CMY-31 and CMY-36 Cephalosporinases Encoded by ColE1-Like Plasmids[▽]

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Two CMY-2 derivatives, CMY-31 (Gln²¹⁵→Arg) from *Salmonella enterica* serotype Newport and CMY-36 (Ala⁷⁷→Cys and Gln¹⁹³→Glu) from *Klebsiella pneumoniae*, were characterized. Both cephalosporinases functionally resembled CMY-2. *bla*_{CMY} alleles occurred as parts of a putative transposon comprising *ISEcp1B* and a *Citrobacter freundii*-derived sequence carried by ColE1-like plasmids similar to CMY-5-encoding pTKH11 from *Klebsiella oxytoca*.

Various plasmid-mediated cephalosporinases, mainly from enterobacteria, have been described. Producers of these enzymes are commonly resistant to penicillins, penicillin-inhibitor combinations, narrow-spectrum cephalosporins, and cefoxitin. Activities of expanded-spectrum cephalosporins (ESCs) are also compromised depending on the amount of cephalosporinase (3, 17). Plasmid-mediated cephalosporinases are classified into five evolutionary groups, the most widespread being the group of *Citrobacter freundii*-derived enzymes, including CMY-2 and more than 30 variants (17; www.lahey.org/Studies/). The *bla*_{CMY-2} alleles are encountered in both hospital- and community-acquired enterobacteria worldwide. Notably, they have emerged also in nontyphoid salmonellae, including *Salmonella enterica* serotype Newport, currently spreading epidemically in the United States (9, 14, 17). The important role of IncA/C and IncI plasmids in the diffusion of CMYs has been documented (10). A CMY-5-encoding ColE1-like plasmid has also been described (24). We report here on two CMY variants, CMY-31 and CMY-36, encoded by ColE1-like plasmids.

CMY-producing microorganisms. Serotype Newport AM17274 was submitted to the National Antibiotic Resistance Monitoring System (CDC, Atlanta, GA) in 2003 from Nevada. *Klebsiella pneumoniae* HP205 was recovered in a hospital in Athens, Greece, in 2005. β -Lactam MICs, determined by agar dilution, indicated cephalosporinase production, i.e., resistance to penicillins, penicillin-inhibitor combinations, cefoxitin, and ESCs and susceptibility to cefepime and imipenem (Table 1). Isolates exhibited resistance also to sulfonamides and streptomycin. *K. pneumoniae* HP205 was, additionally, resistant to trimethoprim, gentamicin, and tobramycin. Isolates were positive in a *bla*_{CMY-2}-specific PCR (6) and produced β -lactamases with alkaline isoelectric points (pIs > 8.5 as determined by isoelectric focusing) that, most likely, represented the CMY cephalosporinases. *K. pneumoniae* HP205 also pro-

duced a β -lactamase with a pI of 7.6, consistent with the resident β -lactamase of this species.

Characterization of CMY-31 and CMY-36. Plasmids from AM17274 and HP205 were isolated with a Midi kit (Qiagen, Hilden, Germany) and introduced into *Escherichia coli* DH5 α by transformation, yielding cephalosporin-resistant clones (Table 1) containing *bla*_{CMY-2}-type genes located in similar plasmids, pA172 from serotype Newport and pH205 from *K. pneumoniae*. Entire plasmid sequences were determined on both strands by a primer walking approach using an API377 sequencer (Applied Biosystems, Foster City, CA). Sequencing data showed that the *bla*_{CMY} gene in pA172 differed by a single nucleotide from *bla*_{CMY-2} (GenBank accession no. X91840), resulting in a Gln²¹⁵→Arg substitution. An identical gene (*bla*_{CMY-31}) has recently been identified in *K. pneumoniae* in Switzerland (GenBank accession no. EF622224) (1). In pH205 the respective gene differed from *bla*_{CMY-2} by 7 nucleotides (nt), and the putative novel cephalosporinase (CMY-36) would include the substitutions Ala⁷⁷→Cys and Gln¹⁹³→Glu compared to CMY-2. Each CMY protein comprised 381 amino acids, including a 20-amino-acid putative signal peptide. The calculated pIs (9.02 for CMY-31 and 8.94 for CMY-36) were in accordance with those determined by isoelectric focusing (9.2 and 9.0, respectively). Based on the structure of the *Enterobacter cloacae* 908R β -lactamase (PDB entry 1Y54) (13), it seems that Arg²¹⁵ is located in the Ω loop that is involved in the interaction with β -lactam substrates (15). Both substitutions in CMY-36 occur at positions distant from the active site cavity (Fig. 1).

The entire *bla*_{CMY}-type genes from pA172 and pH205, along with the *ISEcp1B*-located promoter (8), were amplified by PCR using the primers ecp-F2 (5'-CGGAATTCGTGCTCTGTGGATAACTTG-3') and ampC2 (5'-CGGAATTCCTGCAAGCTTTTCAAGAATGCGC-3') (nt 2084 to 2103 and 3496 to 3517, respectively [GenBank accession no. EU331425 and EU331426, respectively]), which also contained EcoRI restriction sites (underlined). Amplicons (1,434 bp) were restricted with EcoRI and ligated into the high-copy-number vector pBCSK(+) (Stratagene, La Jolla, CA), resulting in plasmids pB-cmy31 and pB-cmy36. Plasmid pB-cmy31 was utilized to construct a similar *bla*_{CMY-2}-carrying plasmid (pB-cmy2) by

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TABLE 1. β -Lactam susceptibility of the CMY-producing clinical isolates serotype Newport AM17274 and *K. pneumoniae* HP205 and *E. coli* DH5 α clones carrying the wild-type plasmids pA172 and pH205 and the respective CMY-encoding recombinant plasmids

β -Lactam(s)	MIC (μ g/ml) for:							
	AM17274 (CMY-31)	HP205 (CMY-36)	DH5 α /pA172 (CMY-31)	DH5 α /pH205 (CMY-36)	DH5 α /pB-c31 (CMY-31)	DH5 α /pB-c36 (CMY-36)	DH5 α /pB-c2 (CMY-2)	DH5 α /pBCSK+ (— ^c)
Amoxicillin	>256	>256	>256	>256	>256	>256	>256	2
Amoxicillin + CL ^a	>64	>64	>64	>64	>64	>64	>64	2
Ticarcillin	>256	>256	>256	>256	>256	>256	>256	2
Piperacillin	64	128	128	128	256	256	256	1
Piperacillin + TZ ^b	16	32	32	32	64	64	64	0.5
Cefoxitin	>256	>256	>256	>256	>256	>256	>256	4
Cefuroxime	>256	>256	>256	>256	>256	>256	>256	2
Ceftazidime	32	64	128	128	256	256	256	0.5
Cefotaxime	32	32	32	32	128	128	128	0.12
Ceftriaxone	16	32	32	64	256	128	128	0.12
Cefepime	0.12	0.12	0.12	0.25	0.25	0.25	0.25	$\leq$ 0.06
Imipenem	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.12

^a CL, clavulanate at a fixed concentration of 2 μ g/ml.

^b TZ, tazobactam at a fixed concentration of 4 μ g/ml.

^c —, no CMY.

site-directed mutagenesis with a QuikChange mutagenesis kit (Stratagene) and the mutagenic primers cmF (5'-TTTC TCCGGGACAACTTGACGCCGA-3') and cmR (5'-TCG GCGTCAAGTTGTCCCGGAGAAA-3') (nt 3066 to 3090 in EU331425; G→A [underlined] in codon 215, resulting in Arg→Gln). Mutations were verified by sequencing at least two independently amplified PCR products.

Plasmids pB-cmy31, pB-cmy36, and pB-cmy2 were introduced into *E. coli* DH5 α . β -Lactam MICs showed that the three CMY variants conferred comparable levels of resistance to cefotaxime, ceftazidime, ceftriaxone, piperacillin, and

piperacillin-tazobactam. MICs of ampicillin, amoxicillin (amoxicilline)-clavulanate, ticarcillin, and cefoxitin, however, exceeded the range of dilutions tested. The strains were susceptible to cefepime and imipenem (Table 1).

Substrate and inhibition profiles of the CMY enzymes were also determined. Cell suspensions of the *E. coli* DH5 α clones carrying pB-cmy31, pB-cmy36, and pB-cmy2 were sonicated, and extracts were clarified by ultracentrifugation. Cephalosporinase purification was performed by two ion-exchange chromatography steps using Q- and S-Sepharose (16). Purity of the preparations was >90%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Kinetic parameters for penicillin G, ampicillin, cephaloridine, cephalothin (cefalotin), nitrocefin, and cefotaxime hydrolysis were studied by spectrophotometry (16). Interaction of CMY-31 and CMY-36 with β -lactam substrates was typical for enterobacterial AmpCs (i.e., rapid hydrolysis of early-generation cephalosporins and significantly lower rates of hydrolysis of penicillins and oximino-cephalosporins). Kinetic parameters of the two CMY enzymes did not differ significantly from those of CMY-2, determined in parallel (Table 2). Inhibitory activities of cloxacillin, aztreonam, and Ro 48-1220 against CMY-31, CMY-36, and CMY-2 were studied by UV spectrophotometry using cephalothin (100 μ M) as a reporter substrate. Results were expressed as 50% inhibitory concentrations (IC₅₀s). Inhibition profiles for the three CMY variants were similar. Both cloxacillin and aztreonam were potent inhibitors (IC₅₀s were 0.004 and 0.006 to 0.007 μ M, respectively). IC₅₀ values for Ro 48-1220 ranged from 0.3 to 0.35 μ M. Taken together with the similar MICs under isogenic conditions, these data indicated that CMY-31 and CMY-36 are functionally similar to CMY-2.

Characteristics of CMY-encoding plasmids. pA172 and pH205 each comprised 8,197 bp and differed at 33 and 40 nt positions, respectively, from pTKH11 (GenBank accession no. Y17716), a ColE1-like *bla*_{CMY-5}-carrying plasmid from *K. oxytoca* isolated in Sweden in 1991 (24). Sequence annotation for pA172 and pH205 (GenBank accession no. EU331425 and EU331426) complements that proposed for pTKH11 (24) by

FIG. 1. Ribbon diagram based on the AmpC from *E. cloacae* 908R (PDB entry 1Y54) (13), indicating the likely locations of amino acid residues Arg²¹⁵ (CMY-31) and Cys⁷⁷ and Glu¹⁹³ (CMY-36) in relation to the active-site cavity indicated by the conserved residues Ser⁶⁴, Tyr¹⁵⁰, and Lys³¹⁵. The image was made using the DeepView/Swiss-Pdb viewer, version 3.7, available at www.expasy.org/spdbv/.

TABLE 2. Kinetic parameters of CMY-type cephalosporinases for various β -lactam substrates^a

Substrate	CMY-31			CMY-36			CMY-2		
	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)
Penicillin G	7.7 $\pm$ 1.0	0.3 $\pm$ 0.04	25.7	9.1 $\pm$ 1.2	0.3 $\pm$ 0.05	30.3	10 $\pm$ 1.5	0.4 $\pm$ 0.05	25.0
Ampicillin	0.8 $\pm$ 0.1	0.1 $\pm$ 0.01	8.0	1.1 $\pm$ 0.2	0.1 $\pm$ 0.01	11.0	0.9 $\pm$ 0.1	0.1 $\pm$ 0.01	9.0
Cephaloridine	310 $\pm$ 30	150 $\pm$ 20	2.1	360 $\pm$ 30	150 $\pm$ 14	2.4	300 $\pm$ 40	120 $\pm$ 20	2.5
Cephalothin	150 $\pm$ 12	5.1 $\pm$ 0.3	29.4	140 $\pm$ 15	4.4 $\pm$ 0.5	31.8	160 $\pm$ 10	5.0 $\pm$ 0.2	32.0
Nitrocefin	960 $\pm$ 40	10 $\pm$ 1.1	96.0	910 $\pm$ 30	7.3 $\pm$ 1.0	125	980 $\pm$ 40	10 $\pm$ 1.5	98.0
Cefotaxime	<0.01	— ^b	—	<0.01	—	—	<0.01	—	—

^a Values for k_{cat} and K_m are means $\pm$ standard deviations of five independent measurements.

^b —, not determined.

including recently characterized *ISEcp1B* (18) as well as the *oriT* and *cer* regions.

Each plasmid carried an *ISEcp1B*-associated *C. freundii*-derived sequence of 2,088 bp (*bla*_{CMY}, *bic* [encoding lipocalin], and *sugE* [encoding a small multidrug resistance protein]) flanked by 5-bp target site duplications (5'-GATTA-3'; nt 604 to 608 and 4634 to 4638 in EU331425 and EU331426) and inserted into a ColE1-like backbone of 4,167 bp. An *oriV* (nt 1599 to 1601) was located 40 bp from the regulatory elements RNA II (511 bases) and RNA I (105 bases) (4). ORF7 (471 bp), downstream of RNA I, resembled a remnant (*mobB*) of the *mob* gene array of ColE1 (23). ORF6 (594 bp), of unknown function, was located between *mobB* and the CMY-encoding element. An AT-rich 227-bp sequence (nt 7094 to 7311) exhibiting 81% homology with the transfer origin (*oriT*) from pIP843 and pJHCMW1 (5, 20) and a 266-bp sequence (nt 7964 to 8182) 95% homologous to the *cer* (ColE1 resolution) recombination site implicated in plasmid monomerization and stability (22) were also identified (Fig. 2).

To test their mobilization capability, plasmids pA172 and

pH205 were introduced into *E. coli* XL1-Blue, which harbors fertility factor F', an IncFIA conjugative plasmid. The resulting transformants were able to transfer the CMY-encoding plasmids to *E. coli* DH5a during conjugation at a relatively low frequency (10^{-7} *bla*_{CMY}-positive clones per donor cell).

Conclusions. Most plasmid-mediated *bla*_{CMY} genes have likely originated from *bla*_{CMY-2} through point mutations, leading to the present array of CMY variants (2). CMY-31 and CMY-36 exhibit activities similar to that of CMY-2. Therefore, the respective amino acid changes, including Gln²¹⁵→Arg in the Ω loop, are probably not associated with selective pressure exerted by drugs such as ESCs. The common-origin hypothesis of the plasmid-borne *bla*_{CMY} alleles is also in line with the similarity of their genetic environments. The elements from the ColE1-like plasmids, as well as a similar CMY-2-encoding transposon from an IncI plasmid (21), occur as shorter versions of *ISEcp1B*-associated sequences from a variety of plasmids (8, 10, 11). It can be hypothesized that these transposon-like sequences are derived from a common element through alterations in the right-hand end that may arise during *ISEcp1B*-mediated transposition (18, 19). Nevertheless, an alternative evolutionary route, such as multiple transposition events in a *bla*_{CMY}-containing sequence from a single *C. freundii* strain, cannot be ruled out.

A possible role for a ColE1-like plasmid in the global spread of CMYs is also indicated. This plasmid species was probably formed through transposition of a *C. freundii*-originating sequence into a ColE1 replicon and diverged by point mutations. It was first identified in clinical *Enterobacteriaceae* in the early 1990s in Sweden (24) and Greece (7; our unpublished data) and, as indicated here, persists in the latter country. Notably, the CMY-encoding ColE1-like plasmid has also been acquired by an important food-borne pathogen, *Salmonella* serotype Newport. ColE1 can be mobilized by plasmids with apparently different conjugation systems such as InFI, IncI1, and IncP. Mobilization efficiency depends on the proteins encoded by the *mob* genes of ColE1 (23). Nevertheless, there have been examples of mobilization of ColE1 derivatives, in which, as in the ones described here, the full array of *mob* genes are deleted. The presence of an *oriT* sequence may play a role in this process (12). Whichever the mechanism, the in vitro mobilization shown here and the carriage by different bacterial species underscore the spreading potential of these CMY-encoding ColE1-like plasmids.

FIG. 2. Genetic organization of the ColE1-like plasmids pA172 from *Salmonella* serotype Newport and pH205 from *K. pneumoniae* encoding CMY-2-type cephalosporinases. Arrows show directions of transcription of open reading frames and regulatory elements. Locations of the ColE1 sequences *oriV*, *oriT*, and *cer* are indicated. The gene *mobB* was reported as ORF7 in the CMY-5-encoding plasmid pTKH11 (21). Target site duplications (TSDs) at the boundaries of the CMY-encoding transposon and the respective inverted repeats (IRR and IRL) are also shown.

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