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Inhibitor-Sensitive AmpC β-Lactamase Variant Produced by an *Escherichia coli* Clinical Isolate Resistant to Oxyiminocephalosporins and Cephamycins

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***Escherichia coli* HKY28, a ceftazidime-resistant strain isolated from a urine specimen in Japan, produced an inhibitor-sensitive AmpC β-lactamase variant. The deduced amino acid sequence of the enzyme contained a number of substitutions and a tripeptide deletion (Gly286-Ser287-Asp288) compared with the sequence of native AmpC of *E. coli*. When the deletion was reverted by a 9-base insertion at the relevant site of *ampC* in the clone, the typical inhibitor-resistant phenotype of AmpC was restored, while at the same time the levels of resistance to ceftazidime, cefpirome, and cefepime were reduced eightfold or more. Molecular modeling studies indicated that a structural change took place in the H-10 helix as a result of the deletion, and this change caused an alteration of the substrate binding site, leading to a unique phenotype analogous to that of inhibitor-sensitive class A extended-spectrum β-lactamases. The degree of inhibition was greater with sulbactam and tazobactam than with clavulanic acid. To our knowledge, this is the first report to have characterized an *E. coli ampC* that encodes chromosomal AmpC β-lactamase sensitive to the available β-lactamase inhibitors.**

The principal and most prevalent mechanism of resistance to β-lactam agents among pathogenic gram-negative bacteria is the production of β-lactamases (3, 17). One approach to overcoming the problem has been the development of β-lactams resistant to the hydrolytic activities of these enzymes. The other has been the development of β-lactamase inhibitors, which protect β-lactams from hydrolysis by β-lactamases when the inhibitors are used in combination with β-lactams (28). At present, three β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam, are available for clinical use in combination with a number of penicillins. These inhibitors mainly target Ambler class A β-lactamases and inactivate their active-site serines, thus potentiating the actions of β-lactamase-sensitive compounds. Clavulanic acid and sulbactam are generally not effective in inhibiting the activities of AmpC β-lactamases, although some are known to be moderately inhibited by tazobactam (4, 14).

In 1994, we isolated an *Escherichia coli* clinical strain, HKY28, which produced a chromosomal AmpC β-lactamase that had an inhibitor-sensitive and extended-spectrum activity profile similar to those of class A extended-spectrum β-lactamases (ESBLs). However, the results of PCR experiments with representative TEM- and SHV-derived ESBLs and CTX-M-type β-lactamases were negative. In the present study we conducted genetic, biochemical, and molecular modeling analyses of this unique AmpC β-lactamase variant.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* HKY28 was isolated from a culture of urine from an inpatient in Japan in 1994. *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) was used as the recipient strain for plasmids. *E. coli* BMH71-18mutS and *E. coli* MV1184 (Takara Bio Inc., Ohtsu, Japan) were used as the hosts in a site-directed mutagenesis experiment. Plasmid vectors pBCKS+ (Stratagene) and pKF18k (Takara Bio) were used for the cloning and site-directed mutagenesis experiments, respectively. For enzyme purification, *ampC*-deficient *E. coli* CS14-2 (7) was used as the host to avoid background AmpC production. Bacteria were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics, unless specified otherwise.

Antibiotics and susceptibility testing. The following β-lactam antibiotics and β-lactamase inhibitors were obtained from the indicated sources: aztreonam, Eizai Co., Ltd., Tokyo, Japan; ampicillin, amoxicillin, and cefminox, Meiji Seika Kaisha, Ltd., Tokyo, Japan; cefepime, Bristol Pharmaceuticals K. K., Tokyo, Japan; cefmetazole and chloramphenicol, Sankyo Co., Ltd., Tokyo, Japan; cefotaxime and cefpirome, Aventis Pharma, Ltd., Tokyo, Japan; cefoxitin and imipenem, Banyu Pharmaceutical Co., Ltd., Tokyo, Japan; ceftazidime and clavulanic acid, GlaxoSmithKline K. K., Tokyo, Japan; cephaloridine and moxalactam, Shionogi & Co., Ltd., Osaka, Japan; sulbactam, Pfizer Pharmaceuticals Inc., Tokyo, Japan; and tazobactam, Taiho Pharmaceutical Co., Ltd., Tokyo, Japan.

MICs were determined by the agar dilution method by the protocol recommended by the National Committee for Clinical Laboratory Standards (18).

PCR amplification. To amplify broad-spectrum β-lactamase genes from HKY28, PCR analysis was performed with sets of primers for various β-lactamases, including TEM- and SHV-derived ESBLs as well as CTX-M-1-, CTX-M-2-, and CTX-M-9-type β-lactamases, as described previously (27).

Transfer of ceftazidime resistance. Conjugation experiments were conducted with *E. coli* CSH2 as the recipient by broth mating and filter mating methods (7). Transconjugants were selected on LB agar supplemented with rifampin (50 μg/ml), nalidixic acid (50 μg/ml), and ceftazidime (4 μg/ml).

Cloning and sequencing of β-lactamase gene. The basic recombinant DNA manipulations were carried out as described by Sambrook et al. (24). The

genomic DNA of HKY28 was prepared and digested with EcoRI. The resultant fragments were ligated with plasmid vector pBCKS+, and electrocompetent *E. coli* XL1-Blue was transformed with these recombinant plasmids. Transformants were selected for resistance to chloramphenicol (30 μg/ml) and ceftazidime (4 μg/ml). For determination of the MICs and use of the transformants for site-directed mutagenesis, the *ampC* gene of HKY28 was amplified with oligonucleotide primers *ampC*-U (5′-CG**G AAT TC**G GTT TTC TAC GGT CTG GC-3′) and *ampC*-L (5′-CG**G GAT CC**G ATG ACA GCA AGG AAA AG-3′), which contained EcoRI and BamHI cleavage sites (indicated in boldface), respectively, at their 5′ ends, by using Pyrobest DNA polymerase (Takara Bio). The EcoRI-BamHI fragment containing the *ampC* gene of *E. coli* HKY28 was ligated with pBCKS+ to yield pBE28W, which was then used to transform *E. coli* XL1-Blue and *E. coli* CS14-2. The coding sequences of the cloned fragments were determined by using custom sequencing primers as well as a BigDye Terminator Cycle Sequencing Ready Reaction kits and an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, Calif.). The enzymes used for gene manipulations were purchased from Nippon Gene Co. Ltd. (Tokyo, Japan) or New England Biolabs, Inc. (Beverly, Mass.).

Reversion of AmpC deletion. Site-directed mutagenesis was performed to revert the 9-nucleotide deletion in the cloned *ampC* gene of *E. coli* HKY28 corresponding to a tripeptide deletion at positions 286 to 288 in AmpC. The reagents and strains contained in the Mutan-Express Km mutagenesis kit (Takara Bio) were used according to the procedures based on the oligonucleotide-directed dual Amber method (9) provided by the manufacturer. The following mutagenic primer containing the 9-nucleotide insertion (in boldface) was used: 5′-CCA GTG CAA TTT TAT TG**T CAC TGC CG**T TAA TGA TGA TGT CAG G-3′. After mutagenesis, the EcoRI-BamHI fragment containing the revertant *ampC* was ligated with pBCKS+ to yield pBE28R, which was then used to transform *E. coli* XL1-Blue and *E. coli* CS14-2.

Enzyme purification. *E. coli* CS14-2 harboring pBE28W or pBE28R was cultured overnight in 2 liters of LB broth supplemented with 30 μg of chloramphenicol per ml. Cells were harvested by centrifugation and washed with and then suspended in 3 ml of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 6.0). The cells were frozen and thawed twice and were then ultracentrifuged at 100,000 × *g* for 4 h at 4°C. For gel filtration, the supernatant containing β-lactamase was chromatographed through a HiLoad 16/60 Superdex 200 prepgrade (Pharmacia Biotech, Uppsala, Sweden) column preequilibrated with 50 mM MOPS buffer (pH 6.0). For cation-exchange chromatography, fractions with activity were then applied to a HiTrap SP HP column (Pharmacia Biotech) preequilibrated with the same buffer. The enzymes were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer. The purity of the enzymes was checked by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Enzyme assays. Purified AmpC enzymes were assayed against various β-lactam substrates at 37°C in 50 mM phosphate buffer (pH 7.0) by using an autospectrophotometer (V-550; Nihon Bunko Ltd., Tokyo, Japan). The specific activity of the enzymes was defined as the activity that hydrolyzed 1 μmol of cephaloridine per min. K_m and k_{cat} values were obtained by a direct-weight fit to the Michaelis-Menten equation by using KaleidaGraph software (Hulinks, Tokyo, Japan). The concentrations of inhibitors giving a 50% reduction in hydrolysis of cephaloridine (IC_{50}) were measured after 10 min of preincubation of the enzymes with the inhibitors at 37°C and cephaloridine as the substrate at 1 mM. The affinities of the enzymes for the inhibitors (K_is) were measured by competition procedures with cephaloridine in the same buffer with no preincubation of the enzyme or the inhibitor. To determine the isoelectric points, 10 μl of enzyme solution was loaded onto an Immobiline DryStrip (pH 3 to 10 and 6 to 11; Pharmacia Biotech), and electrophoresis was carried out with an IPGphor electrophoresis system (Pharmacia Biotech).

Modeling of substrate-enzyme complex structures. The crystal structure of the AmpC β-lactamase (Protein Data Bank accession number 2BLS) was used as the reference to build a model of the AmpC enzyme of *E. coli* HKY28. The tripeptide at the H-10 helix was deleted by the loop search method of the Homology module installed in Insight II software (version 2000; Molecular Simulations Inc., San Diego, Calif.). An initial structure of the enzyme was optimized by use of molecular dynamics calculations at 298 K by the cell multipole method, a distance-dependent dielectric constant, and a time step of 1 fs for 100 ps by sampling the conformation every 1 ps by use of Discover 3 software (version 98.0; Molecular Simulations Inc.). One hundred conformations were minimized until the final root-mean square deviation became less than 0.1 kcal/mol/Å, and the lowest energy conformation was selected for the substrate-docking study. The substrates were roughly docked into the ligand-binding cleft with the guidance of a hydrogen bond of a β-lactam carbonyl oxygen at the oxyanion hole as well as a hydrogen bond of the carboxylate oxygen with Tyr150 (12). The initial complex model was minimized, and then the substrate-binding site was covered by water molecules (sphere thickness, 20 Å). The structure consisted of the substrate and the residues within 10 Å from the substrate, which were energy optimized in the presence of the water molecules by the molecular dynamics and minimization procedure described above. The lowest-energy structures were selected as energy-refined complex models.

Nucleotide sequence accession number. The nucleotide sequence encoding AmpC characterized in this study appears in the EMBL/GenBank/DDBJ databases under accession number AB108683.

RESULTS

Susceptibility of parental strain. The MICs of β-lactams for parental strain *E. coli* HKY28 are shown in Table 1. Strain HKY28 was resistant to ampicillin, amoxicillin, cephaloridine, cefminox, and cefoxitin. It was also resistant to ceftazidime (MIC, 32 μg/ml) but remained susceptible to aztreonam and imipenem. Interestingly, the MIC of ampicillin was reduced by at least 8-fold when it was combined with sulbactam, and the MICs of cefotaxime were reduced by 16- and 8-fold when it was combined with sulbactam and tazobactam, respectively. Addition of sulbactam reduced the MIC of ceftazidime by eightfold. Overall, the reductions in the MICs were the greatest with sulbactam, followed by tazobactam and clavulanic acid.

PCR analysis of β-lactamase genes. By PCR *E. coli* HKY28 was negative for the genes for the TEM-, SHV-, CTX-M-1-, CTX-M-2- and CTX-M-9-type β-lactamases, which are the prevalent types of ESBLs in Japan.

Transfer of ceftazidime resistance. The ceftazidime resistance of *E. coli* HKY28 could not be transferred to recipient *E. coli* strain CSH2 by conjugation, despite repeated attempts.

Cloning and sequencing of resistance gene. A 6-kb EcoRI fragment containing a ceftazidime resistance determinant was cloned into the vector pBCKS+ and was termed pE753. Nucleotide sequencing analysis revealed a chromosomal locus of *E. coli* containing *ampC* flanked by *frdD* and *blc* but without any other β-lactamase gene. PCR-generated recombinant plasmid pBE28W containing *ampC* of *E. coli* HKY28 was found to possess an *ampC* gene identical to that of pE753 and conferred resistance to ceftazidime. The deduced amino acid sequence contained seven amino acid substitutions and three amino acid deletions (Gly286, Ser287, and Asp288) of the AmpC product compared with the sequence of *E. coli* K-12 (10) (Fig. 1). The promoter region of the *ampC* gene contained three mutations (a C-to-T change at position −73, a C-to-T change at position +6, and a G-to-A change at position +34) and a T insertion between positions −14 and −13 compared with the sequence of the corresponding region of the *E. coli* K-12 genome. Recombinant plasmid pBE28R, generated by site-directed mutagenesis, was confirmed to possess *ampC* of *E. coli* HKY28, except for the insertion of the 9-nucleotide sequence designed to restore the tripeptide deleted from *ampC* of *E. coli* HKY28.

Susceptibilities of clones to β-lactams. Both *E. coli* XL1-Blue harboring pBE28W (the HKY28 clone) and that harboring pBE28R (the revertant clone) displayed resistance or reduced susceptibilities to all β-lactams except cefpirome, cefepime, and imipenem; but the degree of resistance varied significantly between the two clones. The cefotaxime and ceftazidime MICs were fourfold or more higher for the HKY28 clone than for the revertant clone. The cefpirome and cefepime MICs were 64-fold higher for the HKY28 clone than

TABLE 1. Results of antibiotic susceptibility testing

β-Lactam	MIC (μg/ml)			
	E. coli HKY28	*E. coli* XL1-Blue(pBE28 W)	*E. coli* XL1-Blue(pBE28 R)	*E. coli* XL1-Blue
Amoxicillin	128	>128	>128	4
Amoxicillin-clavulanate[a]	128	>128	>128	4
Ampicillin	>128	>128	>128	2
Ampicillin-sulbactam[b]	32	64	>128	2
Piperacillin	8	8	8	0.5
Piperacillin-tazobactam[c]	4	2	4	0.5
Cefotaxime	16	32	8	0.06
Cefotaxime-clavulanate[a]	8	8	2	0.06
Cefotaxime-sulbactam[b]	1	1	2	0.06
Cefotaxime-tazobactam[c]	2	4	4	0.06
Ceftazidime	32	128	16	0.06
Ceftazidime-clavulanate[a]	16	32	8	0.13
Ceftazidime-sulbactam[b]	4	8	8	0.06
Ceftazidime-tazobactam[c]	16	8	8	0.13
Cephaloridine	64	128	128	4
Cefminox	32	32	32	0.5
Cefoxitin	16	32	>128	8
Cefmetazole	16	32	128	1
Moxalactam	8	4	8	0.25
Cefpirome	2	4	0.03	0.015
Cefepime	2	4	0.03	0.015
Aztreonam	8	16	16	0.06
Imipenem	0.13	0.13	0.13	0.13

[a] Fixed concentration of clavulanate, 4 μg/ml.
[b] Fixed concentration of sulbactam, 4 μg/ml.
[c] Fixed concentration of tazobactam, 4 μg/ml.

for the revertant clone. On the other hand, the degree of resistance to cefoxitin and cefmetazole conferred by the revertant clone was significantly higher than that conferred by the HKY28 clone. When various β-lactam–β-lactamase inhibitor combinations were tested, the piperacillin, cefotaxime, and ceftazidime MICs for the HKY28 clone were reduced by up to 16-fold. The degree of reduction was the greatest when sulbactam was used as the inhibitor. The reductions in the MICs of the three inhibitors for the revertant clone were fourfold or less.

Isoelectric focusing. The isoelectric points were estimated to be 9.9 for the HKY28 AmpC ($AmpC^D$) and 9.8 for the revertant AmpC ($AmpC^R$). When the crude extract of *E. coli* HKY28 was subjected to analytical isoelectric focusing, only one band corresponding to $AmpC^D$ was visualized with nitrocefin, confirming that $AmpC^D$ is the only β-lactamase produced by *E. coli* HKY28 (data not shown).

Enzyme assays. The specific activites of $AmpC^D$ and $AmpC^R$ were 88 and 220 U/mg of protein, respectively. The kinetic parameters (K_m and k_{cat}) and hydrolytic efficiencies (k_{cat}/K_m) of $AmpC^D$ and $AmpC^R$ against various β-lactams are given in Table 2. The k_{cat} values of $AmpC^D$ were greater than those of $AmpC^R$ for cefpirome and cefepime but lower for the rest of the substrates tested. However, for all substrates with the exception of cefotaxime, $AmpC^D$ exhibited lower K_m values than $AmpC^R$. This difference was approximately 100-fold for ceftazidime, and overall, $AmpC^D$ showed a 2.5-fold greater hydrolytic efficiency for ceftazidime than $AmpC^R$, despite the much poorer k_{cat}. $AmpC^D$ exhibited much lower K_m values and higher k_{cat} values for cefpirome and cefepime than $AmpC^R$, resulting in approximately 40- and 20-fold greater hydrolytic efficiencies, respectively.

The IC_{50}s of the β-lactamase inhibitors for $AmpC^D$ and $AmpC^R$ and the K_i values of the enzymes against the inhibitors are listed in Table 3. $AmpC^D$ exhibited approximately 5- to 10-fold lower K_i values than $AmpC^R$ against all three inhibitors. Tazobactam was the best inhibitor and had the lowest IC_{50} for $AmpC^D$.

TABLE 2. Kinetic activity of $AmpC^D$ and $AmpC^R$

Substrate	$AmpC^D$			$AmpC^R$		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Cephaloridine	100 ± 10	64 ± 4	6.4×10^5	780 ± 40	300 ± 10	3.9×10^5
Ampicillin	6.2 ± 1.6	0.43 ± 0.06	7.0×10^4	13 ± 3	4.0 ± 0.3	3.2×10^5
Cefoxitin	1.2 ± 0.3	0.043 ± 0.006	3.8×10^4	3.9 ± 0.1	0.35 ± 0.01	9.1×10^4
Ceftazidime	5.7 ± 0.8	0.084 ± 0.006	1.5×10^4	550 ± 10	3.5 ± 0.1	6.4×10^3
Cefotaxime	31 ± 8	0.37 ± 0.02	1.2×10^4	13 ± 2	1.2 ± 0.1	9.7×10^4
Cefpirome	21 ± 1	1.5 ± 0.1	7.1×10^4	120 ± 20	0.21 ± 0.03	1.8×10^3
Cefepime	49 ± 5	1.0 ± 0.1	2.1×10^4	200 ± 40	0.21 ± 0.02	1.1×10^3

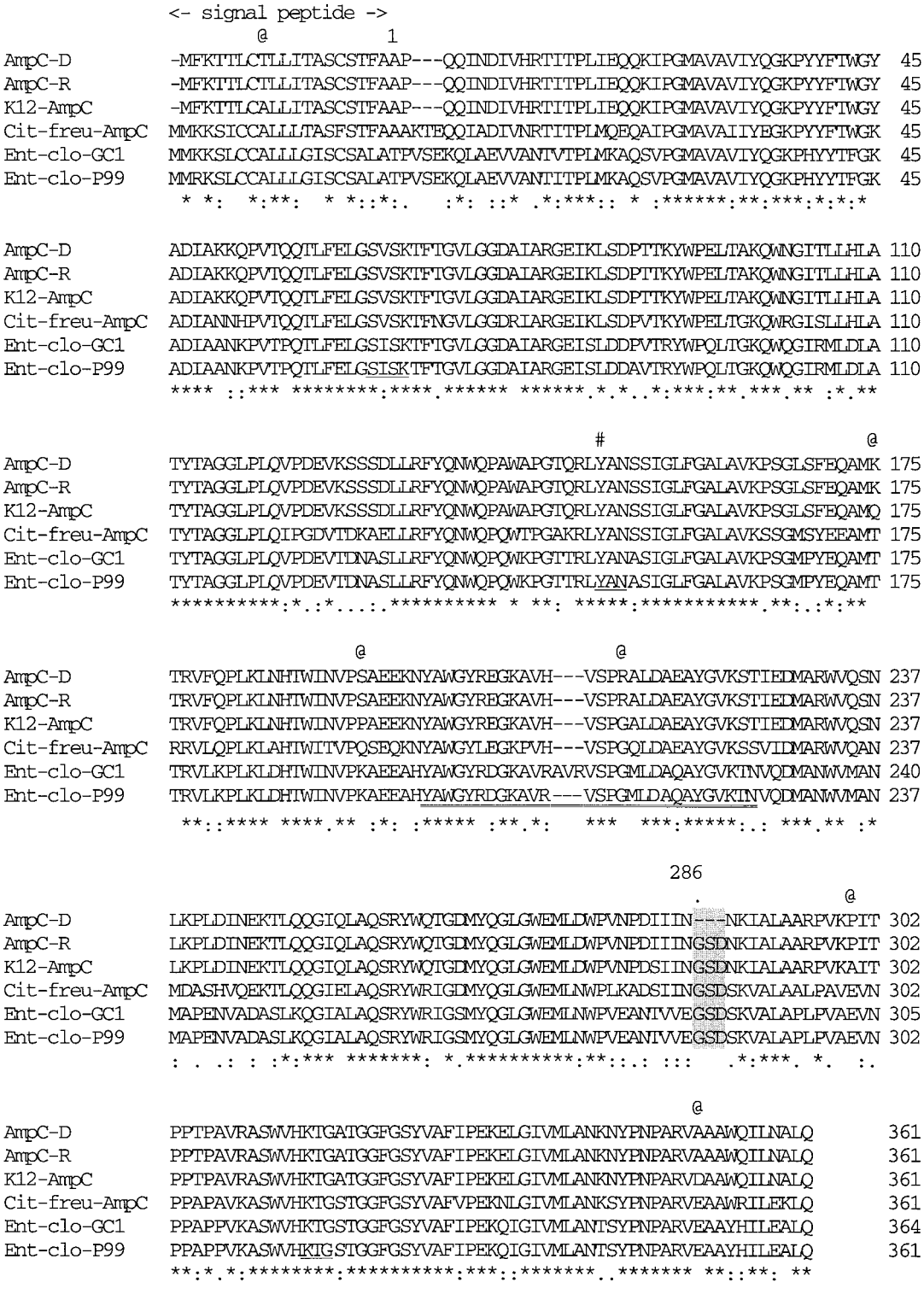


FIG. 1. Predicted amino acid sequence of the AmpC β-lactamase of *E. coli* HKY28 aligned with that of *E. coli* K-12 (10). The 3-amino-acid deletion in the HKY28 AmpC is shaded. Underlines, the β-lactamase active site SVSK, the conserved tripeptide KTG, and the class C motif YXN; #, position of Tyr150; @, positions of the amino acid substitutions observed between the $AmpC^D$ of strain HKY28 and the AmpC of strain K-12; numbers on the right, numbers of amino acid residues from the N terminus of each mature protein; ∗, amino acid residues conserved among the six AmpC-type enzymes; colons and dots, amino acid substitutions that result in homologous amino acid residues; Cit-freu, *Citrobacter freundii*; Ent-clo, *E. cloacae*; double underline, AmpC Ω-loop domain.

TABLE 3. IC_{50}s and K_i values of β-lactamase inhibitors for $AmpC^D$ and $AmpC^R$

β-Lactamase	Clavulanic acid		Sulbactam		Tazobactam	
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
$AmpC^D$	19 ± 1	320 ± 30	3.9 ± 0.2	9.2 ± 0.2	1.4 ± 0.1	8.7 ± 2.4
$AmpC^R$	140 ± 20	4,100 ± 1,600	24 ± 4	780 ± 150	25 ± 1	1,100 ± 120

Molecular modeling study. A molecular modeling study was conducted to elucidate the mechanism for the lower K_m of $AmpC^D$ for ceftazidime (Fig. 2). In the AmpC of *E. coli* K-12, the tripeptide Gly286-Ser287-Asp288 loops out in the direction of ceftazidime (Fig. 2B). Conversely, the tripeptide deletion in $AmpC^D$ creates an open site in the vicinity of the R-2 side chain of ceftazidime (Fig. 2C). Similar models were obtained for cefpirome and cefepime (data not shown).

DISCUSSION

E. coli HKY28 produced an AmpC β-lactamase which conferred resistance to ceftazidime and reduced susceptibility to cefotaxime (MICs, 32 and 16 μg/ml, respectively). This resistance was significantly compromised by the β-lactamase inhibitors sulbactam and tazobactam and to some extent by clavulanic acid. This was an uncommon finding, since *E. coli* rarely acquires resistance to ceftazidime solely by the production of chromosomal β-lactamase. Also, the AmpC β-lactamase, which belongs to Ambler class C β-lactamases, is not usually inhibited well by β-lactamase inhibitors. We therefore investigated the AmpC β-lactamase of the strain.

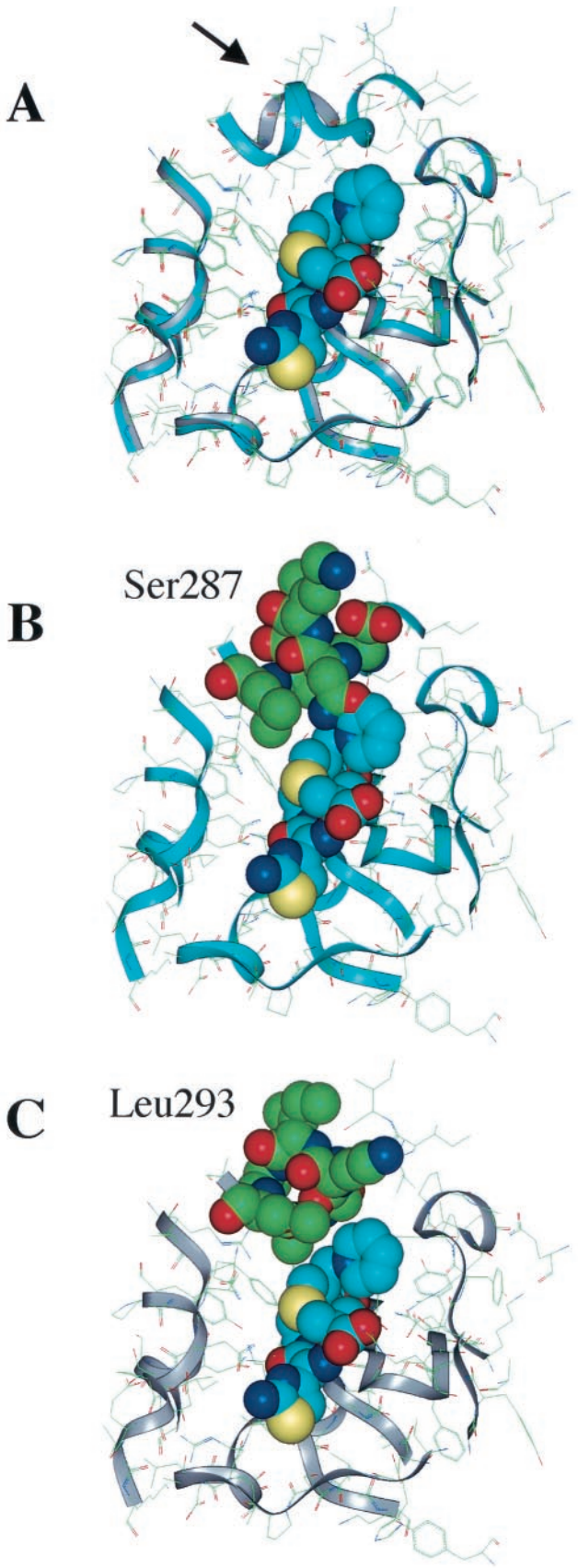
When the *ampC* gene was cloned and expressed in *E. coli* XL1-Blue, it conferred resistance to ceftazidime and cefotaxime, and the resistance could be reversed by any of the three commercially available β-lactamase inhibitors. Sulbactam and tazobactam were much more potent inhibitors in terms of lowering the MICs than clavulanic acid, a distinct profile compared with those of class A ESBLs, which are generally inhibited well by any of the three inhibitors (4).

Sequencing of the entire *ampC* structural gene of *E. coli* HKY28 revealed the presence of seven amino acid alterations and a tripeptide deletion at positions 286 to 288 corresponding to Gly-Ser-Asp in the deduced amino acid sequence of AmpC (Fig. 1). None of the substituted residues has been implicated in playing a functional role in the hydrolysis of β-lactams (23, 25). On the other hand, residues 287 to 289, which overlap the residues deleted from $AmpC^D$, is known to be positioned in close proximity to R-2 substituents of β-lactams (16). The levels of resistance to ceftazidime and cefotaxime were reduced by 4-fold or more, while those of newer oxyiminocephalosporins, such as cefepime and cefpirome, were also reduced by 64-fold for the revertant clone producing $AmpC^R$. The three β-lactamase inhibitors no longer reversed resistance to cefotaxime and ceftazidime in the revertant clone. $AmpC^R$ has a G214R substitution in the so-called Ω loop, and this substitution may have some influence on the expansion of substrate specificity, especially for cephamycins such as cefoxitin and cefmetazole. The kinetic values of $AmpC^R$ for broad-spectrum cephalosporins, including cefotaxime, ceftazidime, cefepime, and cefpirome, as well as cephamycins, such as cefoxitin, indicate that $AmpC^R$ certainly has some unusual properties. Some of the five amino acid substitutions found in $AmpC^R$ might contribute to such a phenotype. In addition, the three amino acid deletions at the H-10 domain observed in $AmpC^D$ might provide this enzyme with a special characteristic, such as enhanced susceptibility to β-lactamase inhibitors and an augmented ability to hydrolyze ceftazidime, cefepime, and cefpirome. However, the deletion might result in a decrease in the ability to hydrolyze cephamycins.

The results of the kinetics studies were very much in accordance with the susceptibility profiles. $AmpC^D$ generally exhibited lower K_m values than $AmpC^R$ against all substrates tested except cefotaxime. These reductions in K_m values were accompanied by compromised k_{cat} values, with the exception of those for cefpirome and cefepime. $AmpC^D$ showed both lower K_m values and greater k_{cat} values for these two agents, resulting in 40- and 20-fold better hydrolytic efficiencies, respectively, compared with those of $AmpC^R$. The kinetic data for cefotaxime did not correlate well with the MICs. A similar observation was reported for an atypical AmpC of an *Enterobacter cloacae* clinical isolate lacking 6 amino acids at positions 289 to 294, located adjacent to the deletion identified in $AmpC^D$ (2). By consideration of the fact that these data were obtained for two clones which differed only by the presence and the absence of the 3 amino acids in AmpC, one possibility is that $AmpC^D$ is unstable.

The results of inhibition studies confirmed the role of the Gly286-Ser287-Asp288 deletion in the increased sensitivity of $AmpC^D$ to all three commercially available β-lactamase inhibitors. The tripeptide deletion in $AmpC^D$ was shown to lower the K_i values against the inhibitors by approximately 10- to 100-fold. In terms of IC_{50}s, sulbactam and tazobactam were potent inhibitors of $AmpC^D$, whereas clavulanic acid only mildly inhibited the enzyme. The AmpC β-lactamase of *E. cloacae* P99 is inhibited well by tazobactam but is inhibited only modestly by sulbactam and is hardly inhibited at all by clavulanic acid (4). In this respect, $AmpC^D$ is an AmpC β-lactamase that is unusually sensitive, especially to sulbactam.

Gly286-Ser287-Asp288 is located in the H-10 helix of AmpC (16). While the functional roles of these residues in the catalytic mechanism have not been clearly elucidated, Asp288 of the *E. coli* AmpC has been suggested to play a role in recognizing the carboxylate group of β-lactams (23, 25). In native AmpC, Ser287 forms hydrogen bonds with Asn346 and Arg349 (23), but these bonds are lost in $AmpC^D$, along with the deletion of Asp288. The result of the molecular modeling study provided a structural explanation for the lowered K_m of $AmpC^D$ for ceftazidime, as shown in Fig. 2. In the *E. coli* K-12 AmpC, the tripeptide Gly286-Ser287-Asp288 impeded access of ceftazidime to the active site of the enzyme, resulting in high K_m values, whereas the tripeptide deletion in $AmpC^D$ was



found to provide an open site where the R-2 side chain of ceftazidime could readily be accommodated. This explains the significantly lower K_m for AmpC^D compared with that for AmpC^R.

While inducible chromosomal AmpC β-lactamases are known to confer resistance to oxyiminocephalosporins and cephamycins by mutations in their regulator genes that lead to derepressed production of the enzymes in many species of gram-negative bacteria, only a few AmpC enzymes with altered substrate specificities have been reported to date (17). The extended-spectrum AmpC produced by *E. cloacae* GC1 contained a tripeptide insertion of a tandem repeat, Ala211-Val212-Arg213, in the Ω loop (6, 20). It was suggested that the conformational flexibility in the expanded Ω loop facilitates hydrolysis of oxyiminocephalosporins (6). It is noteworthy that an AmpC with extended resistance to cefepime and cefpirome was recently described from an *E. cloacae* clinical isolate, as mentioned above (2). A deletion of 6 amino acids (Ser, Lys, Val, Ala, Leu, and Ala) from positions 289 to 294 was likely responsible for the extension of the spectrum of activity. The enzyme showed approximately 10 times higher hydrolytic efficiency for the oxyiminocephalosporins than the P99 β-lactamase did, mostly due to lower K_m values. This amino acid deletion is in close proximity to that in the AmpC^D studied here, both of which are located in the H-10 helix. Therefore, it is not surprising that the two enzymes share similar kinetic characteristics.

E. coli is known to constitutively produce only an insignificant amount of chromosomal AmpC β-lactamase, due to relatively weak promoter activity and the presence of a transcriptional attenuator (11, 13). However, occasional isolates produce large amounts of the enzyme and become resistant to various β-lactams, including ceftazidime. This overproduction could result from gene amplification (8) or the acquisition of a stronger promoter region (21, 22); but most commonly it results from mutations that take place in the promoter region at positions such as −42, −32, and +24, which lead to enhanced transcription of *ampC* (5, 19). These modifications in transcription typically lead to moderately elevated ceftazidime MICs (13). The nucleotide sequence of the promoter and attenuator regions of the *ampC* gene of *E. coli* HKY28 revealed the presence of three mutations (a C-to-T change at position −73, a C-to-T change at position +6, and a G-to-A change at position +34) and a T insertion between positions −14 and −13. The first two mutations have not been implicated as a cause of increased *ampC* transcription, while a nucleotide insertion between −35 and −10 hexamers is known to enhance AmpC transcription, possibly by bringing the distance between the hexamers to the optimal 17 bp (5, 11). Therefore, it is likely that the insertion of a T residue between positions −14 and

FIG. 2. Optimized ribbon structures of ceftazidime docked in the active site of HKY28 AmpC (gray) compared with that of *E. coli* K-12 AmpC (light blue) (10). (A) The two structures are superimposed. The Gly286-Ser287-Asp288 deletion in the HKY28 AmpC creates an open space at the top of the binding site (arrow) that allows the accommodation of the R-2 side chain of ceftazidime in the *E. coli* K-12 AmpC and the R-2 side chain of ceftazidime collides with Ser287(B), but in the HKY28 AmpC (C) it comes near Leu293 but does not make direct contact.

−13 caused the hyperproduction of $AmpC^D$, explaining in part the ceftazidime resistance displayed by *E. coli* HKY28.

Class A ESBLs are inhibited well by the β-lactamase inhibitors clavulanic acid and sulbactam, a characteristic that serves to differentiate them from other β-lactamases, including AmpC (15). The K_i values of class A enzymes for the inhibitors are in the nanomolar range, but those of the $AmpC^D$ enzyme reported in the present study are in the micromolar range. Anyway, $AmpC^D$ acquired considerable sensitivity to inhibition by sulbactam and tazobactam but acquired sensitivity to inhibition by clavulanic acid to a much lower degree, as it extended its spectrum to cephalosporins, including ceftazidime. A few other studies have also reported on the isolation of *E. coli* strains displaying similar inhibitor-sensitive phenotypes, but their mechanisms remain to be described (1, 26). It would be interesting to know if they produce AmpC variants with characteristics similar to those of the AmpC described in the present study.

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