sequence of CMY-1 was performed with other class C β-lactamases described elsewhere (e.g., the plasmidic enzymes MOX-1 [14], FOX-1 [10], and CMY-2 [4] and the chromosomal AmpC β-lactamase of *Pseudomonas aeruginosa* [18]) (Fig. 2 and 3). The result of this analysis is shown in Table 2. It demonstrates that the CMY-1 β-lactamase is most closely related to MOX-1 (88.8% identity of amino acid sequence), followed by FOX-1 (76.1% identity) and AmpC of *P. aeruginosa* (57.5% identity). Because the 2.1-kb insert (pMVP-1-1a) contained only 7 bp upstream of the start codon, the information about the putative ribosome binding site and promoter region was taken from the 2.4-kb subclone (pMVP-1-1b).

K. pneumoniae strains causing nosocomial infections, e.g., pneumonia, were susceptible to β-lactam antibiotics of the

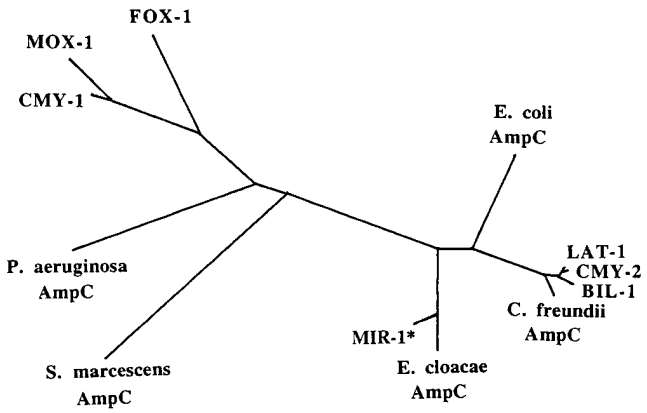

FIG. 3. Dendrogram for 11 mature class C β-lactamases (calculated by Clustal V and the neighbor-joining method of Saitou and Nei [22]). Branch lengths are proportional to the number of amino acid exchanges. *, homology between MIR-1 and AmpC of *E. cloacae* cannot be defined exactly, as the published sequence of MIR-1 is restricted to 150 nucleotides.

oxyimino type until the early 1980s. Since then, various plasmid-encoded β-lactamases conferring resistance to these compounds have emerged (15). These enzymes were, however, unable to hydrolyze cephalosporins with an α-methoxy-group at position 7. These modified cephalosporins remained an option for treatment of infections caused by *K. pneumoniae* strains producing expanded-spectrum β-lactamases of various groups (TEM, SHV [7, 15], CTX [3], or CTI [5, 20] families). Resistance to cephamycins among members of the family *Enterobacteriaceae* was restricted mainly to species producing chromosomal class C β-lactamases constitutively or after induction (24). The appearance of an *ampC*-type gene on a plasmid in *K. pneumoniae* and its transferability to other members of the family *Enterobacteriaceae*, e.g., *E. coli*, signal a risk for spread of these *bla* genes among the human pathogens and indicate a further restriction in the choice of antibiotics for the treatment of infections caused by *K. pneumoniae.*

As shown in Table 1, the CMY-1 β-lactamase increases the MICs for the transconjugant strain in comparison with those for the recipient strain for all β-lactams except temocillin and the carbapenems. The effect is most pronounced for cefotetan and cefotaxime (2,048 times), while the MIC of cefepime is increased only by a factor of 8. The structures of cefotetan and cefotaxime appear to be favorable for hydrolysis by CMY-1, while cefepime may fit much less well into the catalytic site of the CMY-1 enzyme.

Our results on the genetic relationship of the bla_{CMY-1} gene with other *bla* genes demonstrate that a similar *bla* gene, bla_{MOX-1}, appeared 2 years later in the East Asian region (described in 1993, isolated in 1991 [13]). Both the phenotypic and genotypic characterizations of the enzymes and their *bla* genes indicate close relationship. The lower MICs of ceftazidime than of cefotaxime for strains producing both CMY-1 and MOX-1 are unusual because for most cephamycinase-producing strains, MICs of both compounds are equal or MICs of ceftazidime are higher than those of cefotaxime (e.g., CMY-2 [4], MIR-1 [21], BIL-1 [21a], LAT-1 [26], and FOX-1 [10]). This invites speculations on a structure-function relationship, e.g., whether there are common amino acids in these enzymes, different from those in other class C β-lactamases, neighboring the active site which could be responsible for impaired access of the ceftazidime molecule to the active-site region.

Analysis of the genetic relationship among various AmpC cephamycinases indicates that the CMY-1 β-lactamase is related most closely to MOX-1, followed by FOX-1, and that these three plasmidic β-lactamases may be put together with *P. aeruginosa* AmpC into one family. However, the identity between CMY-1 and *P. aeruginosa* AmpC is only about 57%, so most probably CMY-1 did not directly evolve from *P. aeruginosa* AmpC. In addition to the *P. aeruginosa* family of class C β-lactamases, there are two other groups, the *Citrobacter freundii* family, containing the plasmidic enzymes CMY-2 (5), BIL-1 (8), and LAT-1 (26), and the *Enterobacter cloacae* family, containing MIR-1 (21) as the only plasmidic β-lactamase described so far. In these two families the relationship between the chromosomal and the plasmidic genes is much closer (93 to 94% identity of amino acid sequences for the *C. freundii* family and about 90% identity for 50 amino acids in the *E. cloacae* family, with the nucleotide sequence of bla_{MIR-1} only incompletely published) than in the *P. aeruginosa* AmpC family. In these cases, evolution of the plasmidic enzymes from the corresponding AmpC β-lactamases by a few mutations and translocation of the *bla* genes onto a plasmid may be probable.

The number of plasmidic cephamycinases has increased since 1989 up to seven. They appeared in quite different regions of the world—in South Korea, Greece, Pakistan, Japan, Argentina, and the United States. The occurrence of pathogens bearing plasmids encoding cephamycinase activity has been limited so far to a small number of patients; the largest outbreak was observed with MIR-1-producing *K. pneumoniae* in Providence, R.I., including 11 patients at the Miriam Hospital over a period of 9 months (21). For more precise data on the real incidence of cephamycinase producers, careful moni-

TABLE 2. Amino acid sequence identities of nine mature class C β-lactamases[a]

β-Lactamase (reference)	% Identity with:								
	CMY-1	MOX-1	FOX-1	*P. aeruginosa* AmpC	*E. cloacae* AmpC	*C. freundii* OS 60 AmpC	CMY-2	LAT-1	BIL-1
CMY-1	100	88.8	76.1	57.5	45.4	42.9	42.6	42.3	41.8
MOX-1 (14)		100	68.7	52.2	41.6	38.2	38.8	38.5	38.0
FOX-1 (10)			100	56.1	45.9	43.3	43.1	42.8	42.2
P. aeruginosa AmpC (18)				100	44.8	42.6	42.3	42.1	42.1
E. cloacae AmpC (9)					100	73.7	75.3	74.8	73.7
C. freundii OS 60 AmpC (17)						100	95.8	95.3	94.2
CMY-2 (4)							100	99.2	98.3
LAT-1 (26)								100	97.5
BIL-1 (8)									100

[a] MIR-1 is not included as the sequence published so far is incomplete (150 nucleotides).

toring of multiresistant members of the family *Enterobacteriaceae* is necessary, keeping in mind that plasmidic cephamycinases may easily be overlooked when the disk test using neighboring disks loaded with a β-lactam and a β-lactamase inhibitor is applied, because of the low-level susceptibility of cephamycinases to β-lactamase inhibitors.

Plasmidic cephamycinases represent clinically relevant new members of class C β-lactamases which may spread both by translocation of the strains harboring the bla_{CMY} genes and by transfer of the genes among members of the family *Enterobacteriaceae* because of their location on R factors.

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