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Characterization of the Plasmidic β-Lactamase CMY-2, Which Is Responsible for Cephamycin Resistance

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The phenotype of *Klebsiella pneumoniae* HEL-1 indicates a plasmidic cephamycinase gene (bla_{CMY-2}). Its sequence shows one open reading frame coding for a protein of 381 amino acids. CMY-2 is classified as class C β-lactamase that is closely related to the plasmidic enzymes BIL-1 and LAT-1 and the chromosomal AmpC of *Citrobacter freundii*. The bla_{CMY-2} gene possibly was translocated onto a plasmid of *C. freundii* which spread to *K. pneumoniae*.

Resistance of bacterial pathogens to β-lactam antibiotics mediated by β-lactamases has stimulated chemical modifications of the molecules in order to protect them from hydrolysis (e.g., addition of an oxyimino moiety or a methoxy group, as in cephamycins). These efforts were partially counteracted by the emergence of new plasmid-mediated β-lactamases which extended their spectra and hydrolyzed oxyimino-cephalosporins as well, as reported especially for *Klebsiella pneumoniae* (18). However, these plasmid-encoded extended-spectrum β-lactamases, which are classified into Ambler class A (1), remain inactive against cephamycins (e.g., cefoxitin). In 1989, the first report of a plasmidic cephamycin-hydrolyzing β-lactamase was published (4). Data on another plasmidic cephamycinase, CMY-2, were reported in 1990 (7). The nucleotide sequence of the bla_{CMY-2} gene was presented in 1992 (6). We analyzed the sequence data on CMY-2 and compared them with the data on other cephamycinase genes published in the meantime (13, 14, 17, 22, 25). Our results indicate that the plasmidic cephamycinases described so far are members of class C of β-lactamases (19). They can be subclassified by their degree of genetic relationship to *ampC* genes of either *Citrobacter freundii* (CMY-2, LAT-1, and BIL-1), *Enterobacter cloacae* (MIR-1), or *Pseudomonas aeruginosa* (MOX-1 and FOX-1).

Bacterial strains. *K. pneumoniae* HEL-1 is a cefoxitin-resistant isolate from a urine culture of a male patient suffering from pyelonephritis in 1990 in Athens, Greece. *Escherichia coli* C600 (MIC for nalidixic acid, 1,024 mg/liter) was the recipient strain to investigate transfer of resistance determinants from the *K. pneumoniae* wild-type. *E. coli* DH5α was the host for the cloning experiments. *E. cloacae* M6300 (24) was used as a reference strain for a β-lactamase with a pI of 8.8.

Antibiotics. Cefoxitin and imipenem (MSD Sharp & Dohme, Haar, Germany); clavulanate and temocillin (SmithKline Beecham Pharma, Munich, Germany); sulbactam (Pfizer, Karlsruhe, Germany); cefotetan and meropenem (Zeneca, Plankstadt, Germany); cefmetazole (Sankyo Europe, Düsseldorf, Germany); moxalactam (Eli Lilly, Bad Homburg, Germany); flomoxef (Shionogi & Co., Osaka, Japan); cefotaxime and cefpirome (Hoechst, Frankfurt am Main, Germany); ceftazidime (Cascan, Wiesbaden, Germany); ceftibuten (Schering-Plough Corp., Kenilworth, N.J.); aztreonam (Bristol-Myers Squibb, Munich, Germany); carumonam (Hoffmann-La Roche, Grenzach-Wyhlen, Germany); and piperacillin and tazobactam (Lederle, Wolfratshausen, Germany). Combinations of β-lactams with clavulanate, sulbactam, or tazobactam were used at proportions of 1 + 4, 1 + 1, or 1 + 7, respectively.

MICs. MICs were determined by an agar dilution technique with Mueller-Hinton agar (Difco, Ausburg, Germany). The inoculum was 10^4 CFU per spot 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 per ml of strain) were suspended in Mueller-Hinton broth (Difco) and incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Oxoid, Wesel, Germany) supplemented with nalidixic acid (64 mg/liter) to inhibit the growth of the donor strain and cefoxitin (64 mg/liter) to inhibit the growth of the recipient strain.

Plasmid DNA preparation. Plasmid preparation was performed by the alkaline lysis method (10). Plasmid DNA in the lysate was purified with an anion-exchange column (Qiagen, Hilden, Germany) according to the protocol of the supplier.

Isoelectric Focusing of β-lactamases. Crude homogenates of β-lactamases were prepared as described previously (5). For isoelectric focusing, the procedure described by Matthew et al. (20) was modified (5).

Assignment of the β-lactamase activity within the lane. After isoelectric focusing, the gel was covered with a tryptic soy agar (Difco) overlay containing cefoxitin (16 mg/liter), and the mixture was incubated for 2 h at 35°C. A second layer with *E. coli* (10^7 CFU/ml) susceptible to cefoxitin (MIC, 2 mg/liter) was then applied. After overnight incubation, visible growth at the spot on the gel where cefoxitin had been hydrolyzed allowed specific localization of the cephamycinase band.

Cloning and sequencing of the bla_{CMY-2} gene. A 3.2-kb *Sac*I-*Cla*I fragment was cloned into vector pSelect-1 according to the basic procedure described by Sambrook et al. (23). The DNA sequence of the insert (pMVP-2-1) was determined by dideoxy sequencing. The sequence reactions were performed with nested deletions.

Sequence analysis. Related β-lactamases were identified by comparison with the EMBL database (Fasta). Multiple alignment was calculated by Clustal V (15, 16).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL database under accession number X91840.

Antibiotic susceptibility. The MICs of selected β-lactams for the wild-type strain *K. pneumoniae* HEL-1, the transconjugant

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TABLE 1. Antibiotic susceptibilities of *K. pneumoniae* HEL-1, its transconjugant, its transformant, and the *E. coli* C600 recipient

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

E. coli C600 R^+, the transformant *E. coli* DH5α T^+ (pMVP-2), and the *E. coli* recipient strain are shown in Table 1. For all strains, the MICs of 7-methoxy-cephalosporins and of oxacephems are between 32 and 512 mg/liter, except for moxalactam (2 mg/liter). Reduction of the MICs of cefoxitin by β-lactamase inhibitors is weak for clavulanate (mostly one step of dilution) and is more expressed for sulbactam (three steps of dilution); the MIC-reducing effect of tazobactam is between those of clavulanate and sulbactam. The MICs of ceftazidime are four to eight times higher than those of cefotaxime. CMY-2 is active against both monobactams, the MICs of aztreonam being two to four times higher than those of carumonam. The MICs of moxalactam, cefpirome, and cefepime for the transconjugant and transformant strains are 16 times higher than the MICs for the *E. coli* recipient; however, the strains are considered susceptible to these three antibiotics as defined by the National Committee for Clinical Laboratory Standards (≤8 mg/liter for moxalactam and cefepime [21]; no data are available for cefpirome). The MICs of temocillin and carbapenems remain unchanged for CMY-2 producers, in comparison with nonproducers (*E. coli* C600 recipient).

Transfer of the plasmid carrying the bla_{CMY-2} gene. Transconjugants resistant to cefoxitin were selected at a frequency of 2.5×10^{-5} per donor cell. Resistance genes to the following non-β-lactams were cotransferred: chloramphenicol, tetracycline, sulfamethoxazole, trimethoprim, gentamicin, and tobramycin.

Identification of the plasmid carrying the bla_{CMY-2} gene. Plasmid preparation of the patient isolate *K. pneumoniae* HEL-1 contained plasmids of different sizes (Fig. 1). Only the largest of them was transferred to the *E. coli* C600 transconjugant strain.

Isoelectric focusing. On polyacrylamide gels run with crude homogenates, different bands were visualized by nitrocefin. The major band was found at a pI of higher than 8.8 (*E. cloacae* M6300), i.e., at approximately 9.0 (Fig. 2a).

Assignment of β-lactamase activity within the lane. Among the nitrocefin-hydrolyzing bands on the lanes of the polyacrylamide gel, the one with specific activity against cephamycins

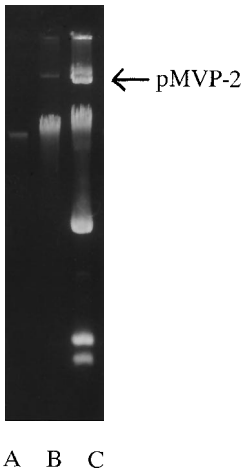

FIG. 1. Agarose gel electrophoresis of plasmid DNAs of *K. pneumoniae* HEL-1 (lane C), its transconjugant *E. coli* C600 R^+ (pMVP-2) (lane B), and the *E. coli* C600 R^- recipient strain (lane A). The transferable plasmid pMVP-2 is indicated by an arrow.

was identified by a bioassay (see above). Only at the pI 9.0 band was the *E. coli* strain susceptible to cefoxitin able to grow on account of previous hydrolysis of cefoxitin at this spot (Fig. 2b).

Analysis of the bla_{CMY-2} gene. The nucleotide sequence of a 3.2-kb fragment, which was cloned into vector pSelect, contained one large open reading frame of 1,146 nucleotides which corresponds to a putative protein of 381 amino acids (Fig. 3). Further analysis of the amino acid sequence revealed a relationship to class C β-lactamases. As expected for this class of β-lactamases, the active-site serine is located at position 64. Multiple sequence alignment of the amino acid sequence of CMY-2 with those of the other class C β-lactamases described so far (e.g., the plasmidic enzymes LAT-1 and BIL-1 and the chromosomal AmpC β-lactamases of *C. freundii* and *E. cloacae*) was performed (Fig. 4). The results of this analysis are shown in Table 2. This alignment demonstrates the closest relationship of the CMY-2 β-lactamase to be that to LAT-1

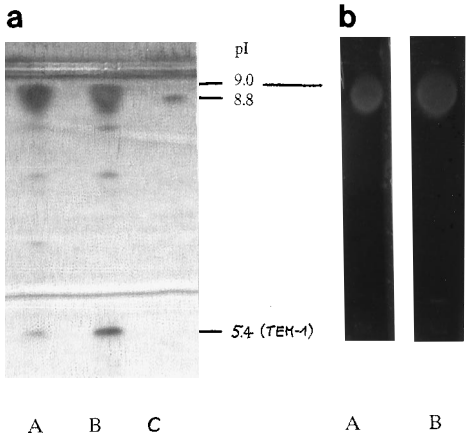

FIG. 2. Isoelectric point of β-lactamase CMY-2. Isoelectric focusing of the wild-type and transconjugant strains producing CMY-2 revealed several bands, the most distinct at a pI higher than 8.8, at about 9.0 (a). Only this band at pI 9.0 was able to hydrolyze cefoxitin, as shown by a bioassay (b). Lanes for panel a: A, *K. pneumoniae* HEL-1; B, *E. coli* C600 R^+; C, *E. cloacae* M6330 pI 8.8. Lanes for panel b: A, *K. pneumoniae* HEL-1; B, *E. coli* C6000 R^+.

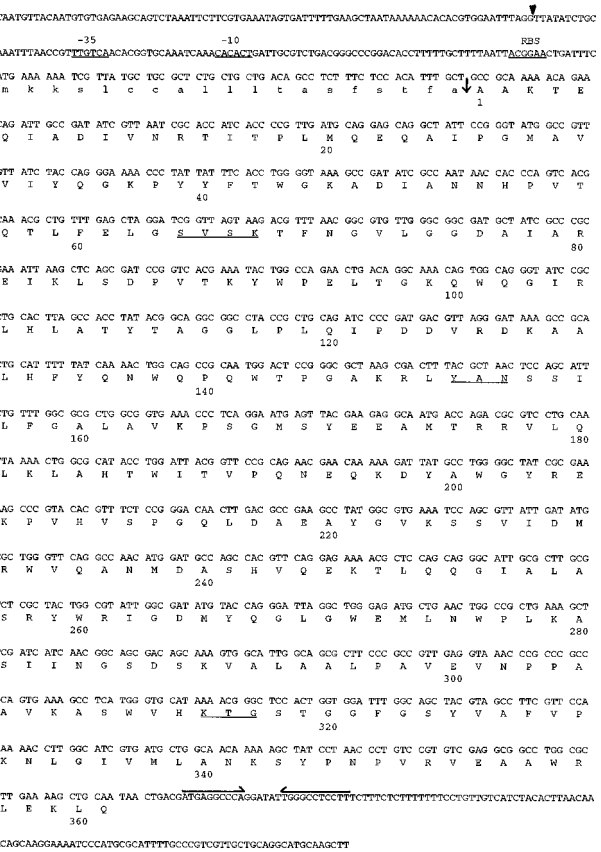

FIG. 3. Nucleotide sequence of the bla_{CMY-2} (pMVP-2-1) gene. The deduced amino acid sequence of CMY-2 is shown in the lines below the nucleotide triplets. Amino acids of the signal peptide are in lowercase letters; the putative cleavage site of signal peptidase is indicated by an arrow. The β-lactamase active site S-V-S-K (64 to 67), the conserved triad K-T-G (315 to 317), and the class C-typical motif Y-X-N (150 to 152) are underlined. Possible promoter (−35 and −10) and the ribosome-binding site (RBS) upstream the start codon are underlined. ▼ marks the end of sequence homology to *ampC* of *C. freundii*. A terminator hairpin following the stop codon is marked by two arrows.

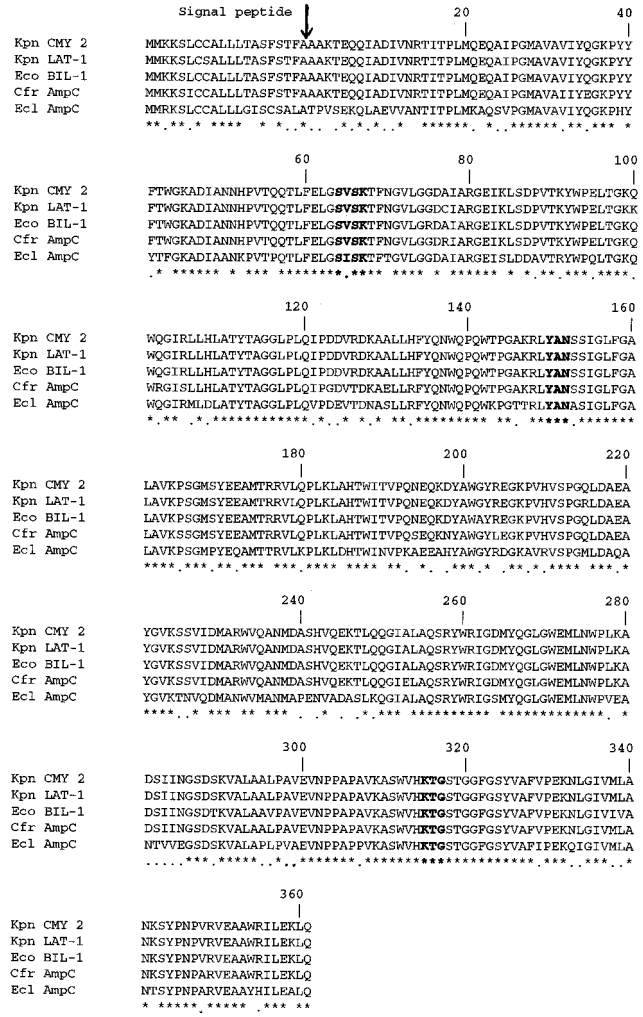

FIG. 4. Multiple sequence alignment of the amino acid sequences of CMY-2, LAT-1, BIL-1, and AmpC of *C. freundii* OS 60 and *E. cloacae*. Asterisks, identical amino acids; dots, conservative exchanges; boldface letters, conserved amino acid motives among which YAN is characteristic of class C β-lactamases. Kpn, *K. pneumoniae*; Eco, *E. coli*; Cfr, *C. freundii*; Ecl, *E. cloacae.*

(99.0% amino acid sequence homology [Ala for Cys at position 77, Gln for Lys at position 100, and Gln for Arg at position 215]), which is followed by BIL-1 (98.4% homology) and AmpC of *C. freundii* (95.8% homology for *C. freundii* OS 60 and 96.6% homology for *C. freundii* GN 346), while AmpC of *E. cloacae* shares only 75.1% homology with CMY-2.

TABLE 2. Percent homology of amino acid sequences of various class C β-lactamases (calculated by Clustal V)

	Homology (%)							
	MOX-1	FOX-1	*P. aeruginosa* AmpC	*C. freundii* OS 60 AmpC	CMY-2	LAT-1	BIL-1	*E. cloacae* AmpC
MOX-1		66.7	49.5	38.3	38.3	38.3	37.5	40.2
FOX-1			53.4	42.7	42.4	42.2	41.6	44.9
P. aeruginosa				41.8	41.6	41.1	41.3	44.0
C. freundii					95.8	95.0	94.2	73.3
CMY-2						99.0	98.4	75.1
LAT-1							97.4	74.3
BIL-1								73.5
E. cloacae								

Resistance to 7-methoxy-cephalosporins (cephamycins) in gram-negative rods is widely spread among species or genera which carry *ampC* genes in their chromosomes (e.g., *Enterobacter* species, *Citrobacter* species, *Serratia* species, *Providentia* species, and *P. aeruginosa*). The activities of these chromosomally encoded class C β-lactamases are not or are only weakly inhibited by the established β-lactamase inhibitors clavulanate, sulbactam, and tazobactam. This also holds true for some class B enzymes which hydrolyze cefoxitin (11). However, inhibitors active against class C β-lactamases were recently described (8, 9). Inhibition of class C β-lactamases by cloxacillin was observed previously (12) but was found not to be useful clinically. Therefore, resistance to cephamycins and inactivity of β-lactamase inhibitors established in therapy have usually been interpreted as indicators for the chromosomal location of the *bla* gene. This is still valid for the majority of cases. However, it may have contributed to obscure and postpone the detection of plasmidic cephamycinases, since screening for extended-spectrum β-lactamases by a disk test, in which extension of the inhibition zone around a β-lactam disk in the

neighborhood of clavulanate is evaluated, does not work satisfactorily with plasmidic class C β-lactamases.

The plasmidic cephamycinases described so far were characterized either by phenotypic markers only (e.g., spectrum of antibiotic resistance, isoelectric point, and enzyme kinetic parameters) or both by phenotypic and genotypic analyses. So far, no screening methods applicable routinely in a laboratory for clinical microbiology are available. Thus, the initial suspicion of a new enzymatic resistance comes only from the unusual resistance phenotype (e.g., cefoxitin resistance in *K. pneumoniae*). The plasmidic localization of the gene can be demonstrated by the transferability of the resistance gene; the classification may be confirmed by genetic analysis later on (as performed for CMY-2).

Comparison of the amino acid sequence of CMY-2 β-lactamase with those of other plasmidic cephamycinases clearly shows that all of them are closely related with chromosomal class C β-lactamases and may therefore have spread from the chromosome onto plasmids, e.g., with transposons as vehicles. In fact, we could demonstrate recently an R factor carrying a cephamycinase gene (CMY-2) in *C. freundii* which was transferable to *K. pneumoniae* and *E. coli*. This may indicate the natural route taken by chromosomal genes on their way to plasmids (2).

The plasmidic cephamycinases described so far—although belonging all to the class C β-lactamases—are nevertheless different. Their relationship among one another and to AmpC enzymes of various organisms allows their classification into three groups (C-1, C-2, and C-3), namely, the *C. freundii* group (C-1) with CMY-2, LAT-1, and BIL-1; the *P. aeruginosa* group (C-2) with MOX-1 and FOX-1; and the *E. cloacae* group (C-3), with MIR-1 being the only member identified thus far. For groups C-1 and C-3, the relationship between the plasmidic enzymes and the chromosomal β-lactamases is very close (more than 94% homology within the *C. freundii* group and about 90% homology within the *E. cloacae* group [22]; based on only a provisional 150-bp sequence near the C terminus of the MIR-1 β-lactamase). However, in group C-2, the plasmidic β-lactamases MOX-1 and FOX-1 show only about 50% homology to AmpC of *P. aeruginosa*; thus, direct evolution of these enzymes from *P. aeruginosa* AmpC may be questionable.

Only members of group C-1, the *C. freundii* group, have been observed in a larger number of pathogens over extended periods of time (3). Their occurrence has been limited so far to Europe (Greece and Turkey) and Asia (Pakistan).

It remains unclear whether this is due to the type of gene or plasmid or to some local epidemiological conditions. Nevertheless, infections caused by *K. pneumoniae* producing CMY-2 β-lactamase are no longer treatable by broad-spectrum generation cephalosporins, monobactams, combinations of them with β-lactamase inhibitors, or cephamycins. As shown, resistance genes for non-β-lactams may be linked to the *bla* gene in the same R factor, which further restricts the therapeutic alternatives. Furthermore, some of the strains are resistant to quinolones. Thus far, however, the activity of carbapenems remains unimpaired.

We conclude that there exists a reservoir in *K. pneumoniae* strains of plasmidic genetically related but distinct *bla* genes derived from *ampC* genes of either *C. freundii*, *P. aeruginosa*, or *E. cloacae*. Selective pressure may contribute to a wider spread of these multiresistant pathogens.

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