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Plasmid-Encoded ACC-4, an Extended-Spectrum Cephalosporinase Variant from *Escherichia coli*▽

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ACC-4, an omega loop mutant (Val^{211}→Gly) of the *Hafnia alvei*-derived cephalosporinase ACC-1, was encoded by an *Escherichia coli* plasmid. The genetic environment of bla_{ACC-4} shared similarities with plasmidic regions carrying bla_{ACC-1}. Kinetics of β-lactam hydrolysis and levels of resistance to β-lactams showed that ACC-4 was more effective than ACC-1 against expanded-spectrum cephalosporins.

A variety of plasmid-determined β-lactamases belonging to Ambler class C (cephalosporinases) have been identified for members of *Enterobacteriaceae*, mainly *Klebsiella pneumoniae*, *Escherichia coli*, and nontyphoid-causing *Salmonella*. Based on sequence similarities to species-specific AmpC enzymes, plasmidic cephalosporinases are classified into five evolutionary groups, the most widespread being the group of *Citrobacter freundii*-originating CMY/LAT variants. The remaining groups include enzymes related to the chromosomal cephalosporinases of *Enterobacter cloacae* (MIR-1 and ACT-1), *Morganella morganii* (DHA variants), *Hafnia alvei* (ACC-1), and *Aeromonas* spp. (FOX, MOX, and various CMY enzymes) (18).

Plasmid-encoded CMY/LAT cephalosporinases have been repeatedly identified among clinical enterobacteria in Greece (5, 25). A MOX-2-producing *K. pneumoniae* strain that originated in a Greek hospital has also been reported (21). In a previous screening for acquired *bla* genes among oxyimino-cephalosporin-resistant *E. coli* isolates in Athens hospitals, an ACC producer (*E. coli* EC-3521r) was detected for the first time. *E. coli* EC-3521r was isolated in 2002 from a urine sample from a patient treated in a general hospital. ACC was encoded by pR3521, a self-transferable plasmid of approximately 80 kb that also mediated production of TEM-1 and a novel carbenicillinase designated SCO-1 (17). It is shown here that the detected ACC-type β-lactamase was a point mutant of ACC-1 with increased activity against expanded-spectrum cephalosporins.

Identification of bla_{ACC-4}. The ACC-encoding plasmid pR3521 was extracted from an *E. coli* K-12(pR3521) transconjugant clone (17) using a Nucleobond BAC100 kit (Macherey-Nagel, Duren, Germany) and partially digested with Sau3A. Fragments were ligated into the BamHI site of pBCSK(+) (Stratagene, La Jolla, CA), and recombinant plasmids were introduced into *E. coli* MC4100 (Δ*ampC*) (10) by electroporation. Clones were selected with ampicillin (40 μg/ml) and chloramphenicol (20 μg/ml) and screened for bla_{ACC} by PCR using the bla_{ACC}-specific primers a-F1 (5′-GACACCGTTGATGACCTGAT-3′) and a-R1 (5′-CACCGAAGCCGTTAGTTGAT-3′) (4). A recombinant plasmid of approximately 5.9 kb (pS18-d) with a bla_{ACC} insert was identified. Plasmid pS18-d was purified using a QIAGEN Plasmid Midi kit (QIAGEN, Hilden, Germany), and the bla_{AAC}-containing insert was sequenced on both strands using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). The insert was 2,479 bp long and included a bla_{ACC} gene (1,161 bp) that differed from bla_{ACC-1} (1) in a T-to-G transversion (nucleotide [nt] 1,350 in sequence under GenBank accession no. AJ133121), resulting in a Val→Gly substitution at position 211 of ACC-1 (numbering is as in reference 1). This novel *ampC* variant was designated bla_{ACC-4} (nt 1,995 to 3,155 in the sequence under GenBank accession no. EF504260). The likely secretory signal sequence of the 387-amino-acid-long ACC-4 polypeptide comprised 23 residues. The mature ACC-4 β-lactamase (364 amino acids; molecular weight, 39,673) had an apparent isoelectric point (pI) of 7.8 as determined by analytical isoelectric focusing of cell extracts from *E. coli*(pS18-d) in ampholine-containing polyacrylamide gels (pH range, 3.5 to 9.5) and using β-lactamases with known pIs as controls (TEM-1, 5.4; TEM-3, 6.3; SHV-3, 7.0; SHV-1, 7.6; SHV-5, 8.2) (data not shown). This pI corresponded to the values reported for ACC-1 and the chromosomal cephalosporinase of *H. alvei* (1, 7).

Genetic environment of bla_{ACC-4}. The regions flanking bla_{ACC-4} were determined by sequencing of the entire insert of pS18-d, as well as PCR mapping of pR3521 with primers used for the characterization of ACC-1-encoding plasmids (4) and sequencing of the respective amplicons (Fig. 1). An intact glutamate dehydrogenase gene (GDHA gene) of 915 bp was located downstream of bla_{ACC-4} in the opposite orientation. The remaining left-hand sequence comprised a 175-bp segment homologous to an internal part of *tnpA* from Tn*2* (GenBank accession no. AY123253) and an IS*26* element. At an 87-bp distance upstream of bla_{ACC-4}, a sequence of 1,306 bp corresponding to an IS*Ecp*1 element was identified. A putative promoter for bla_{ACC-4} was located within IS*Ecp*1 as in the ACC-1-encoding plasmids (4). This segment shared extensive homology with the bla_{ACC-1}-carrying region of pSLK54, representing a group of similar plasmids spread among *K. pneumoniae* and *Proteus mirabilis* in French hospitals (4, 14) (Fig. 1). Additionally, both pR3521 and pSLK54 carried bla_{SCO-1}, a

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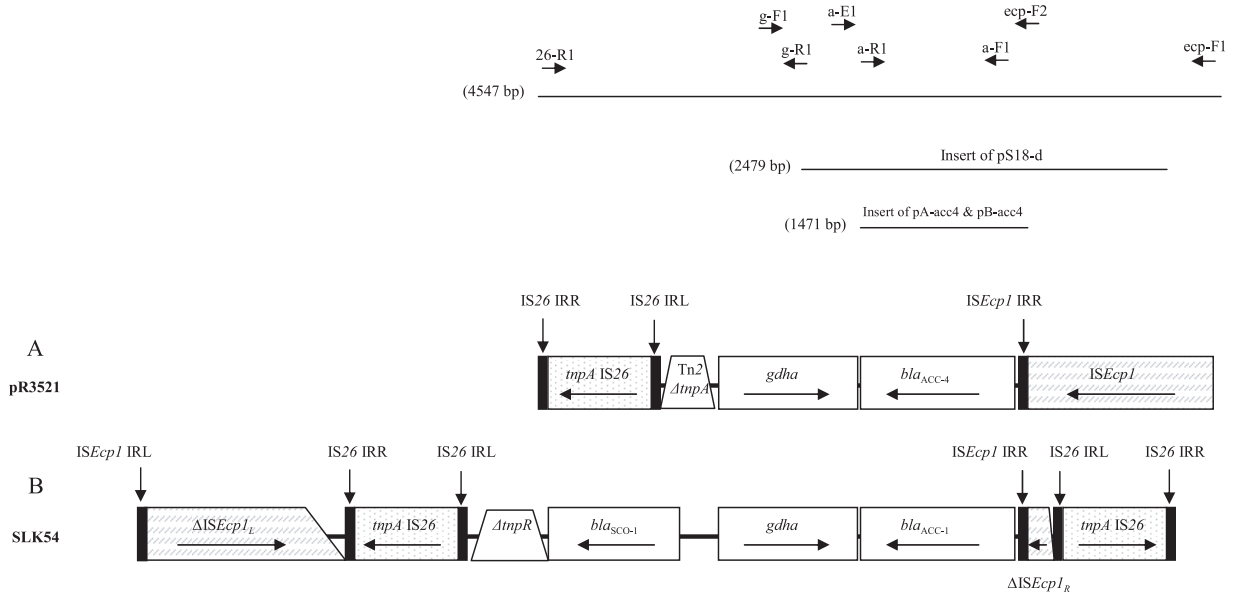

FIG. 1. Structures of a bla_{ACC-4}-containing segment of plasmid pR3521 (A) and the respective region of the ACC-1-encoding plasmid pSLK54 (B). Arrows indicate the translational orientation of the genes. A tilted line indicates a truncation of the respective gene end. The insert of pS18-d and the locations of primers used in PCR mapping experiments are shown at the top of panel A. Sizes of the respective nucleotide sequences are also indicated.

novel carbenicillinase gene initially reported as *orf1* (GenBank accession no. AJ870922 and EF104648), as well as bla_{TEM-1} (14, 17). However, in the bla_{ACC-1}-containing sequence from pSLK54, bla_{SCO-1} was adjacent to the GDHA gene while it was missing from the respective region in pR3521. PCR assays using primers specific for bla_{SCO-1} combined with primers for bla_{ACC} and the GDHA gene were negative, suggesting that the previously characterized bla_{SCO-1}-containing segment (17) was not in the vicinity of bla_{ACC-4} in pR3521. Also, IS*Ecp1* of pR3521, unlike that of pSLK54, was not interrupted by IS*26* (Fig. 1). It is probable that pR3521 and the group of pSLK54-like plasmids were derived from a common ACC-encoding replicon that diverged through insertion sequence-mediated rearrangements. Association of bla_{SCO-1} with IS*26* in pR3521 as described previously (17) is consistent with this hypothesis.

Kinetic properties of ACC-4. *E. coli*(pS18-d) was used as a source of ACC-4. Mid-log-phase cultures (5 liters of tryptone soy broth supplemented with 100 μg of ampicillin per ml) were harvested by centrifugation, and pellets were resuspended in 50 ml Tris buffer (20 mM; pH 7.5). Bacterial cell suspensions were sonicated, and the cell debris was removed by ultracentrifugation. β-Lactamases were purified by ion exchange chromatography essentially as described for chromosomal AmpC of *H. alvei* (7). Clarified extracts were loaded onto a Q-Sepharose column, and the β-lactamase preparations recovered in the flowthrough were dialyzed against 50 mM phosphate buffer (pH 6.8) and then loaded onto an S-Sepharose column. The proteins were eluted with a linear gradient of NaCl (0 to 0.6 M) in 50 mM phosphate buffer (pH 6.8). β-Lactamase-containing fractions were dialyzed against 50 mM phosphate buffer (pH 7.0). The purity of the final preparation was higher than 95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Kinetic parameters of penicillin G, piperacillin, cephalothin, cefoxitin, cefotaxime, ceftazidime, and cefepime hydrolysis at 30°C and pH 7.0 were determined by UV spectrophotometry. The respective wavelengths and extinction coefficients were as described previously (19). Results are presented in Table 1. ACC-4 hydrolyzed cephalothin efficiently due to a high k_{cat} value. Hydrolysis of penicillin G and piperacillin was characterized by low k_{cat} and K_m values, which is typical for a class C β-lactamase. ACC-4 exhibited unusually high hydrolytic efficiencies for cefotaxime and ceftazidime, which are considered poor substrates for class C β-lactamases. This was apparently due to a significant increase in the k_{cat} value compared to the respective values reported for plasmid-mediated ACC-1 and chromosomal ACC-2 (1, 7). Measurable hydrolysis of cefepime was also noted. Measurable hydrolysis of cefoxitin by ACC-4 was not observed, as has also been reported for ACC-1 and ACC-2 (1, 7).

Activities of β-lactamase inhibitors against ACC-4 were assessed at 30°C and pH 7.0 by UV spectrophotometry using cephalothin (100 μM) as a reporter substrate. Enzyme-inhibitor mixtures were preincubated for 5 min before the addition of cephalothin. Results were expressed as 50% inhibitory concentrations (IC_{50}s). Clavulanic acid, sulbactam, and tazobactam exhibited weak inhibitory activities against ACC-4 (IC_{50}s were >500, 250, and 171 μM, respectively). The most potent inhibitors, in descending order, were cloxacillin (IC_{50}, 0.026 μM), aztreonam (IC_{50}, 0.032 μM), and Ro 48-1220 (IC_{50}, 10.3 μM). The inhibitory profile of ACC-4 was characteristic of a class C β-lactamase.

Resistance conferred by ACC-4. A DNA fragment of 1,471 bp comprising the bla_{ACC-4} gene and its promoter sequence (nt 1,952 to 3,422 in sequence under GenBank accession no. EF504260) was produced by PCR using pS18-d as a template and the primers ecp-F2 (5′-GTTGCTCTGTGGATA ACTTG-3′) and a-E1 (5′-ACTCAACATATCGCCTCTCC-

TABLE 1. Kinetic parameters of ACC-4 and ACC-1 for various β-lactam substrates[a]

Substrate	Value for hydrolysis of substrate by:					
	ACC-4			ACC-1		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)	V_{max}[b]	K_m (μM)	V_{max}/K_m
Cephalothin	96 ± 6	30 ± 3.7	3.2	—	—	—
Penicillin G	0.59 ± 0.05	3.8 ± 0.4	0.16	—	—	—
Piperacillin	0.13 ± 0.01	1.1 ± 0.15	0.12	0.17 ± 0.05	1.4 ± 0.6	0.12
Cefoxitin	<0.01	—[c]	—	<0.002	—	—
Cefotaxime	2.7 ± 0.05	9.4 ± 1.0	0.29	<0.01	—	—
Ceftazidime	1.5 ± 0.1	15 ± 1.9	0.1	<0.025	17	<0.01
Cefepime	0.14 ± 0.01	73 ± 12	0.002	—	—	—

[a] Values for ACC-4 are the means of four independent measurements. Values for ACC-1 are from reference 1.
[b] Expressed as nmol of substrate per min per μg of protein.
[c] —, not determined.

3′) (Fig. 1). The fragment was directly ligated into the polycloning site of a Topo TA vector (Invitrogen, Carlsbad, CA). The resulting plasmid was utilized to construct bla_{ACC-1} by site-directed mutagenesis using a mutagenesis kit (Stratagene) and the mutagenic primers MT-1 (5′-AGC CAGTGCACG**T**GAATATGGAGAT-3′) and MT-2 (5′-AT CTCCATATTC**A**CGTGCACTGGCT-3′) (boldface and underlining indicates nucleotides different from those in the original sequence). For an isogenic comparison of ACC-4 and ACC-1, the similar bla_{ACC}-containing segments were cloned into the EcoRI sites of the plasmid vectors pACYC184 (low copy number) and pBCSK(+) (high copy number). Identity and orientation of the inserts were confirmed by sequencing. Recombinant plasmids were introduced into *E. coli* MC4100, and the MICs of β-lactams were determined by using an agar dilution technique. The pBCSK-derived plasmids, pB-acc4 and pB-acc1, mediated higher resistance levels to most β-lactams tested than the respective pACYC184 recombinants (pA-acc4 and pA-acc1), likely reflecting differences in the bla_{ACC} copy number, as also indicated by a six- to sevenfold increase in specific activity against cephalothin. Differences in the β-lactam resistance levels between ACC-4- and ACC-1-producing strains were in line with the hydrolysis data (Table 2). MICs of cefuroxime, cefotaxime, ceftazidime, and aztreonam for the ACC-4 producers were consistently higher than those observed for the respective ACC-1-producing *E. coli* strains. ACC enzymes conferred similar levels of resistance to the remaining β-lactams, except piperacillin and piperacillin-tazobactan, which were slightly more effective against ACC-4 producers. MICs of cefoxitin, cefepime, and imipenem were low, which is typical for *E. coli* strains expressing an ACC-type cephalosporinase (1, 7, 14), and were not affected by the moderately increased production of the enzymes mediated by the pBCSK-derived recombinant plasmids (Table 2).

Conclusions. An ACC-1-producing *K. pneumoniae* strain was first isolated in Germany in 1997 (1). Subsequently bla_{ACC-1}-positive enterobacteria were detected in Tunisia, France, Spain, and The Netherlands (2, 6, 8, 11, 13, 14, 16, 18, 22). Isolation of an ACC-producing strain in Greece, as well, indicates an ongoing spread of bla_{ACC} mostly in the Mediterranean region due at least in part to an epidemic plasmid.

β-Lactam hydrolysis and susceptibility data indicated significant differences between ACC-4 and ACC-1. Val^{211} is common among intrinsic and acquired AmpCs from mem-

TABLE 2. β-Lactam susceptibilities of *E. coli* strains producing ACC-4 and ACC-1 under isogenic conditions

β-Lactam	MIC (μg/ml) of drug for *E. coli* MC4100 clones harboring plasmid[b]:					
	pA-acc4	pA-acc1	pACYC184	pB-acc4	pB-acc1	pBCSK
Amoxicillin	>512	>512	4	>512	>512	4
Piperacillin	32	64	0.5	128	256	0.5
Piperacillin + TZ[a]	16	32	0.5	32	64	0.5
Cephalothin	>512	>512	2	>512	>512	2
Cefaclor	>512	>512	2	>512	>512	2
Cefoxitin	4	4	2	4	4	2
Cefotetan	1	1	0.5	1	1	0.5
Cefuroxime	>512	128	1	>512	128	1
Ceftazidime	256	32	0.25	512	128	0.25
Cefotaxime	32	8	≤0.06	64	16	≤0.06
Ceftriaxone	32	16	0.12	128	32	0.12
Aztreonam	2	0.5	≤0.06	2	1	≤0.06
Cefepime	0.12	0.12	≤0.06	0.25	0.12	≤0.06
Imipenem	0.12	0.12	0.12	0.12	0.25	0.12

[a] TZ, tazobactam at a fixed concentration of 4 μg/ml.
[b] Specific activities (units of activity against cephalothin [1 U corresponded to hydrolysis of 1 μmol of substrate/min/mg of protein]) were as follows: for pA-acc4, 6.3; for pA-acc1, 9.7; for pB-acc4, 43.1; for pB-acc1, 58.8; for pACYC184 and pBCSK, not determined.

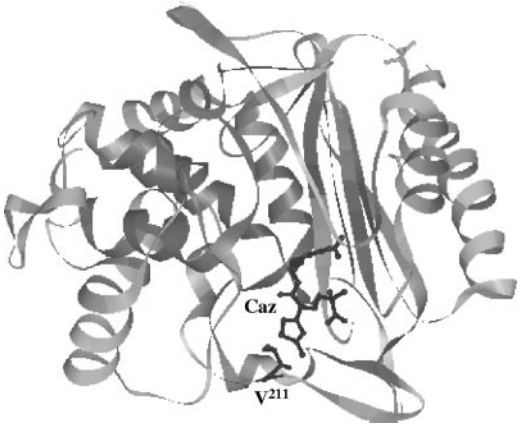

FIG. 2. Ribbon diagram of AmpC from *E. coli* in complex with acylated ceftazidime (Caz) (PDB entry 1IEL) (20), displaying the proximity of the side chain of Val^{211} to the aminothiazole group of the antibiotic. The image was made using the DeepView/Swiss-Pdb Viewer, version 3.7, available at www.expasy.org/spdbv/.

bers of *Enterobacteriaceae*, with the exception of *Providencia stuartii* AmpC, which nevertheless also contains an amino acid with an aliphatic side chain (Leu^{211}) and two FOX variants, FOX-4 and FOX-7, containing Ala^{211} (GenBank accession no. Y17315, AJ277535, and AJ703796, respectively). Val^{211} is also encountered in the majority of the AmpC enzymes from *Pseudomonas*, *Aeromonas*, and *Acinetobacter*, while Gly^{211} has not been observed before. Val^{211} in ACC-1 is most probably located in the Ω loop, as indicated by a Clustal W alignment (not shown) as well as the extensive similarities to other enterobacterial AmpCs with known structures (e.g., 40% identity and 64% similarity with AmpC from *E. coli*). Mutational alterations, including substitutions and insertions in this domain in natural and in vitro-selected enterobacterial AmpC variants, facilitate hydrolysis of expanded-spectrum cephalosporins, mainly by increasing the k_{cat} values, as also observed for ACC-4 (9, 12, 15, 23, 24, 26). Such functional changes have been associated with a wider active site and/or more flexibility of the Ω loop (3, 9, 26). Notably, the low hydrolytic efficiencies of AmpCs against expanded-spectrum cephalosporins have been partly attributed to a steric clash of the bulky R1 substituent of these substrates with the side chain of Val^{211} (Fig. 2). Ω-Loop mutations may alter the position of this residue, thus allowing catalytically more-efficient substrate docking (20). The effect of Gly-for-Val^{211} substitution in ACC as described here is compatible with an important role of Val^{211} in determining the substrate specificity of AmpC β-lactamases. Additionally, these findings also underscore the role of the Ω loop as a hot spot for extended-spectrum mutations in the *H. alvei*-originating cephalosporinases.

Nucleotide sequence accession number. The GenBank accession number of the nucleotide sequence reported in this study is EF504260.

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