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Identification of a New Allelic Variant of the *Acinetobacter baumannii* Cephalosporinase, ADC-7 β-Lactamase: Defining a Unique Family of Class C Enzymes‡

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***Acinetobacter* spp. are emerging as opportunistic hospital pathogens that demonstrate resistance to many classes of antibiotics. In a metropolitan hospital in Cleveland, a clinical isolate of *Acinetobacter baumannii* that tested resistant to cefepime and ceftazidime (MIC = 32 μg/ml) was identified. Herein, we sought to determine the molecular basis for the extended-spectrum-cephalosporin resistance. Using analytical isoelectric focusing, a β-lactamase with a pI of ≥9.2 was detected. PCR amplification with specific *A. baumannii* cephalosporinase primers yielded a 1,152-bp product which, when sequenced, identified a novel 383-amino-acid class C enzyme. Expressed in *Escherichia coli* DH10B, this β-lactamase demonstrated greater resistance against ceftazidime and cefotaxime than cefepime (4.0 μg/ml versus 0.06 μg/ml). The kinetic characteristics of this β-lactamase were similar to other cephalosporinases found in *Acinetobacter* spp. In addition, this cephalosporinase was inhibited by meropenem, imipenem, ertapenem, and sulopenem (K_i < 40 μM). The amino acid compositions of this novel enzyme and other class C β-lactamases thus far described for *A. baumannii*, *Acinetobacter* genomic species 3, and *Oligella urethralis* in Europe and South Africa suggest that this cephalosporinase defines a unique family of class C enzymes. We propose a uniform designation for this family of cephalosporinases (*Acinetobacter-d*erived *c*ephalosporinases [ADC]) found in *Acinetobacter* spp. and identify this enzyme as ADC-7 β-lactamase. The coalescence of *Acinetobacter ampC* β-lactamases into a single common ancestor and the substantial phylogenetic distance separating them from other *ampC* genes support the logical value of developing a system of nomenclature for these *Acinetobacter* cephalosporinase genes.**

Acinetobacter spp. are commonly associated with serious nosocomial infections (10, 14, 23, 37, 40, 41). Most recently, American troops wounded in Iraq (Operation Iraqi Freedom) and Afghanistan (Operation Enduring Freedom) have suffered severe infections from antibiotic-resistant *Acinetobacter baumannii*, making this organism an important pathogen in military health (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5345a1.htm). Unfortunately, antibiotic treatment of *Acinetobacter* infections has been challenging (11, 33, 46). Resistance against potent β-lactam antibiotics (extended-spectrum cephalosporins and carbapenems) is reported from many centers, and infections caused by antibiotic-resistant *Acinetobacter* spp. lead to significant morbidity and mortality (6, 23, 31, 32). Ampicillin-sulbactam, cefoperazone-sulbactam, carbapenems, and antibiotic combinations (e.g., polymyxin B, rifampin, and doxycycline) are among the only therapeutic options effective against multidrug-resistant *Acinetobacter* infections (12, 13, 22, 33, 46, 48). Randomized control trials to define the best regimen are still forthcoming (22).

A growing number of β-lactamases that confer resistance to extended-spectrum cephalosporins have been found in *Acinetobacter* spp. Recently, a group of AmpC-type cephalosporinases with highly alkaline isoelectric points (pI ~ 9.0) have been described (4, 7, 24, 31, 32, 47). Here, we describe a novel class C β-lactamase of *A. baumannii* found in a clinical isolate recovered from a hospital in Cleveland, Ohio. Based upon phylogenetic analysis, we propose a uniform designation for this family of β-lactamases: ADC, for *Acinetobacter-d*erived *c*ephalosporinases. Since six related cephalosporinases have thus far been described, five from *Acinetobacter* spp. and one from *Oligella urethralis*, this enzyme is identified as ADC-7 β-lactamase.

MATERIALS AND METHODS

Bacterial isolate identification and susceptibility testing. The isolate originated from a patient hospitalized at MetroHealth Medical Center, an 800-bed Case Western Reserve University School of Medicine-affiliated hospital. API strips (BioMerieux, Durham, NC) were used to identify *A. baumannii*, and initial susceptibility testing was performed using Kirby Bauer disks (Becton Dickinson, Cockeysville, MD) according to criteria established by the National Committee for Clinical Laboratory Standards (NCCLS) (27). β-Lactam agar dilution susceptibility tests (MICs) were performed using a 10^4 inoculum delivered by a Steers replicator. The agar dilution MIC determinations were done a minimum

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‡ Supplemental material for this article may be found at http://aac.asm.org/.

TABLE 1. PCR amplification and sequencing primers used in these experiments

Primer use and name	Sequence	Reference or source
PCR		
Class C-1	5′-ATC-AAA-ACT-GGC-AGC-CG-3′	21
Class C-2	5′-GAG-CCC-GTT-TTA-TGG-ACC-CA-3′	21
ABAMPC-1	5′-ATG-CGA-TTT-AAA-AAA-ATT-TCT-TGT-3′	6, 7
ABAMPC-2	5′-TTA-TTT-CTT-TAT-TGC-ATT-CAG-3′	6, 7
EBF bla_{ADC-7}	5′-GGG-ATA-TCA-TGC-GAT-TTA-AAA-AAA-TTT-C-3′	This paper
EBR bla_{ADC-7}	5′-AAG-GAT-CCT-TAT-TTC-TTT-ATT-GCA-TTC-3′	This paper
Sequencing (Cy5 labeled)		
M13 Reverse	5′-CAG-GAA-ACA-GCT-ATG-AC-3′	
M13 Universal	5′-GTA-AAA-CGA-CGG-CCA-G-3′	
ABAC 481F	5′-GAC-TGG-AAA-CCT-AAA-AAC-CCA-AT-3′	This paper
ABAC 628R	5′-GTT-TTA-AGC-CAA-GGG-CCG-GAA-A-3′	This paper

of three times against each β-lactam. The following β-lactams were tested: ampicillin (Sigma Chemical Co., St. Louis, MO), sulbactam (a kind gift of Pfizer Research, Groton, CT), ampicillin-sulbactam (in a 2/1 ratio), tazobactam (a kind gift of Wyeth Pharmaceuticals, Pearl River, NY), piperacillin (Sigma Chemical Co.), piperacillin-tazobactam (in an 8/1 ratio, which is an exception to the NCCLS guidelines for testing piperacillin-tazobactam at a fixed concentration of 4 μg/ml), cephalothin (Sigma Chemical Co.), cefoxitin, imipenem-cilistatin and ertapenem (Merck Research Laboratories, Rahway, NJ), meropenem (AstraZeneca, Wilmington, DE), sulopenem (a kind gift of Pfizer Research), ceftazidime (GlaxoSmithKline, Research Triangle Park, NC), and cefepime (Bristol-Myers Squibb, Princeton, NJ). *Escherichia coli* DH10B was used as a control and host strain in these experiments (20).

Analytical isoelectric focusing. The pI of the β-lactamase was estimated by liberating the enzyme from the periplasmic space using lysozyme and EDTA, according to a method developed in our laboratory (29). The extracted β-lactamase was resolved on Ampholine PAG plates, pI 3.5 to 9.5 (Amersham Biosciences, Piscataway, NJ), with analytical isoelectric focusing standards as controls (Bio-Rad, Hercules, CA). To visualize the β-lactamase, a 1 mM solution of nitrocefin (Becton Dickinson) was used in an overlay method as previously described (29).

PCR amplification, cloning, and DNA sequencing. Two sets of oligonucleotides for PCR amplification and identification of the cephalosporinase were used (Table 1). The first set (class C-1 and class C-2) amplifies AmpC β-lactamases with significant homology to CMY-2 β-lactamases (e.g., ACT-1, P99, and the CMY β-lactamases). This class C primer set produces a 549-bp product (19–21). The second set of primers (ABAMPC-1 and ABAMPC-2) is specific for *Acinetobacter* sp. β-lactamases and produces a 1,152-bp product (6, 7).

PCR amplification of bla_{ADC-7} was performed in the following manner. A 1:10 dilution of an overnight culture was boiled for 10 min. Amplification was then performed with 10 μl of this dilution as the DNA template. PCR conditions included 28 cycles of amplification under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Cycling was followed by a final extension at 72°C for 10 min. PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and photographed with UV illumination. ΦX174 replicative-form DNA HaeIII fragments (Gibco BRL Life Technologies, Rockville, MD) were used to assess PCR product size.

To accurately determine the bla_{ADC-7} sequence, high-fidelity PCR amplification (GeneAmp XL PCR kit; Applied Biosystems, Foster City, CA) on the *A. baumannii* isolate was performed as described above, using the ABAMPC-1 and ABAMPC-2 primers.

The PCR product generated was cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). Using an ALF Express automated DNA sequencer (Amersham Biosciences) with a Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham Biosciences), we determined the sequence of the bla_{ADC-7} gene from three independent TOPO clones that were created using the high-fidelity PCR amplification product. bla_{ADC-7} in the TOPO clones was cycle sequenced under the following conditions: DNA was heated to 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55°C for 1 min, and 60°C for 2 min. The Cy5-labeled sequencing primers are listed in Table 1.

Cloning of bla_{ADC-7} into pET24a (+) plasmid for β-lactamase characterization. For large-scale protein expression and β-lactamase characterization, the bla_{ADC-7} gene was cloned into the pET24a (+) vector (kanamycin resistance) (Novagen, Madison, WI) according to the following method. Using the GeneAmp XL PCR kit (Applied Biosystems), high-fidelity amplification of bla_{ADC-7} in the TOPO clone was performed with primers EBF bla_{ADC-7} and EBR bla_{ADC-7}, listed in Table 1. The cycling conditions used were 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 3 cycles and then 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min for 25 cycles, after which there was final extension at 72°C for 10 min. A restriction digest of the pET24a (+) vector was done using NdeI, after which it was treated with Klenow to blunt the ends and then digested with BamHI. The amplification product was purified using the Promega Wizard kit (Madison, WI) and digested using EcoRV and BamHI. The resulting digest was gel purified. This product was ligated to the digested pET24a (+) vector and electroporated into *E. coli* DH10B. The resulting construct was sequenced with the Cy5-labeled primers ABAC 481F and ABAC 628R. After sequencing verification, the correct construct was transformed into *E. coli* BL21(DE3) cells for protein expression. This approach permitted us to express ADC-7 β-lactamase without the accompanying C-terminal His tag.

Cloning of bla_{ADC-7} into pBC SK (+) phagemid for MIC determinations. For MIC determinations, bla_{ADC-7} was cloned into the pBC SK (+) phagemid vector (Stratagene, La Jolla, CA). First, bla_{ADC-7} from the pET24a (+) bla_{ADC-7} construct was digested with XbaI and BamHI in Multi-Core buffer (Promega) so as to maintain the stop codon. The digest mixture was supplemented with BamHI halfway through the reaction to further enhance cutting. This allowed us to maintain the 5′ upstream flanking region from the pET24a (+) vector in front of the insert when ligated into pBC SK (+). The digests were gel purified and ligated to the XbaI- and BamHI-cut pBC SK (+) vector. This plasmid construct was transformed into electrocompetent *E. coli* DH10B cells (Invitrogen) and selected on plates containing 100 μg/ml of ampicillin and 20 μg/ml of chloramphenicol. Select colonies from ampicillin-chloramphenicol plates were isolated. Plasmids were extracted from these isolates, and bla_{ADC-7} was sequenced using M13 Universal, M13 Reverse, and the two internal primers listed above, which were all Cy5 labeled.

β-Lactamase induction. The induction of *A. baumannii* cephalosporinase by cefoxitin was tested using the disk approximation method (35). A single colony of the *A. baumannii* isolate was inoculated into 1 ml of fresh antibiotic-free Mueller-Hinton broth until turbidity approximating a 0.5 McFarland standard was obtained. This suspension was then used to create a lawn culture on Mueller-Hinton agar using a sterile cotton swab. Antibiotic disks containing cefoxitin and ceftazidime were placed onto the lawn culture at distances of 10, 15, and 20 mm apart. The plates were incubated for 18 to 24 h at 37°C. Induction of β-lactamase by cefoxitin in *A. baumannii* was assessed by visual inspection for a flattening of the zone of inhibition between the disks containing cefoxitin and ceftazidime. This was done in triplicate.

β-Lactamase purification. After successful cloning of bla_{ADC-7} into pET24a (+), ADC-7 cephalosporinase was expressed and purified to homogeneity. Induction of a log-phase culture of *E. coli* BL21(DE3) possessing pET24a (+) bla_{ADC-7} with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was performed at 37°C for 4 h in 500 ml Luria-Bertani broth. These cells were pelleted and resuspended in 50 ml of 50 mM Tris (pH 7.4), and β-lactamase was liberated with lysozyme and EDTA according to the established method (see above).

Preparative isoelectric focusing was next performed using a Sephadex G100 gel matrix (Amersham) and commercially prepared ampholines, pH 3.5 to 10.0 (Amersham Biosciences). The run conditions used for preparative isoelectric focusing have been described previously (20). After overnight isoelectric focusing, areas of the gel demonstrating β-lactamase activity by nitrocefin overlay were

TABLE 2. MICs of β-lactams for the *A. baumannii* clinical isolate and cloned bla_{ADC-7} compared to *E. coli* DH10B control

Antibiotic	MIC (μg/ml)			
	A. baumannii clone 9	*E. coli* DH10B with pBC SK (+) bla_{ADC-7}	*E. coli* DH10B pBC SK (+)	*E. coli* DH10B
Ampicillin	1,024	512	4	4
Sulbactam	4	>16	>16	>16
Ampicillin-sulbactam[a]	4/2	8/4	4/2	4/2
Piperacillin	128	32	2	2
Tazobactam	16	>32	>32	>32
Piperacillin-tazobactam[b]	64/8	32/4	4/0.5	4/0.5
Cephalothin	>4,096	512	4	2
Cefoxitin	>128	32	4	4
Cefepime	32	0.06	0.06	0.06
Ceftazidime	32	4	0.13	0.13
Cefotaxime	128	4	0.06	0.06
Ertapenem	2	0.06	0.06	0.06
Imipenem-cilistatin	0.25	0.06	0.06	0.06
Meropenem	0.25	0.06	0.06	0.06
Sulopenem	0.5	0.06	0.06	0.06

[a] Ampicillin-sulbactam is in a 2/1 ratio.
[b] Piperacillin-tazobactam is in an 8/1 ratio.

cut from the gel, eluted with phosphate-buffered saline, pH 7.4, on polyethylene glycol columns (Amersham Biosciences), and concentrated using an Amicon concentrator.

Protein concentrations were determined by a Bio-Rad protein assay using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gels were stained using Coomassie blue R-250, and protein molecular weight was estimated using prestained low-molecular-weight standards supplied by Bio-Rad.

MALDI-TOF mass spectrometry. ADC-7 β-lactamase was desalted and eluted using C_{18} Millipore Ziptips according to the manufacturer's recommended protocol (Millipore Corp., Billerica, MA). Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) spectra were acquired on a BiFlex III MALDI-TOF spectrometer (Bruker Daltonics, Billerica, MA) equipped with a pulsed N_2 laser (3-ns pulse at 337 nm) and XTOF acquisition software. Aliquots of the loaded samples were eluted onto a stainless steel MALDI target plate with the wetting solution saturated with sinapinic acid, the matrix compound. The ion source was operated with an accelerating voltage of −25 kV and had an ion deflector to deflect interfering low-mass ions that reduce sensitivity. Linear mode was used with a laser power attenuation ranging from 70 to 85 and an average of 300 scans acquired. Data were analyzed using Bruker software (42).

Kinetics. Steady-state kinetic parameters (K_m, k_{cat}, V_{max}, and k_{cat}/K_m) were measured by continuous assays at room temperature and determined using nonlinear least squares employing the program Enzfitter (Sigma) (20). Each rate determination was performed in triplicate in 20 mM phosphate-buffered saline at pH 7.4 in an Agilent 8453 diode array spectrophotometer with a 1-cm path length. Direct velocity measurements were obtained using cephaloridine ($\Delta\varepsilon_{260} = -10{,}200\ M^{-1}\ cm^{-1}$), cephalothin ($\Delta\varepsilon_{262} = -7{,}660\ M^{-1}\ cm^{-1}$), and nitrocefin ($\Delta\varepsilon_{482} = 17{,}400\ M^{-1}\ cm^{-1}$).

A competition assay was performed to determine the dissociation constant for the preacylation complex, K_i, of the inhibitors (clavulanic acid, sulbactam, tazobactam, imipenem, meropenem, ertapenem, and sulopenem), cefoxitin, extended-spectrum cephalosporins (ceftazidime, cefotaxime, and cefepime), and aztreonam. We used a final concentration of 100 μM nitrocefin as the indicator substrate and 1.2 nM ADC-7 β-lactamase. The apparent K_m or K_i was calculated using the following formula: true K_m or K_i = apparent K_m or $K_i/(1 + [S]/K_{m\ ncf})$, where [S] is the substrate concentration and ncf is nitrocefin.

BLAST search. The *ampC* and *ampC* homolog DNA sequences were identified with a pBLASTn (1, 2) search of the nonredundant National Center for Biotechnology Information sequence database and the completed microbial genomes database using characterized *ampC* genes as query sequences.

Alignment. The protein sequences of the identified AmpC β-lactamases and their homologs were aligned with ClustalX 1.8 (45) using the Gonet 250 similarity matrix with a gap opening penalty of 35 and a gap extension penalty of 0.75 for the pairwise alignment stage and a gap opening penalty of 15 and a gap extension penalty of 0.3 for the multiple-alignment stage.

The corresponding DNA coding sequences were aligned by introducing triplet gaps between codons corresponding to gaps in the aligned protein sequences by using the program CodonAlign (16). CodonAlign for Macintosh and for PC (Windows) computers, and source code that can be compiled for other platforms, is available at no charge from Sinauer.

Phylogenetic reconstruction. Phylogenies were constructed by the Bayesian method (25, 26, 34) as implemented by the program MrBayes (18). MrBayes is available at no charge from www.mrbayes.net. The evolutionary model used was the General Time Reversible model (44). Because evolutionary rates are not homogeneous for every site in a gene, site variation in evolutionary rate was estimated separately for first, second, and third positions of sites within codons. Four chains, with a "temperature" of 0.2 for the heated chains, were run for each tree. Trees were sampled every 100 generations. A total of 2,500,000 generations were run with a burn-in of 500 trees. The length of burn-in was set at a value that exceeded twice the number of trees required for convergence of the ln likelihood. Because the consensus trees calculated by MrBayes do not include the posterior probabilities of the clades, each entire set of trees was imported into PAUP* (43), and the same trees used by MrBayes to calculate a consensus were used to calculate a 50% majority rule consensus in PAUP* (43). The resulting tree shows the posterior probabilities of the clades, i.e., the percentage of time that those taxa are included in the clade.

The consensus tree calculated by MrBayes was imported into PAUP* for the

TABLE 3. Comparison of DNA sequences of class C cephalosporinases described for *Acinetobacter* spp. and *O. urethralis*

Isolate and cephalosporinase	Origin, year	GenBank accession no.	Reference or source	Revised designation	% Identity compared with ADC-1
A. baumannii AmpC	Spain, 2000	AJ009979	7	ADC-1	
O. urethralis ABA-1	France, 2003	AY177427	24	ADC-2	98.4
A. baumannii ABAC-1	France, 2003	AY178995	24	ADC-3	98.2
A. baumannii ABAC-2	France, 2003	AY178996	24	ADC-4	99
Acinetobacter genomic species AG3	Spain, 2004	AJ575184	6	ADC-5	97.9
A. baumannii AmpC	South Africa, 2004	AY325306	38	ADC-6	98.4
A. baumannii AmpC	Cleveland, Ohio	AY648950	This paper	ADC-7	97.7

purposes of displaying and printing the tree. The tree was rooted using experimentally determined penicillin binding proteins as the outgroup.

Nucleotide sequence accession number. The nucleotide sequence generated for bla_{ADC-7} was deposited in GenBank (AY648950).

RESULTS AND DISCUSSION

Antimicrobial susceptibility profile of *A. baumannii*. Agar dilution antimicrobial susceptibility testing revealed that the representative *A. baumannii* isolate (clone 9) was resistant to ampicillin, piperacillin, and all cephalosporins tested (Table 2). Most notably, resistance was demonstrated against cephalothin, ceftazidime, cefepime, cefoxitin, and cefotaxime (MIC ≥ 32 μg/ml). This isolate was fully susceptible to sulbactam (4 μg/ml), ampicillin-sulbactam (4/2 μg/ml), imipenem-cilistatin (0.25 μg/ml), and meropenem (0.25 μg/ml). As expected, the MIC of ertapenem (2 μg/ml) was elevated for clone 9 of *A. baumannii* compared to the MICs of other carbapenems. The MIC for this isolate of sulopenem, a penem antibiotic formerly known as CP 65,207, was 0.5 μg/ml (NCCLS susceptibility standards are not yet available for this penem). To our surprise, MICs were 64/8 μg/ml for piperacillin-tazobactam and 16 μg/ml for tazobactam alone.

Susceptibility profile of *E. coli* DH10B expressing the ADC-7 β-lactamase. To evaluate the β-lactamase activity of bla_{ADC-7} in a uniform and defined genetic background (*E. coli* DH10B), we cloned bla_{ADC-7} from pET24a (+) into the pBC SK (+) vector. This construct possessed the promoter used in pET24a (+) (see above).

It was evident that ADC-7 β-lactamase expressed in *E. coli* DH10B contributed to ampicillin, piperacillin, cephalothin, and cefoxitin resistance (MICs ≥ 32 μg/ml) (Table 2). The MICs of ceftazidime and cefotaxime were both 4 μg/ml. Unlike with the parent *A. baumannii* isolate, cefepime resistance was not observed (MIC = 0.06 μg/ml). Furthermore, for *E. coli* DH10B, sulbactam and tazobactam MICs were >16 μg/ml. Their activities improved in β-lactam–β-lactamase inhibitor combinations with ampicillin or piperacillin (ampicillin-sulbactam MIC = 8/4 μg/ml and piperacillin-tazobactam MIC = 32/4 μg/ml). Meropenem, ertapenem, imipenem, and sulopenem remained active against *E. coli* DH10B possessing pBC SK (+) bla_{ADC-7} (MIC = 0.06 μg/ml). It is clear that even behind a strong promoter, resistance to cefepime, the penems, and carbapenems was not due to the β-lactamase.

***bla*$_{ADC-7}$ identification and analysis.** PCR primers used for detecting bla_{AmpC} enzymes related to CMY-2 (class C-1 and class C-2 primers) were unable to amplify AmpC-like β-lactamases. However, primers based upon the nucleotide sequence of bla_{AmpC} of *A. baumannii* described by Bou et al. (ABAMPC-1 and ABAMPC-2 primers) successfully amplified the bla_{ADC-7} gene (Tables 1 and 3) (6, 7).

DNA sequencing using primers specific for the *A. baumannii* cephalosporinase gene that we cloned into the pCR 2.1-TOPO vector revealed a β-lactamase with 8 amino acid differences compared to ABAC-1 (ADC-3) (Table 3) and *Acinetobacter* genomic species 3 (ADC-5) AmpC and 7 amino acid differences compared to ABAC-2 (ADC-4) (Table 3) (Fig. 1). The importance of these changes in amino acid sequence and their impact on the hydrolytic profile of ADC-7 are unknown. Given the high degree of similarity of the *bla* gene detected in Cleveland to the sequences of six *bla* genes present in *A. baumannii*, *Acinetobacter* genomic species 3, and *O. urethralis* and the resultant phylogenetic analysis, we propose a uniform designation for this family of cephalosporinases (*Acinetobacter-d*erived *c*ephalosporinases [ADC]) and identify this enzyme as ADC-7 β-lactamase (see below) (Table 3) (4, 7, 24, 38).

TABLE 4. Substrate and inhibitor profile of the ADC-7 β-lactamase

β-Lactam	K_m (or K_i)[a] (μM), corrected	V_{max} (μM s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Nitrocefin	53 ± 8	2.1 ± 0.1	1,750 ± 83	33 ± 3
Cephaloridine	45 ± 17	1.43 ± 0.2	1,189 ± 166	26 ± 6
Cephalothin	40 ± 11	1.5 ± 0.1	1,154 ± 80	29 ± 6
Cefoxitin	0.34 ± 0.09	0.09 ± 0.01	0.92 ± 0.03	2.7 ± 0.6
Cefepime	(594 ± 6)	NM		
Aztreonam	(0.7 ± 0.1)	NM		
Ceftazidime	(11.6 ± 1.6)	NM		
Cefotaxime	(0.10 ± 0.02)	NM		
Tazobactam	(91 ± 21)			
Sulbactam	(109 ± 3)			
Clavulanic acid	(4,275 ± 253)			
Imipenem	(2.0 ± 0.3)			
Sulopenem	(10.7 ± 2.0)			
Meropenem	(38 ± 4)			
Ertapenem	(3.8 ± 0.3)			

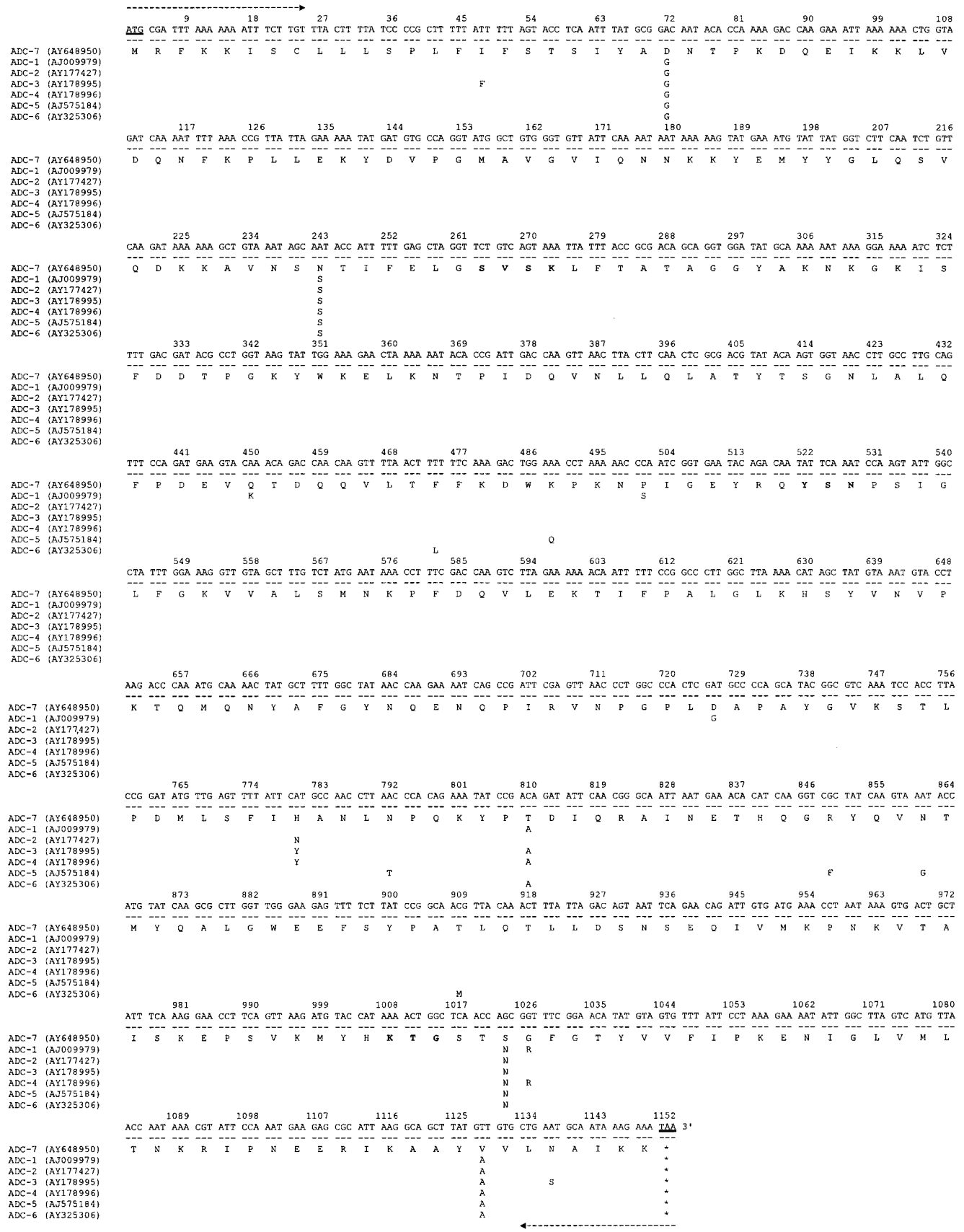
[a] The affinity of each of these substrates for the ADC-7 β-lactamase ("true K_i") was determined by performing a competition reaction using nitrocefin as the indicator substrate. NM, not measurable using 100 nM ADC-7 β-lactamase.

β-Lactamase induction. The induction of *A. baumannii* AmpC ADC-7 by cefoxitin was evaluated using a disk approximation method. As others have found, we were not able to show that the ADC-7 β-lactamase from this isolate was inducible (7).

β-Lactamase purification and characterization. The β-lactamase possessed a pI of ≥9.2. Purification of ADC-7 β-lactamase was possible from *E. coli* BL21(DE3) cells containing pET24a (+) bla_{ADC-7} grown in 500 ml Luria-Bertani broth. The estimated molecular mass based upon comparison to prestained protein standards was 41,000 Da. The calculated molecular mass of *A. baumannii* AmpC ADC-7 β-lactamase is 40,631 Da, and the predicted pI is 9.22 (www.expasy.org). The MALDI-TOF mass spectrum indicated a molecular mass of 40,540 Da (±0.2% error).

Substrate profile of ADC-7 β-lactamase. The substrate profile of ADC-7 β-lactamase closely resembled other AmpC

FIG. 1. Nucleotide sequence of the 1,152-bp amplification product of the ADC-7 β-lactamase. The deduced amino acid sequence of the ADC-7 β-lactamase is shown in single letter code below the nucleotide triplets. ATG and TAA represent the initiation and termination codons, respectively. The positions of the primers used to amplify bla_{ADC-7} are indicated by arrowheads. We have represented the ADC-7 β-lactamase active site as S-V-S-K in bold, the conserved triad K-T-G in bold, and the class C typical motif Y-X-N in bold. The predicted signal peptide cleavage site is between amino acids 23 and 24 (www.expasy.org). The GenBank accession numbers for each of the comparison cephalosporinase genes found in *Acinetobacter* spp. and *O. urethralis* are listed.


ADC-7 (AY648950)
ADC-1 (AJ009979)
ADC-2 (AY177427)
ADC-3 (AY178995)
ADC-4 (AY178996)
ADC-5 (AJ575184)
ADC-6 (AY325306)

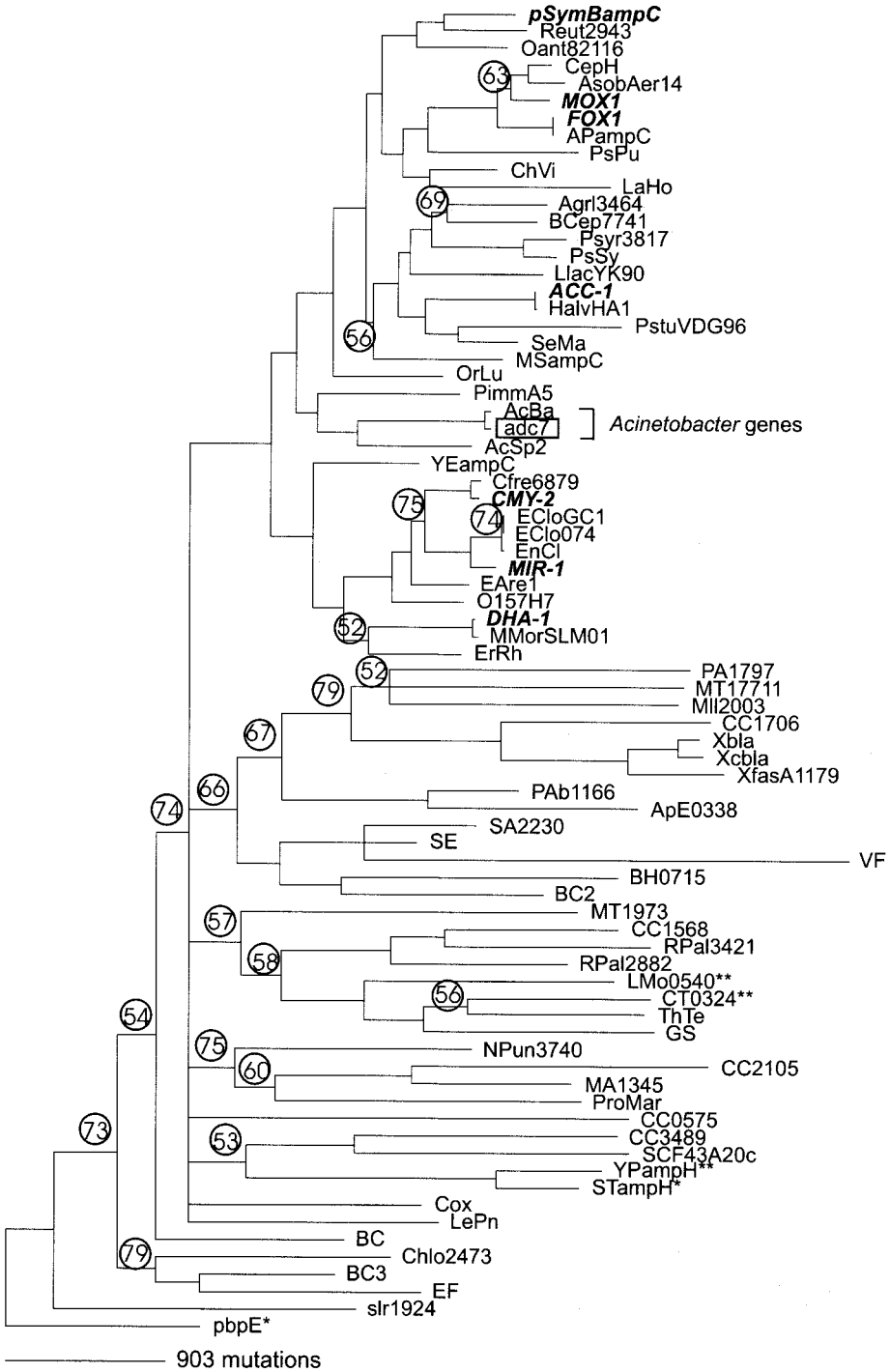


FIG. 2. Phylogenetic analysis of the ADC-7 β-lactamase. This phylogram, inferred through Bayesian analysis, represents an estimate of the relationships that exist among the class C β-lactamases and their homologs. Branch lengths are representative of the number of nucleotide mutations that have occurred since the divergence of the genes represented in this tree. Posterior probabilities for groupings that occurred in less than 80% of the trees sampled are indicated by a circle that contains the percentage of time during which that grouping did occur. Plasmidic resistance genes are indicated in boldface italics. A single asterisk represents a known penicillin binding protein, and a double asterisk represents putative penicillin binding proteins. The phylogram was based upon sequence data generated from AmpC β-lactamases in the supplemental material.

β-enzymes found in *Acinetobacter* spp. (30) (Table 4). Narrow-spectrum cephalosporins were the most efficiently hydrolyzed substrates. It is remarkable that the k_{cat}/K_m ratio for the hydrolysis of nitrocefin, cephaloridine, and cephalothin approaches the upper limits of catalytic efficiency for substrates by class C β-lactamases (30×10^6 M^{-1} s^{-1}) (8). These kinetic data are consistent with the determined MICs showing high-level cephalothin resistance.

Using the Agilent 8453 diode array spectrophotometer with a 1-cm path length, the hydrolysis of aztreonam, ceftazidime, cefepime, and cefotaxime was not measurable compared to the narrow-spectrum cephalosporins assayed. To assess the apparent affinity of these agents for ADC-7 β-lactamase, we employed a competition assay using nitrocefin as the indicator substrate. In this manner, we found that cefotaxime and aztreonam possessed the greatest affinities for ADC-7 β-lactamase (0.1 and 0.7 μM, respectively), while the affinity for ceftazidime was less (11.6 μM). Cefepime, a zwitterionic cephalosporin, demonstrated the most elevated K_i (>500 μM).

Since imipenem, meropenem, and ertapenem are β-lactams that contain large, ethoxy substituents in the 6α position, we reasoned that these substrates would serve as effective inhibitors of ADC-7 β-lactamase (3). As expected, our experiments revealed that imipenem, ertapenem, and meropenem were superior inhibitors of ADC-7 β-lactamase when compared to tazobactam, clavulanate, and sulbactam (Table 4). It is noteworthy that tazobactam and sulbactam demonstrated a modest ability to inhibit ADC-7 β-lactamase activity (5, 9). Sulopenem, a penem antibiotic that combines features of penicillin and cephalosporins, also has a low K_i against ADC-7 β-lactamase (15, 17). The singular property of carbapenems, sulfones, and penems to serve as inhibitors of penicillin binding proteins and class C enzymes merits consideration.

Phylogenetic reconstruction. Phylogenetic reconstruction of the AmpC β-lactamases shows the relationship of the *Acinetobacter ampC* genes to other *ampC* genes (Fig. 2). Because this tree is represented as a phylogram, the lengths of the branches represent the number of DNA mutations that have occurred since the divergence of the genes represented in this phylogeny. The number of mutations separating any two genes in this phylogeny can be estimated by summing the lengths of the horizontal lines necessary to draw a path from one gene to the other. Vertical lines do not represent the occurrence of mutations within genes; rather, vertical lines indicate that a divergence has occurred, and they serve as a marker for the relative point during *ampC* evolution at which that divergence occurred. The number of mutations separating any two genes is greater than the number of sites that differ between genes because multiple mutations can occur at any given site. Because of the way in which phylograms represent the evolutionary history of a gene, the vertical proximity of taxon labels to each other is not an accurate measure of the relatedness of taxa.

As illustrated by the phylogeny, the *Acinetobacter ampC* genes are descended from a common ancestor and are more closely related to each other than *ampC* genes found in other species of bacteria. Furthermore, the *Acinetobacter ampC* genes are separated from their closest relative, PimmA5, by nearly 2,000 mutations. The coalescence of *Acinetobacter ampC* genes to a single common ancestor and the substantial distance separating them from other *ampC* genes support the logical value of developing a system of nomenclature for these genes (see the supplemental material).

In conclusion, we have found a new *Acinetobacter* spp. cephalosporinase, ADC-7 β-lactamase. Among *Acinetobacter* genospecies, it is likely that this cephalosporinase is widespread and that many separate alleles exist. Based upon the kinetic behavior of this enzyme, it is becoming clear that extended-spectrum-cephalosporin resistance seems to be a consequence of the interplay of this enzyme and other inherent permeability properties related to the *Acinetobacter* sp. outer cell membrane (7, 30, 36). The resistance to cefepime in *A. baumannii* compared to what was seen in *E. coli* DH10B containing bla_{ADC-7} supports this notion. Investigations are under way to examine for the presence of integrons and insertion sequence elements (28, 39). Insertion sequence elements can cause increased expression of this class C β-lactamase and result in very high levels of cephalosporin resistance (24, 38).

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