CMY-13, a Novel Inducible Cephalosporinase Encoded by an *Escherichia coli* Plasmid

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An IncN plasmid (p541) from *Escherichia coli* carried a *Citrobacter freundii*-derived sequence of 4,252 bp which included an *ampC-ampR* region and was bound by two directly repeated IS26 elements. *ampC* encoded a novel cephalosporinase (CMY-13) with activity similar to that of CMY-2. *AmpR* was likely functional as indicated in induction experiments.

The most widespread plasmid-mediated AmpC β -lactamases among enterobacteria are those derived from the chromosomal cephalosporinases of *Citrobacter freundii*. The respective *bla* genes are carried by various plasmids that may differ in size, self-transfer capability, and antibiotic resistance patterns (15). Recently, we reported on a self-transferable, multiresistant plasmid of 50 kb (p541) from *Escherichia coli* that encoded an AmpC-type enzyme and the VIM-1 metallo- β -lactamase. The *bla*_{VIM-1} gene was located in a class 1 integron (In-e541) (12). The presence of two *bla* genes encoding potent β -lactamases in the same plasmid prompted us to further characterize p541.

Sequencing of the *ampC*-carrying region. Plasmid p541 was transferred by transformation into *E. coli* DH5 α . Replicon typing was performed by Southern blot hybridization using *inc-rep* probes specific for the major incompatibility groups as previously described (4). Plasmid p541 reacted with only the *repN* probe and was consequently assigned to the IncN group.

Fragments of p541, produced after partial digestion with HindIII, were ligated into the chloramphenicol-resistant phagemid pBC-SK(+) (Stratagene), and the resulting plasmids were used to transform *E. coli* DH5 α . A recombinant plasmid (p541-H) with a 10,850-bp insert conferred the antibiotic resistance pattern of p541. Based on the sequence of In-e541 (12), the insert was further restricted by PvuII yielding a fragment of 7,021 bp (Fig. 1). A pBC-SK(+) derivative containing the latter fragment (p541-HP) conferred a cephalosporinase phenotype (Table 1).

The sequence of the HindIII-PvuII insert was determined on both strands by a primer walking approach using an ABI PRISM 377 sequencer (Applied Biosystems). It included 4,252 bp that resembled a segment from *C. freundii* OS60 (GenBank accession no. U21727) containing seven open reading frames (ORFs) (Fig. 1). The first two ORFs encoded putative polypeptides identified as SugE (105 amino acids $\rightleftharpoons$, 100% identity) and Blc (177 aa, 98.3% identity) from *C. freundii* (accession no. U21727). The third ORF (1,143 bp) exhibited >92%

homology with the *ampC* genes of *C. freundii* and the respective plasmidic cephalosporinases (1). The encoded polypeptide (381 aa) possessed the characteristic motifs of class C β -lactamases (14) and was most similar to the plasmid-mediated CMY-2 (97.4% identity, 98.2% similarity) (3) and the AmpC of *C. freundii* GN346 (96.5% identity, 98.6% similarity) (accession no. X91840 and D13207, respectively). The calculated isoelectric point (pI) of the putative mature polypeptide (361 aa) was 9.1, in agreement with that determined by isoelectric focusing (pI of 9.0) (12). This novel AmpC variant was designated CMY-13. An ORF of 873 bp, showing an opposite direction of transcription, was identified upstream of *bla*_{CMY-13}. The putative polypeptide (291 aa) differed by eight residues (97% identity) from the AmpR of *C. freundii*, the transcriptional regulator of *ampC* expression (accession no. AY125469) (10). None of these differences was located in the helix-turn-helix region or in other positions important for AmpR function (reviewed in reference 7). The *bla*_{CMY-13}-*ampR* intercistronic sequence was identical to that of *C. freundii* OS60 (accession no. U21727). The remaining three ORFs were identified as *frdD*, *frdC*, and a 5'-end-deleted version of *frdB* (Δ *frdB*) constituting part of the fumarate operon that is adjacent to *ampC-ampR* in *C. freundii*.

Comparison of CMY-13 with CMY-2. CMY-13 and CMY-2 were expressed under isogenic conditions. p541-HP and the *bla*_{CMY-2}-carrying plasmid pMEL (1, 6) were used as templates in PCR with primers P1 (5'-CCGGAATTCTAAGTGTAGATGACAACAGGAAAA-3') and P2 (5'-CCGGAATTCTTATATCTGCTGCTAAATTTAACCG-3') containing EcoRI restriction sites (shown in boldface type). Amplicons (1,324 bp) comprising identical parts of the *ampC-ampR* intercistronic region and the respective *bla*_{CMY} genes were restricted with EcoRI and ligated into pBC-SK(+). The resulting plasmids, pCMY-13 and pCMY-2, were introduced into *E. coli* DH5 α . Determination of MICs by an agar dilution method (14) showed that CMY-13 and CMY-2 conferred comparable resistance levels to β -lactams, suggesting similar substrate specificities (Table 1).

Inhibition profiles were compared by using tazobactam and Ro 48-1220 as inhibitors and nitrocefin as a reporter substrate

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FIG. 1. Schematic representation of the HindIII-PvuII fragment of plasmid p541 including a *C. freundii* chromosomal fragment encompassed by IS26 elements. Arrows indicate the direction of transcription. This structure is included in the sequence presented for GenBank accession no. AY339625. ITR_R, right ITR; ITR_L, left ITR.

as described previously (18). Tazobactam 50% inhibitory concentration values for CMY-13 and CMY-2 were 10.5 and 12 μ M, respectively. The 50% inhibitory concentration of Ro 48-1220 was 0.7 μ M for both enzymes. Therefore, CMY-13 was considered to be functionally equivalent with the plasmid-mediated, *C. freundii*-derived AmpC variants represented by CMY-2 (1).

Induction of CMY-13. The presence of an intact *ampC-ampR* in p541 implied that CMY-13 production could be inducible. To test this hypothesis, *E. coli* DH5 α cells carrying plasmids p541, p541-H, and p541-HP were used. An *E. coli* DH5 α (p541) transformed with pNH5, a plasmid encoding AmpD from *Enterobacter cloacae* (8), was also used. Induction was performed by adding imipenem at 1 μ g/ml in broth cultures 3 h before harvesting the cells. β -Lactamases were extracted by ultrasonic treatment. The protein concentration was determined by a protein assay kit (Bio-Rad). Hydrolysis of nitrocefin was assessed by spectrophotometry. VIM-1 β -lactamase was blocked by adding EDTA in the respective extracts at a final concentration of 1 mM. Preliminary testing with *E. coli* DH5 α (p541-HP) extracts had indicated that the use of EDTA as described above did not significantly affect cephalosporinase activity.

Induction of *E. coli* DH5 α (p541) caused a 3.5-fold increase in cephalosporinase activity. Lower levels of induction were observed for the clones harboring p541-H and p541-HP (2.0- and 1.7-fold increase, respectively). Also, the transformation of *E. coli* DH5 α (p541) with pNH5 resulted in lower basal amounts

of AmpC which were marginally induced by imipenem (1.2-fold increase) (Table 2). AmpD processes mucopeptides that act as cofactors for AmpR activation of *ampC* transcription (7). Increased AmpD activity is expected to reduce AmpC production in the presence of a functional AmpR. These results indicated that *ampR* in p541 was likely functionally contributing to the regulation of CMY-13 production. Plasmid-mediated, inducible cephalosporinases from *Morganella morganii* (DHA-1 and DHA-2) and *Enterobacter* (ACT-1) have been reported previously (2, 5, 16). In this study, the plasmidic location of a functional *ampC-ampR* system originating from *C. freundii* is described for the first time.

Genetic environment of the *C. freundii*-derived sequence. The *C. freundii*-derived segment was bounded by two directly repeated IS26 elements (Fig. 1). Each 820-bp-long IS26 comprised a transposase gene (*tnpA*) (705 bp) flanked by characteristic inverted terminal repeats (ITRs) (11, 13). The left-hand IS26 was preceded, up to the HindIII site, by a 755-bp sequence identical to the 5' end of an *aphA7* gene (9). *aphA* alleles code for aminoglycoside phosphotransferases and occur as parts of various IS26-containing transposons (17, 21). The right-hand IS26 was inserted within *frdB*, causing its deletion at the 5' end. The sequence immediately after the right-hand ITR corresponded to the 5'CS of the VIM-1-encoding integron (12). The 5' conserved sequence (CS) lacked the first 113 bp of the common 5' end due to the IS26 insertion.

Although the above-described structure resembles a composite transposon, the absence of target site duplications sug-

TABLE 1. MICs of β -lactam antibiotics

Antibiotic ^a	MIC (μ g/ml) for <i>E. coli</i> DH5 α strains carrying plasmid (β -lactamase[s])						pBC-SK (+)
	p541 (CMY-13 + VIM-1)	p541 + pNH5 (CMY-13 + VIM-1)	p541-H (CMY-13 + VIM-1)	p541-HP (CMY-13)	pCMY-13 (CMY-13)	pCMY-2 (CMY-2)	
AMP	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	2
TIC	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	4
PIP	512	256	512	$\geq 1,024$	512	512	1
TZP	256	128	256	128	128	128	1
CXM	$\geq 1,024$	$\geq 1,024$	$\geq 1,024$	512	$\geq 1,024$	512	4
FOX	$\geq 1,024$	512	$\geq 1,024$	512	$\geq 1,024$	$\geq 1,024$	4
CTX	128	64	256	256	256	256	0.06
CRO	256	256	256	256	512	512	≤ 0.03
CAZ	512	256	512	256	512	256	0.12
FEP	64	32	64	1	2	1	≤ 0.03
CPO	64	64	64	2	2	2	0.06
ATM	2	1	8	64	64	32	0.06
IPM	8	8	8	0.25	0.5	0.5	0.12
MEM	1	1	1	≤ 0.03	0.06	0.06	≤ 0.03

^a AMP, ampicillin; TIC, ticarcillin; PIP, piperacillin; TZP, piperacillin-tazobactam (inhibitor fixed at 4 μ g/ml); CXM, cefuroxime; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; FEP, cefepime; CPO, ceftiofime; ATM, aztreonam; IPM, imipenem; MEM, meropenem.

TABLE 2. Induction of β -lactamase activity in CMY-13-producing strains

Strain	Cephalosporinase activity (U/mg of protein) ^a		
	Uninduced	Induced	Inducibility (fold)
<i>E. coli</i> DH5 α (p541) ^b	1.04	3.62	3.5
<i>E. coli</i> DH5 α (p541, pNH5) ^b	0.63	0.76	1.2
<i>E. coli</i> DH5 α (p541-H) ^b	2.49	5.05	2.0
<i>E. coli</i> DH5 α (p541-HP)	3.19	5.52	1.7
<i>E. coli</i> DH5 α	<0.01	ND ^c	

^a One unit of activity was defined as 1 μ mol of substrate hydrolyzed per min. Each value was the mean of three independent measurements not differing more than 10%.

^b Extracts were pretreated with EDTA as described in the text.

^c ND, Not determined.

gests that the two elements might be inserted independently. Notably, a *bla*-associated IS26 located in the 5' CS of a class 1 integron as in p541 has been described in three additional multiresistant plasmids: pSEM (an IncL/M plasmid from *Salmonella enterica* serovar Typhimurium encoding SHV-5; GenBank accession no. AJ245670) (19), p1658/97 (an IncF plasmid from *E. coli* also encoding SHV-5; accession no. AF550679), and pAK33 (an *E. coli* plasmid encoding IBC-1 β -lactamase; accession no. AY260546) (20). No marked target selectivity has been described for IS26 (11). It can be hypothesized that the IS26- Δ 5' CS sequence was part of a common mobile structure which spread among distinct plasmids and evolved through the acquisition of gene cassettes and IS26-mediated transposition or recombination events.

Nucleotide sequence accession number. The described nucleotide sequence has been deposited in GenBank under accession no. AY339625.

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