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A Novel Type of AmpC β-Lactamase, ACC-1, Produced by a *Klebsiella pneumoniae* Strain Causing Nosocomial Pneumonia

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A *Klebsiella pneumoniae* strain resistant to oxyimino cephalosporins was cultured from respiratory secretions of a patient suffering from nosocomial pneumonia in Kiel, Germany, in 1997. The isolate harbors a *bla* resistance gene located on a transmissible plasmid. An *Escherichia coli* transconjugant produces a β-lactamase with an isoelectric point of 7.7 and a resistance phenotype characteristic of an AmpC (class 1) β-lactamase except for low MICs of cephamycins. The *bla* gene was cloned and sequenced. It encodes a protein of 386 amino acids with the active site serine of the S-X-X-K motif at position 64, as is characteristic for class C β-lactamases. Multiple alignment of the deduced amino acid sequence with 21 other AmpC β-lactamases demonstrates only very distant homology, reaching at maximum 52.3% identity for the chromosomal AmpC β-lactamase of *Serratia marcescens* SR50. The β-lactamase of *K. pneumoniae* KUS represents a new type of AmpC-class enzyme, for which we propose the designation ACC-1 (Ambler class C-1).

Resistance of bacterial pathogens to β-lactam antibiotics is frequently mediated by β-lactamases. *bla* genes located on transmissible plasmids have been observed for Ambler class A β-lactamases since 1963 (TEM-1 [15]). In contrast, *bla* genes coding for class C β-lactamases (class 1 in the classification by Bush et al. [12]) are primarily chromosomally located. Plasmid-borne transmissible *ampC* genes were first discovered about 25 years after the plasmidic class A *bla* genes (MIR-1 in 1988 [32] and CMY-1 in 1989 [4]). Since then, plasmid-borne *ampC* genes have been detected in many regions of the world (2, 6, 8, 10, 11, 16, 18, 19, 22, 27, 37, 38). They contribute to the spread of multidrug resistant *Klebsiella pneumoniae*, *Escherichia coli*, and other *Enterobacteriaceae*.

We report the first plasmid-encoded AmpC-type β-lactamase originating in Germany (Kiel, Schleswig-Holstein, 1997). The gene designated $bla_{ACC\text{-}1}$ (Ambler class C) has a unique nucleotide sequence with only distant homology of its deduced amino acid sequence with known AmpC β-lactamases and an unusual resistance phenotype due to its low activity against cephamycins. It therefore presents a new and so far solitary type among the AmpC β-lactamases.

(Part of this work was presented at the 38th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, Calif., 24 to 27 September 1998.)

MATERIALS AND METHODS

Case history. (i) Clinical background. *K. pneumoniae* KUS was isolated in 1997 from a 31-year-old male patient. The patient was diabetic and had been admitted to the Kiel University Hospital because of convulsive seizures due to hypo-osmolaric coma. He developed multiple pulmonary complications that necessitated mechanical ventilation for a total of 8 weeks. After 3 months of hospitalization, the patient had recovered and was transferred to a rehabilitation center.

(ii) Bacteriological findings and antibiotic therapy. Early onset pneumonia was caused by *Streptococcus pneumoniae*, which was eradicated by therapy with tazobactam. Consecutively, a late-onset pneumonia caused by *K. pneumoniae* was treated by combined antibiotic therapy of imipenem plus ciprofloxacin for 3 weeks. As *K. pneumoniae* was not eradicated, therapy was changed to meropenem plus ciprofloxacin and continued for another 20 days. Although *K. pneumoniae* persisted in throat and skin cultures, antibiotic treatment was discontinued as signs and symptoms of inflammation were no longer diagnosed.

Bacterial strains and vectors. Strains and vectors used in this study are characterized in Table 1.

Antibiotics. The following antibiotics were obtained from the indicated manufacturers: ceftazidime (Cascan GmbH & Co. KG, Wiesbaden, Germany); cefotaxime, cefpirome, and tetracycline (Hoechst AG, Frankfurt on the Main, Germany); clavulanate and BRL 42715 (SmithKline Beecham Pharmaceuticals, London, United Kingdom); sulbactam (Pfizer, Karlsruhe, Germany); tazobactam (Lederle, Münster, Germany); cefepime and aztreonam (Bristol-Myers Squibb, Munich, Germany); cefoxitin and imipenem (Merck Sharp & Dohme, Munich, Germany); cefoteten and meropenem (Zeneca GmbH, Plankstadt, Germany); Ro 47-8284, sulfamethoxazole, and trimethoprim (Hoffmann-La Roche Inc., Basel, Switzerland); moxalactam and tobramycin (Lilly Deutschland GmbH, Bad Homburg, Germany); flomoxef (Shionogi, Düsseldorf, Germany); and chloramphenicol (Boehringer, Mannheim, Germany). Combinations of ceftazidime or cefotetan with β-lactamase inhibitors were used at the following proportions: clavulanate, 1/4; sulbactam, 1/2; tazobactam, 1/8; and BRL 42715 (14) and Ro 47-8284 (53) at a constant concentration of 4 μg/ml.

Susceptibility testing. For determination of MICs, a standard procedure described by the National Committee for Clinical Laboratory Standards was followed (29). *E. coli* ATCC 25922 was used as the MIC reference strain.

Transfer of resistance determinants. The procedure for conjugation experiments was described previously (8). Transconjugants were selected on MacConkey agar (Unipath GmbH, Wesel, Germany) supplemented with rifampin (128 μg/ml) and ceftazidime (2 μg/ml).

Inducibility of the synthesis of ACC-1. Inducibility of ACC-1 production was tested by a double disk test with cefoxitin as the inducing compound. An *Enterobacter cloacae* strain producing an inducible AmpC β-lactamase was used as a reference.

Identification of the isoelectric point (pI) of ACC-1. Sonicates of strains were prepared as described previously (5). For isoelectric focusing of their β-lactamases, the procedure of Matthew et al. (28) was modified (5). After isoelectric focusing, the polyacrylamide gel was covered by a 0.75% tryptic soy agar (TSA) (Difco, Augsburg, Germany) overlay containing the β-lactam to be tested for inactivation and incubated for 2 h at 35°C. A second TSA layer with a strain (1.2×10^7 CFU/ml) susceptible to the β-lactam used was then applied. After overnight incubation at 35°C, growth of the indicator strain on the gel identifies the position at which the β-lactam had been inactivated.

Kinetic analysis of the ACC-1 β-lactamase. The ACC-1 β-lactamase for the kinetic analysis was obtained from the transformant strain MV1190 T^+. Bacteria from a 1-liter overnight culture of tryptic soy broth (TSB) supplemented with 100 μg of ampicillin/ml were harvested by centrifugation and washed with 50 mM phosphate buffer (pH 7.0). The pellet was resuspended in 2 ml of 0.2 M sodium acetate, and subjected to five freeze-thaw cycles. ACC-1 was partially purified by Sephadex G-100 chromatography in 50 mM phosphate buffer (pH 7.0). Fractions containing nitrocefin-hydrolyzing activity were precipitated with 90% ammonium

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TABLE 1. Bacterial strains used in this study

Strain	Plasmid	Characteristics	pI(s) of β-lactamases
K. pneumoniae KUS	pMVP-8	Clinical isolate from sputum, Kiel, Germany	5.4, 7.7
E. coli C600		Rifampin resistant	None
E. coli MV1190		*lac*$^-$	None
E. coli C600	pMVP-8	Transconjugant of KUS	5.4, 7.7
E. coli MV1190	pBC	Chloramphenicol-resistant cloning vector	None
E. coli MV1190		Transformant of KUS containing a 2.3-kb fragment cloned into pBC	7.7

sulfate; pellets were resuspended in 50 mM phosphate buffer (pH 7.0) and dialyzed in the same buffer overnight at 4°C. Initial hydrolysis rates were measured on a Shimadzu UV-1601 spectrophotometer at 25°C in 50 mM phosphate buffer (pH 7.0). K_m and V_{max} values were obtained by using Lineweaver-Burk, Eadie-Hofstee, Hanes-Woolf, and direct linear plot analyses. Substrates were assayed on at least two separate days, with cephaloridine included as a reference each day. Due to the slow hydrolysis rate detected with ceftazidime, a K_i value was determined: cephaloridine was used as the substrate and ceftazidime was used as the competing substrate following a 5-min preincubation of enzyme with ceftazidime to establish a steady state.

Plasmid DNA preparation. Cells were grown overnight in 150 ml of TSB (Oxoid, Wesel, Germany) supplemented with ceftazidime (1 μg/ml). DNA preparation was performed by alkaline lysis (9). Plasmid DNA in the lysate was purified with an anion exchange column (tip 100; Qiagen, Hilden, Germany) according to the recommendations of the manufacturer.

Cloning and sequencing of the bla_{ACC-1} gene. Cloning experiments were performed by following standard procedures (35). The plasmid DNA of an *E. coli* transconjugant strain carrying the bla_{ACC-1} gene was partially digested with *Sau*IIIa and subsequently ligated into vector pBC. DNA fragments were purified with the QIAquick purification kit (Qiagen). For ligation, vector and insert DNA were mixed in ratios of 1:3 and 1:6. Ligation buffer and 1 U of T4 ligase were added, and the mixture was incubated at 16°C overnight. The ligase was then inactivated at 70°C for 10 min. Cells of *E. coli* MV1190 were transformed by electroporation. Transformants were selected on TSA containing 2 μg of ceftazidime/ml, 20 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml, and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After overnight incubation at 37°C, white colonies were isolated and screened for inserts in vector pBC. From two transformants, inserts of 2.0 or 2.3 kb were sequenced. Sequencing was performed with consecutive primers by the dideoxy chain ter-

TABLE 2. Antibiotic susceptibilities of the wild-type *K. pneumoniae* KUS, the transconjugant *E. coli* C600 R$^+$, the transformant *E. coli* MV1190 T$^+$, and the recipient *E. coli* C600 R$^-$

Antibiotic	MIC (μg/ml) for:				
	Wild-type *K. pneumoniae* KUS	Transconjugant *E. coli* C600 R$^+$	Recipient *E. coli* C600 R$^-$	Transformant *E. coli* MV1190 T$^+$	Host *E. coli* MV1190 T$^-$
Ceftazidime	64	16	0.13	32	0.06
Plus clavulanate	32	16	0.13	16	0.06
Plus sulbactam	32	16	0.13	16	0.06
Plus tazobactam	32	16	0.13	32	0.06
Plus BRL 42715	1	0.5	0.13	1	0.06
Plus Ro 47-8284	1	0.5	0.13	0.5	0.06
Cefotaxime	16	4	0.06	8	0.03
Cefotetan	4	1	0.13	2	0.06
Plus clavulanate	4	1	0.06	2	0.06
Plus sulbactam	4	1	0.06	2	0.06
Plus tazobactam	4	1	0.06	2	0.06
Plus BRL 42715	1	0.25	0.06	0.5	0.06
Plus Ro 47-8284	0.5	0.25	0.06	0.5	0.06
Cefoxitin	4	2	2	4	2
Moxalactam	1	0.25	0.06	1	0.13
Flomoxef	2	0.5	0.06	1	0.13
Cefpirome	4	0.25	0.03	1	0.03
Cefepime	0.25	0.13	0.016	0.25	0.016
Aztreonam	2	0.5	0.06	1	0.06
Piperacillin	512	256	0.5	32	0.5
Plus tazobactam	64	16	0.5	32	0.5
Temocillin	2	4	4	4	4
Imipenem	0.13	0.25	0.25	0.13	0.25
Meropenem	0.03	0.03	0.03	0.03	0.03

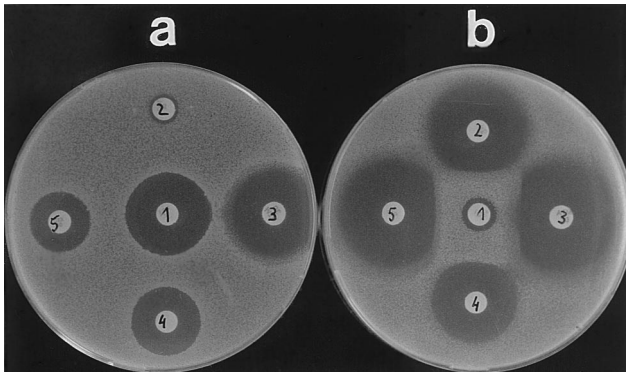

FIG. 1. Double-disk test to check inducibility of ACC-1 synthesis. (a) Inducibility test with *K. pneumoniae* KUS. (b) Inducibility test with *Enterobacter cloacae* WG7250. Disks: 1, cefoxitin; 2, ceftazidime; 3, cefotaxime; 4, cefotetan; 5, aztreonam.

mination procedure of Sanger (36) with an automatic sequencer (model 373A; Applied Biosystems, Weiterstadt, Germany).

Sequence analysis. β-Lactamase relatedness was investigated by comparison with EMBL and Swissprot databases (Fasta). Multiple alignment was calculated with Clustal V (20, 21).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL database under accession no. AJ133121.

RESULTS

Phenotypic characterization of the ACC-1 β-lactamase of *K. pneumoniae* KUS. The bla_{ACC-1} gene was conjugated into an *E. coli* recipient strain together with the bla_{TEM-1} gene. The expression of the bla_{ACC-1} gene in transconjugant and transformant strains significantly increased the MICs (equal to or above fourfold) of a variety of different β-lactam structures, namely oxyimino cephalosporins (ceftazidime and cefotaxime, 64- to 128-fold), 7-methoxy-cephalosporins (cefotetan, moxalactam, and flomoxef, four- to eightfold), dipolar cephalosporins (cefepime and cefpirome, four- to eightfold), the monobactam aztreonam (eightfold), and piperacillin (512-fold in the transconjugant, 64-fold in the transformant). No increase of MICs by acquisition of the bla_{ACC-1} gene was detectable against cefoxitin, the 6-methoxy-penicillin temocillin, and the carbapenems (Table 2). MICs of transformants were reduced only by β-lactamase inhibitors active against class C β-lactamases (BRL 42715 [14], Ro 47-8284 [33], 8- to 32-fold). Tazobactam reduced the MICs of piperacillin of the wild type and the transconjugant strains but not of the transformant strain, as bla_{TEM-1} was not cloned into the transformant. Inducibility of ACC-1 synthesis could not be demonstrated by a double-disk test, in which AmpC production of an *Enterobacter cloacae* was induced by cefoxitin (Fig. 1).

TABLE 3. Kinetic data for ACC-1

Substrate	V_{max} (nmol of substrate/min/μg of protein)	Relative V_{max}	K_m (μM)	V_{max}/K_m
Cephaloridine	30.7 ± 3.0	100	122 ± 16	0.25
Nitrocefin	63.7 ± 2.2	208	28 ± 3.7	2.3
Piperacillin	0.17 ± 0.05	0.55	1.4 ± 0.6	0.12
Ceftazidime	<0.025	≤0.1	17[a]	<0.01
Cefotaxime	<0.01	<0.02	ND[b]	ND
Cefotetan	<0.001	<0.01	ND	ND
Cefoxitin	<0.002	<0.01	ND	ND

[a] K_i value was determined by using cephaloridine as substrate.
[b] Rates were too slow to determine a K_m from steady-state hydrolysis rates.

Isoelectric focusing of crude homogenates demonstrated two bands at pIs of 5.4 and 7.7 for the *K. pneumoniae* KUS wild type and the transconjugant strain and only one band at 7.7 for the transformant strain (Fig. 2A). Ceftazidime, cefotetan, and cefoxitin were inactivated only at the pI 7.7 band as demonstrated by growth of the susceptible indicator strain (Fig. 2B) at this position. So the hydrolytic activity of the β-lactamase ACC-1 was assigned to the band focusing at pI 7.7.

Kinetic data of the ACC-1 β-lactamase. Measurable hydrolysis rates were not observed for cephalosporins except for cephaloridine and nitrocefin; however, in a competitive assay, slow hydrolysis of ceftazidime was detectable (Table 3).

Characterization of the bla_{ACC-1} gene. A 2,263-bp DNA fragment excised from plasmid DNA of a transconjugant strain

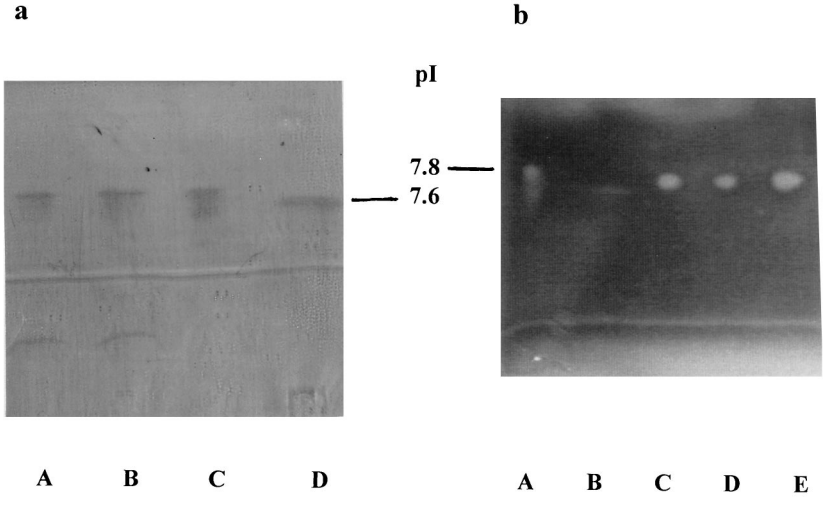

FIG. 2. Isoelectric focusing of β-lactamase ACC-1. The ACC-1 producing wild-type, transconjugant, and transformant strains revealed a band at a pI lower than 7.8 (SHV-4) but slightly above 7.6 (SHV-2), at about 7.7 (a). This band was able to inactivate ceftazidime, as shown by a bioassay (b). Lanes for panel a: A, *K. pneumoniae* KUS producing ACC-1; B, *E. coli* C600 R^+ producing ACC-1; C, *E. coli* DH5α T^+ producing ACC-1; D, *E. coli* R^+ producing SHV-2. Lanes for panel b: A, *E. coli* R^+ producing SHV-4; B, *E. coli* R^+ producing SHV-2; C, *K. pneumoniae* KUS producing ACC-1; D, *E. coli* C600 R^+ producing ACC-1; E, *E. coli* DH5α T^+ producing ACC-1.

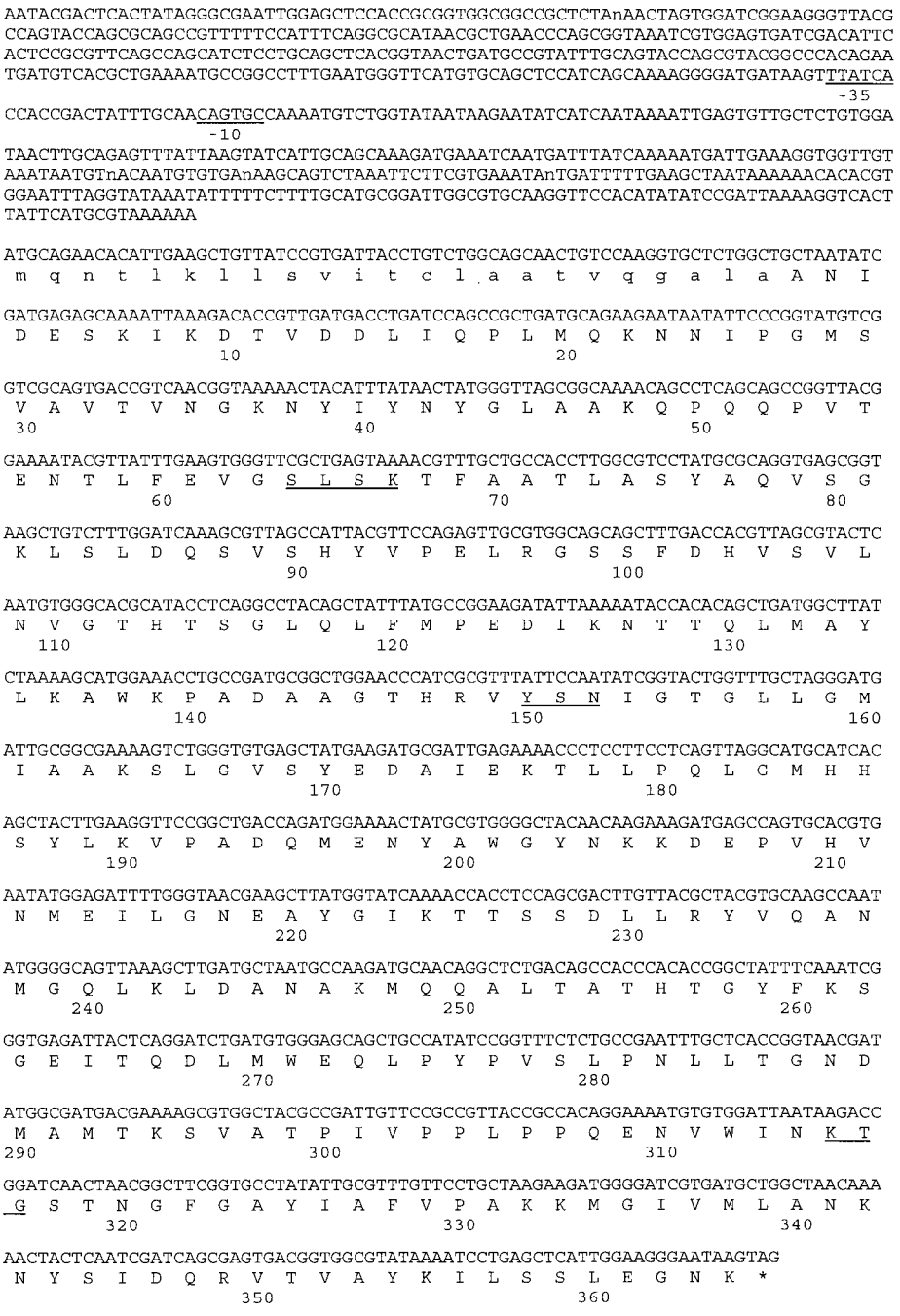

FIG. 3. Nucleotide sequence of the bla_{ACC-1} gene (pMVP-8). The deduced amino acid sequence of ACC-1 is shown in the line below the nucleotide triplets. Amino acids of the signal peptide are written in small letters. The β-lactamase active site S-L-S-K, the conserved triad K-T-G, and the class C-typical motif Y-X-N are underlined. The putative −10 and −35 promoter regions upstream of the start codon are underlined. The asterisk indicates the stop codon.

was cloned into *E. coli* MV1190 (pBC) and sequenced. An open reading frame of 1,161 nucleotides encoding a protein of 386 amino acids could be identified (Fig. 3). The deduced amino acid sequence carries the catalytic residues S-X-X-K (here serine-leucine-serine-lysine) with the initial serine at position 64 as is typical of class C β-lactamases, the motif Y-S-N (tryptophan-serine-asparagine) at position 150, and the K-T-G (lysine-threonine-glycine) motif at position 315. In the 639-bp nucleotide sequence upstream of the start codon no *ampR* motif could be detected. This finding is concordant with the noninducibility of ACC-1 β-lactamase synthesis.

The deduced amino acid sequence of the enzyme was compared with that of other chromosomal or plasmid-encoded AmpC β-lactamases. The percentage of amino acid identity was below 50% for all β-lactamases included except for the chromosomal *Serratia marcescens* SR50 AmpC β-lactamase (31) with 52.3% (Table 4). Multiple alignment of the amino acid sequence of AmpC β-lactamases demonstrates enzyme-

TABLE 4. Identity of amino acid sequences of ACC-1 to other class C β-lactamases

β-Lactamase (reference)[a]	% Identity with:								
	ACC-1	*S. marcescens* AmpC	FOX-2	CMY-1	*P. aeruginosa* AmpC	*C. freundii* AmpC	*E. coli* AmpC	*Enterobacter cloacae* AmpC	*M. morganii* AmpC
ACC-1	100	52.3	46.4	43.9	43.7	39.5	38.8	37.9	37.2
S. marcescens AmpC (31)		100	45.6	44.5	44.9	38.3	38.9	41.5	39.8
FOX-2 (8)			100	74.9	53.7	41.8	42.0	43.9	46.3
CMY-1 (7)				100	54.4	41.2	41.1	43.1	43.5
P. aeruginosa AmpC (26)					100	41.9	42.7	44.1	54.4
C. freundii AmpC (25)						100	76.7	73.2	57.2
E. coli AmpC (24)							100	69.8	57.9
Enterobacter cloacae AmpC (17)								100	55.1
M. morganii AmpC (1)									100

[a] One representative of each group of the seven AmpC types (Fig. 5) was selected for comparison.

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                                  1        10        20        30
                                  |        |         |         |
Sma_AmpC          MTKMNRCAALIAALIL-PTAHAA----QQQ-DI-DAVIQPLMKKYGVPGMAI
Kpn_CMY-2         .M.-KSLCCAL-LLTASFSTF.A.KTE..IA..VNRT.T...QEQAI....V
Eco_FOX-2         .QQRRALALLTLGS.L-LA.CTY.SGEAPLTAAV.GI.Q.ML.E.RI....V
Kpn_ACC-1         .QNTLKLLSV.TC.AATVQ.L..NIDESKIK.TV.DL.....Q.NNI...SV

                           40        50        60        70        80
                           |         |         |         |         |
Sma_AmpC          AVSVDGKQQIYPYGVASKQTGKPITEQTLFEVGSLSKTFTATLAVYAQQQ
Kpn_CMY-2         ..IYQ..PYYFTW.K.DIANNH.V.Q.....L..V....NGV.GGD.IAR
Eco_FOX-2         ..LK...AHYFN....NRES.QRVS......I..V...L....GA..AVK
Kpn_ACC-1         ..T.N..NY..N..L.A..PQQ.V..N............A....S...VS

                          90       100       110       120       130
                          |         |         |         |         |
Sma_AmpC          SKLSFKDPASHYLPDVRGSAFDGVSLLNLATHTS-GLPLFVPDDVTNNAQ
Kpn_CMY-2         GEIKLS..VTK.W.ELT.KQWQ.IR..H...Y.AG....QI....RDK.A
Eco_FOX-2         GGFELD.KV..HA.WLK.......TMAE...YSAG....QF..E.DS.DK
Kpn_ACC-1         G...LDQSV...V.EL...S..H..V..VG....-..Q..M.E.IK.TT.

                         140       150       160       170       180
                          |         |         |         |         |
Sma_AmpC          LMAYYRAWQPKHPAGSYRVYSNLGIGMLGMIAAKSLDQPFIQAMEQGMLP
Kpn_CMY-2         .LHF.QN...QWTP.AK.L.A.SS..LF.AL.V.PSGMSYEE..TRRV.Q
Eco_FOX-2         MQT...S.S.VY...TH.Q...PS..LF.HL..N..G...EKL.S.TL..
Kpn_ACC-1         ....LK..K.ADA..TH.....I.T.L.........GVSYED.I.KTL..

                         190       200       210       220       230
                          |         |         |         |         |
Sma_AmpC          ALGMSHTYVQVPAAQMANYAQGYSKDDKPVRVNPGPLDAESYGIKSNARDL
Kpn_CMY-2         P.KLA..WIT..QNEQKD..W..-REG...H.S..Q....A..V..SVI.M
Eco_FOX-2         K..LH...I...ESA.....Y....E...I..T..V.A..A....TGSA..
Kpn_ACC-1         Q...H.S.LK...D..E...W..N.K.E..H..MEI.GN.A....TTSS..

                         240       250       260       270       280
                          |         |         |         |         |
Sma_AmpC          IRYLDANLQQVKV---ASVARRWPRRTSVITSAGAFTQDLMWENYPYPVKL
Kpn_CMY-2         A.WVQ..MDASH.-QEKTLQQGIALAQ.RYWRI.DMY.G.G..MLNW.L.A
Eco_FOX-2         LKFVE..M-GYQG--D.ALKSAIALTHTGFY.V.DM..G.G..S.A...TE
Kpn_ACC-1         L..VQ..MG.L.LDAN.KMQQALTATHTGYFKS.EI.......QL....S.

                         290       300       310       320       330
                          |         |         |         |         |
Sma_AmpC          SRLIEGNNAGMIMNGTPATAITPPQPELRAGWYNKTGSTGGFSTYAVFIP
Kpn_CMY-2         DSI.N.SDSKVALAAL..VEVN..A.AVK.S.VH........GS.VA.V.
Eco_FOX-2         QA.LA..SPAVSFQAN.V.RFAV.KAMGEQRL..........GA.VA.V.
Kpn_ACC-1         PN.LT..D--.A.TKSV..P.V..L.PQENV.I......N..GA.IA.V.

                         340       350       360
                          |         |         |
Sma_AmpC          AKNIAVEMLANKWFPNDDRVEAAYHIIQALEKR
Kpn_CMY-2         E..LGIV.....SY..PV.....WR..EK.Q
Eco_FOX-2         .RG..IV....RNY.IEA..K..HA.LSQ.AE
Kpn_ACC-1         ..KMGIV.....NYSI.Q..TV..K.LSS..GNK
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FIG. 4. Multiple alignment of amino acid sequences of the chromosomal AmpC β-lactamase of *S. marcescens* (32), CMY-2 (6), FOX-2 (8), and ACC-1 (this study).

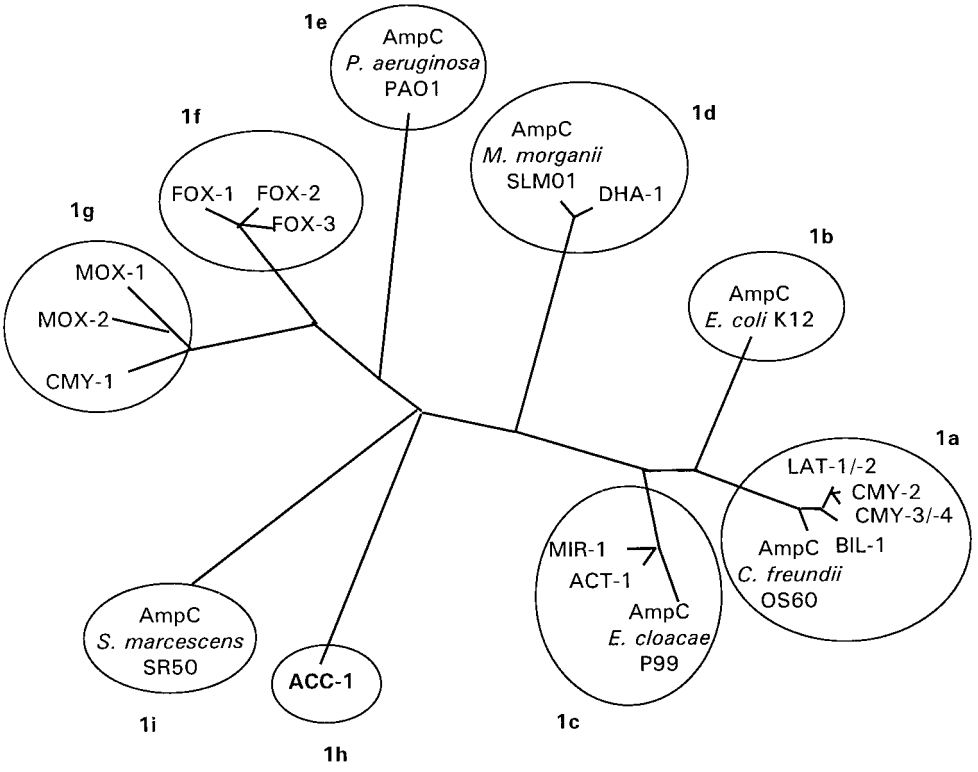

FIG. 5. Dendrogram for 22 AmpC (group 1) β-lactamases (calculated by Clustal V using the neighbor-joining method of Saitou and Nei [34]). According to the identity of their amino acid sequences with CMY-2, the group 1 β-lactamases might be subclassified into 1a to 1i.

specific amino acid fingerprints as well as amino acid positions conserved in all enzymes (Fig. 4).

DISCUSSION

The majority of the plasmid-borne *ampC* genes have been described in *K. pneumoniae* (6, 7, 10, 11, 18, 19, 22, 23, 37). Many among them have close homology with chromosomal AmpC β-lactamases, e.g., of *Citrobacter freundii* (6, 18, 37), or *Enterobacter cloacae* (11, 23). So *K. pneumoniae* may have acquired these genes from other species at sites where *K. pneumoniae* lives close to the ecological neighborhood of *ampC*-carrying organisms, e.g., other *Enterobacteriaceae*, namely in the intestine. The degree of homology between some of the chromosomal and the plasmid-encoded β-lactamases appears to be high enough to assume a phylogenetic lineage. There remain, however, both chromosomal and plasmid-mediated AmpC enzymes without a currently identified counterpart, e.g., the chromosomal β-lactamase of *Pseudomonas aeruginosa* (26) or the plasmid-encoded β-lactamases forming the CMY-1 cluster (Fig. 5).

The enzyme described here as well is highly dissimilar to known AmpC β-lactamases (Table 4, Fig. 5). The closest amino acid sequence homology of the mature enzyme is with the chromosomal AmpC β-lactamase of *S. marcescens* (31). The degree of homology is very low (52.3%). There are altogether 181 amino acid substitutions between both enzymes (92 conservative, 89 nonconservative). Thus, the phylogenetic relationship of this enzyme to others remains unclear. The phenotypic characteristics of the β-lactamase (antibiotic susceptibilities and pI) as well indicate a distinct type of AmpC β-lactamase. In particular, the resistance phenotype expressed by bla_{ACC-1}-carrying strains is unusual among AmpC-type β-lactamases, as cefoxitin appears to be a poor substrate. The MIC of cefoxitin for transformants is elevated by a factor of only two, in comparison with a 64- to 128-fold increase for transformants producing CMY-1 (4), CMY-2 (6), FOX-2 (8), or ACT-1 (11). Accordingly, inactivation of cefoxitin as determined in the bioassay is lower in comparison with ceftazidime. To obtain an equivalent effect, the same homogenate has to be used undiluted (for cefoxitin) or at a dilution of 1:32 (for ceftazidime), indicating a good correlation between MICs and the inactivation of these substrates. This reaction proceeds at a rather low rate, while the affinity for binding appears to be high. Slow hydrolysis of cephamycins and third generation cephalosporins has been observed for chromosomal cephalosporinases (30).

The low inactivation level of cefoxitin by ACC-1 stimulates speculation on the role of amino acids at specific positions in the hydrolysis of cephamycins. We identified 11 positions with amino acids identical in all AmpC β-lactamases hydrolyzing cephamycins at which ACC-1 carries a different amino acid. Five of them (P 118 Q, Y 135 L, I 155 T, P 213 M, and G 214 E) are nonconservative exchanges and may be associated with structural modifications which impair the hydrolysis of methoxy-cephalosporins. The functional significance of the five nonconservative amino acid exchanges by site-directed mutagenesis is being analyzed.

The ensemble of phenotypic and genetic characteristics indicates that the enzyme produced by *K. pneumoniae* KUS represents a new type within class C β-lactamases. Nomenclature of plasmid-encoded AmpC β-lactamases—similar to that of TEM-β-lactamases (13)—has not been systematically standardized. They were designated mainly by their preferred substrates (the MOX, FOX, and CMY enzymes) or by the place

where strains producing them were first isolated (MIR-1 or BIL-1). For the new enzyme, neither a preferred substrate nor an outbreak of infections caused by *K. pneumoniae* KUS was observed. We propose the designation Ambler class C, number 1 (ACC-1).

At this time, the number of plasmid-mediated AmpC β-lactamases that has been described is 17. For their classification, phenotypic characteristics such as preferred substrates and inhibitory compounds are of less discriminatory power than for class A enzymes (e.g., low MIC of ceftazidime of CMY-1, or of cefoxitin of ACC-1). However, the amino acid sequence data are available for all of them. This allows a genetic subclassification as proposed in Fig. 5. This tentative subclassification follows the degree of amino acid sequence identity with CMY-2 (3). Maximum discrimination of β-lactamases could be based on amino acid fingerprints. Positions in the enzyme occupied by amino acids not found in any other AmpC β-lactamase may be used to define the molecular individuality of the respective enzyme. The positions occupied with a unique amino acid describe the sequence individuality for an enzyme. CMY-2 and ACC-1 are located at the extreme ends on this scale. ACC-1 carries amino acids not found in any other AmpC β-lactamase at 83 positions. So, this β-lactamase is most distant from the comparable enzymes.

The future evolution of plasmid-encoded AmpC β-lactamases might proceed at a rate comparable to that of the last decade where on average 1.5 new enzymes were identified per year (3). The novel β-lactamases were either closely related to already described enzymes (CMY-2 or CMY-1 groups) or solitary types (DHA-1, ACC-1). The selection of strains producing AmpC β-lactamases may be more effective in comparison with ESBL due to their additional resistance to cephamycins (except ACC-1). So, monitoring for AmpC β-lactamases by including a cephamycin in the panel of antibiotics used for susceptibility testing is of major clinical relevance. There is a risk of accumulation of additional resistance mechanisms in one strain, e.g., impaired expression of outer membrane proteins or enhanced efflux, which may add up to pleiotropic resistance including non-β-lactams (11).

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