

Extended-Spectrum Cephalosporinases in *Enterobacteriaceae*

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Abstract: Extended-spectrum AmpC β -lactamases are derived from chromosomal cephalosporinases by amino acid deletion, insertion and replacement. These structural changes are responsible for an increased catalytic efficiency against extended-spectrum cephalosporins, such as ceftazidime, cefotaxime, cefepime, and ceftipime. An overview of the molecular and biochemical characterization of these identified β -lactamases in *Enterobacteriaceae* is provided. The structural modifications that account for the broadening substrate specificity and the phenotypes of resistance of the clinical isolates are detailed.

Keywords: ESAC, extended-spectrum cephalosporinase, cefepime, ceftazidime.

1. INTRODUCTION

Most class C β -lactamases produced by Gram-negative bacteria hydrolyze many β -lactam antibiotics *in vitro*, including cephamycins (cefotaxime, cefotetan) and oxyiminocephalosporins, such as ceftazidime (**1**), cefotaxime (**2**), and ceftriaxone (**5**) (Fig. 1), and monobactams, such as aztreonam but usually to a less extent [1,2].

In many species of the *Enterobacteriaceae* family, including *Enterobacter sp.*, *Citrobacter freundii*, *Providencia sp.*, *Morganella morganii*, *Hafnia alvei*, *Serratia marcescens* the expression of chromosomal *ampC* genes is low and inducible in response to β -lactam addition, which is related to a LysR regulator located upstream and divergently from the cephalosporinase gene [3]. *Escherichia coli* behaves differently since its chromosomal AmpC is normally undetectable [4,5] due to a weak promoter as well as a transcriptional attenuator.

The production of AmpC β -lactamases at low level conferred resistance to aminopenicillins and early generation cephalosporins (such as cephalothin). Spontaneous mutations affecting the regulatory genes, most frequently *ampD* [6,7], that codes for an amidase, induce constitutive overproduction of the enzyme and an increase resistance to several extended-spectrum β -lactams, such as oxyiminocephalosporins (cefotaxime (**2**), cefuroxime (**6**), ceftriaxone (**5**), and ceftazidime (**1**)) [8,9]. Most of these compounds are hydrolyzed efficiently in presence of overproduction of the enzymes because of their high affinity for the AmpC enzyme that compensates their low deacylation rates [10]. AmpC-overproducing strains have also usually reduced susceptibilities to expanded-spectrum cephalosporins such as cefotaxime (**2**), ceftriaxone (**5**), and ceftazidime (**1**).

Zwitterionic cephalosporins (cefepime (**3**) and ceftipime (**4**)) and carbapenems (imipenem, ertapenem, and mero-

penem), which penetrate very efficiently through the outer-membrane of Gram-negative bacteria [11] and are poor substrates for AmpC β -lactamases, are active *in vitro* against organisms producing high levels of cephalosporinases.

Recently, a novel mechanism of resistance due to the production of cephalosporinases with broadened substrate activity has been reported among clinical enterobacterial isolates [12-23]. Those extended-spectrum AmpC β -lactamases (ESACs) confer reduced susceptibility to all cephalosporins including not only ceftazidime (**1**), but also cefepime (**3**) and ceftipime (**4**). These enzymes are structurally related to cephalosporinases by either amino acid insertions [22-24], deletions [13, 14, 20], or substitutions [12, 15, 16, 18].

This review summarizes the data available concerning the ESACs.

2. STRUCTURE OF CEPHALOSPORINS

It is mandatory, before detailing the hydrolysis spectrum of ESACs, to introduce the structure of the extended-spectrum cephalosporins. To protect the β -lactam ring from hydrolysis by β -lactamases, cephalosporins with a 7-oxyimino moiety on the side chain of the cephem nucleus have been synthesized, and these have been shown to be stable.

Whereas different in size, electrostatic charge, and side chain stereochemistry, ceftazidime (**1**) is the β -lactam antibiotic that is most similar to cefepime (**3**) (Fig. 1) [25]. Ceftazidime (**1**), exceptionnally among the oxyiminocephalosporins introduced in the 1980s, has a relatively low affinity even against *E. cloacae* and *E. coli* cephalosporinases (with K_m values of around 4.0 mM [26,27] and 16 mM [28] for the two enzymes, respectively). This is probably due at least in part to the presence of the pyridinium side chain at the 3' position, a feature similar to those uniformly present in the oxyiminocephalosporins developed to exhibit lower affinity toward AmpC enzymes, such as ceftipime (**4**) and cefepime (**3**) [29].

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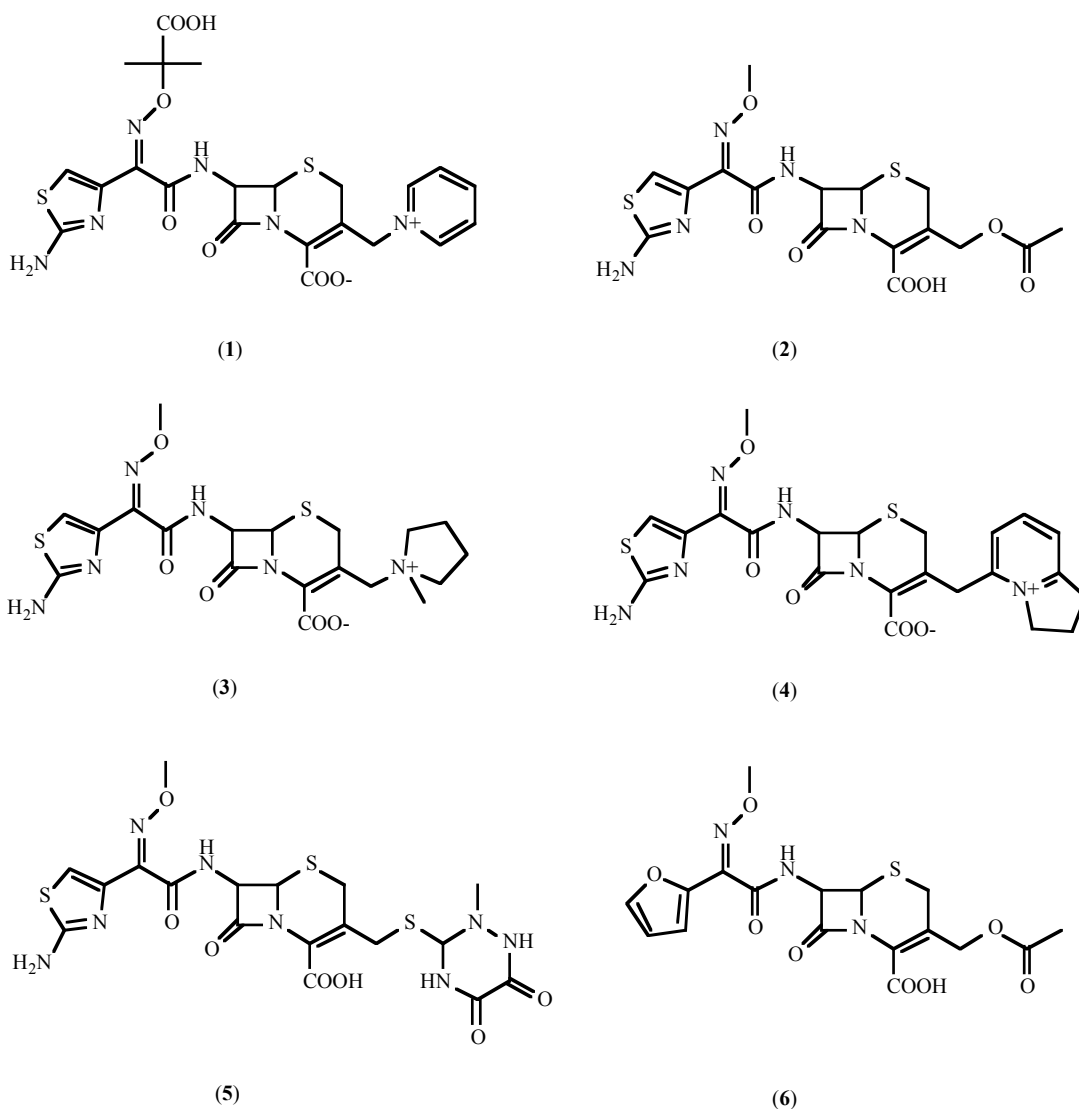


Fig. (1). Structures of the oxyminocephalosporins. Ceftazidime (1); Cefotaxime (2); Cefepime (3); Cefpirome (4); Ceftriaxone (5); Cefuroxime (6).

3. DESCRIPTION OF ESAC-PRODUCING CLINICAL ISOLATES

Fourteen enterobacterial isolates producing ESAC β -lactamases have been so far reported. Their clinical features and corresponding recombinant clones are summarized in the Table 1. *K. pneumoniae* HKY363, HKY466, HKY474, that had been isolated in April 1995, June 1995, and October 1996, were not presented in the Table 1 since they shared the same characteristics with *K. pneumoniae* HKY327 [19].

4. GENETICS

Most ESAC enzymes are very likely chromosomally encoded. Repeated attempts to transfer *bla*_{AmpC} genes from *E. coli* KL and *E. coli* HKY28 into recipient *E. coli* strains using conjugation or transformation experiments failed [12,20]. No further molecular experiment was performed to confirm the location of *bla*_{AmpC} genes. However, the identification using PCR of acquired mutations in the natural chromosome-borne *ampC* genes of the isolates constituted a strong evi-

dence that *bla*_{AmpC} genes were very likely chromosomally located. The presence of the *bla*_{ESAC} genes on non-transferable genetic elements does not allow dissemination of these resistant determinants and may explain, in part, sporadic identification.

Recently, the extended-spectrum cephalosporinase CMY-19 that is carried by the conjugative plasmid pCMXR1 [30], has been characterized [19]. The *bla*_{CMY-19} gene, that derived from the *bla*_{CMY-9} gene by point mutation, was located onto a *sull*-type integron [30, 31] that included the 2.1-kb so-called common region (CR) containing *orf513*. The *bla*_{CMY-19} gene is closely related to *bla*_{CMY-8}, *bla*_{CMY-1}, and *bla*_{MOX-1} genes [30]. The origin of these plasmid-borne *ampC* genes remains unclear, although the degree of homology with chromosomally-encoded *ampC* genes of *Aeromonas sobria* and *Aeromonas hydrophila* appears to be high enough to assume a phylogenetic lineage. It is believed that MOX-1, CMY-1, CMY-8, CMY-9, CMY-19 were mobilized from the *Aeromonadaceae* group, although the precise reservoir species is not yet determined [32]. It has been speculated that these

Table 1. Clinical Features and Cephalosporinase Identification of the ESAC-Producing Clinical Isolates

Clinical isolates	Corresponding recombinant clones	AmpC denomination	Date and country of isolation	Isolate source	Infection or colonization	Antibiotic treatment prior to isolation	References
<i>E. cloacae</i> GC1	<i>E. coli</i> AS226-51 (pCS902)	AmpC GC1	1992, Japan	Unknown	Unknown	Unknown	[22]
<i>E. coli</i> HKY28	<i>E. coli</i> XL1-Blue (pBE28W)	AmpC ^D	1994, Japan	Urine	Unknown	Unknown	[20]
<i>S. marcescens</i> HD	<i>E. coli</i> DH10B (pBK-HD)	AmpC HD	2001, France	Urine	Infection	Unknown	[13]
<i>E. coli</i> KL	<i>E. coli</i> DH10B (pBK-KL)	AmpC KL	2001, France	Urine	Infection	Unknown	[12]
<i>E. aerogenes</i> Ear2	<i>E. coli</i> DH5/p-Ear2	AmpC Ear2	2001, France	Rectal swab	Colonization	Cefepime	[15]
<i>E. cloacae</i> CHE	<i>E. coli</i> JM101/pBK-CHE	AmpC CHE	1998, France	Stool sample	Colonization	Cefepime	[14]
<i>S. marcescens</i> SMSA	<i>E. coli</i> JM101 (pBK-SerR)	AmpC SMSA	2000, France	Intraabdominal wound	Infection	Ceftazidime	[16]
<i>S. marcescens</i> GN16694	<i>E. coli</i> JM83/pGFR5 (SRT-1)	AmpC SRT-1	1985, Japan	Urine	Unknown	Unknown	[18]
<i>E. coli</i> 1740	None	AmpC 1740	1995, United States	Abcess iliacus muscle	Infection	Unknown	[21]
<i>K. pneumoniae</i> HKY327	<i>E. coli</i> DH5 α (pBC-CMY-19)	CMY-19	1995, Japan	Sputum	Unknown	Unknown	[19]

genes have been mobilized by site-specific recombination likely to be catalyzed by the produce of *orf513* [30,31]. Moreover, characterization of the *sull*-type integron harbored by plasmid pQR1 revealed that the promoter of the *qnrA* gene was located inside the right end boundary of the common region [33]. This result supports the hypothesis that the expression of the *bla*_{CMY-19} gene may be related to the CR element.

5. STRUCTURE AND FUNCTION RELATIONSHIP

5.1. General Consideration

The three-dimensional structures of all known cephalosporinases are very similar [34]. These β -lactamases are a mixed α - β structure with an all-helical domain on the left and a mixed α/β domain on the right (Fig. 2) [35]. Between the α domain on the left and the α/β domain on the right lies the site of β -lactam binding which is constituted by residues Ser64, Lys67, Gln120, Tyr150, Asn152, Lys315, Thr316, and the variable residue at position 318 [35-37]. The β -lactam substrate is likely to be oriented in an oxyanion binding pocket between helix H2 (NH of Ser-64) and β -strand B3 (NH of residue 318). The reactive Ser-64, at the N terminus of the H2 helix, is adjacent to Lys-67.

Above the active site, a loop laying from residues 286 to 298, just at the end of the H-10 helix, participates in the formation of a secondary substrate binding site (Fig. 2) [38,39].

Indeed, the hydrophobic patch formed by the Leu293 interacts with the methyl group C19 of the cephalosporins (Fig. 1 and Fig. 2). Moreover, Asn346 and Arg349, which interact with the carboxylate group at the C3(4) position of the β -lactams, also act as secondary binding sites [38].

Below the active site, the Ω loop lays from residues 178 to 226 (Fig. 2). This loop is much longer in the class C enzymes than in class A enzymes, and this added length is thought to facilitate the accomodation of larger cephalosporins [40]. Moreover, the Ω loop interacts through hydrogen bonding with the helix H-2 that is closed to the active Ser64 [40].

5.2. Structure of Extended-spectrum cephalosporinases

The expansion of the substrate specificity in ESAC β -lactamases toward extended-spectrum cephalosporins is attributable to amino acids changes in specific locations of the proteins. These changes are represented in the (Fig. 3) that shows amino acid alignment of all the ESACs known.

Replacement of threonine by isoleucine at position 70 in the ESAC produced by the laboratory-obtained mutant *Serratia marcescens* 520R [40] was responsible for hydrolysis spectrum extension. The crystal structure of β -lactamase from *S. marcescens* is not yet available. However, as the three-dimensional structures of all these enzymes are very similar [34], crystal structure of *E. cloacae* P99 was used for comparison. Although it is not in the substrate-binding

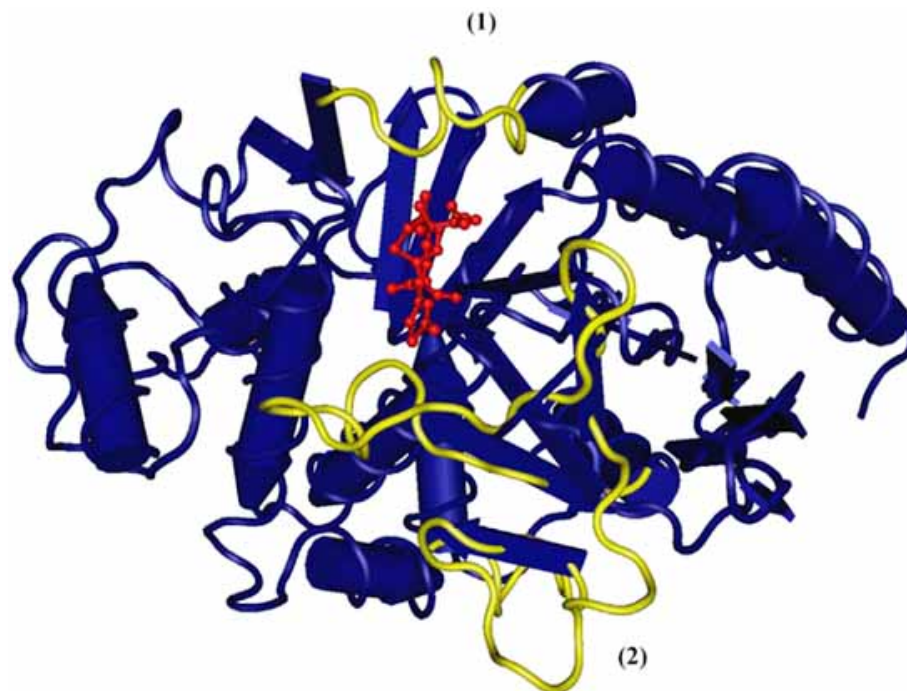


Fig. (2). Ribbon representation of the crystallographic structure of the *E. coli* K-12 β -lactamase bound to ceftazidime [36]. The atomic coordinates are available on the internet (www.ncbi.nlm.nih.gov/entrez) with the accession number 1IEL. The representation has been determined using the software Cn3D 4.1 available on the internet (www.ncbi.nlm.nih.gov/entrez). The antibiotic is represented in red inside the active site. The loop, which is located above the active site at the C terminus end of the H-10 helix from residues 286 to 298 (A), and the Ω loop, which is located below the active site (B) from residues 178 to 226, are represented in yellow.

cleft, the side chain hydroxyl oxygen of the Thr70 is within hydrogen bonding distance to the main chain carbonyl oxygen of Gln219. The replacement of threonine with isoleucine abolishes this hydrogen-bonded structure and also introduces a more bulky side chain. The substitution will make helix2, which is closed to the active-site serine, more movable or pliable, and this will make the attack on compounds like ceftazidime (1) easier.

Gln219 is located near the end of the long Ω loop (from residue 189 to residue 226) at the entrance of the substrate-binding site. In the SRT-1 β -lactamase produced by *S. marcescens* GN16694 [18] and in the AmpC produced by *S. marcescens* SMSA [16], the change of Glu219 to Lys [18] and the changes of nearby residues Ser220 to Tyr [16], respectively, accounted for the broadened substrate specificity.

Extension of the Ω loop in the middle of both natural and laboratory-generated *E. cloacae* mutant enzymes was shown to result in an increase of the hydrolysis spectrum [22, 23]. In this case, the crystal structure of the mutant enzyme indeed showed the increased opening of the entrance of the substrate-binding pocket [24].

Another hot spot for mutations in AmpC of *Enterobacteriaceae* conferring resistance to extended-spectrum cephalosporins is the region laying from residues 286 to 298, which constitutes a loop above the binding site. The replacement of Leu-293 with proline, described in the laboratory mutant ESAC obtained from the AmpC of *E. cloacae* P99 [41], in the laboratory mutants 5 and 7 obtained from CMY-2 [25], and in the ESAC produced by the clinical isolate *E. aerogenes* Ear2, may have structural consequences

producing a conformational change in the 287 to 298 loop above the binding site [35, 41]. Mutagenic replacement of Leu-293 revealed that only proline substitution accounted for a reduced susceptibility to cefepime (3) [41]. The replacement of the neighboring amino acid Ile292 to serine, which occurred in the plasmid-borne CMY-19, also lead to an increase activity against oxyimino-cephalosporins. The authors speculated that a more flexible loop, or a more open one, results from the mutations, such that more space is available to an approaching substrate [19, 41].

The tripeptide deletion (Gly286-Ser287-Asp288) in AmpC^D produced by the clinical isolate *E. coli* HKY28 also altered the enzymatic properties of the enzyme [20]. Result of modeling studies provided a structural explanation for this modification. In the *E. coli* K-12 AmpC, the tripeptide impeded access of ceftazidime (1) whereas the tripeptide deletion in AmpC^D was found to provide an open site where the R-2 side chain of ceftazidime could be accommodated.

The hexapeptide deletion (Ser289-Lys290-Val291-Ala292-Leu293-Ala294) in the AmpC produced by the clinical *E. cloacae* CHE [14], and the four-amino-acid deletion (Met293-Asn294-Gly295-Thr296) in the AmpC produced by the clinical strain *S. marcescens* HD [13] accounted for the broaden substrate specificity, but no modeling or crystallographic studies were performed to provide a structural explanation.

The substitution Val298Glu in the laboratory-obtained mutant ESAC obtained from the AmpC of *E. cloacae* MHN should disrupt the local structure nucleated by a mini-hydrophobic core [42]. This modification may increase the

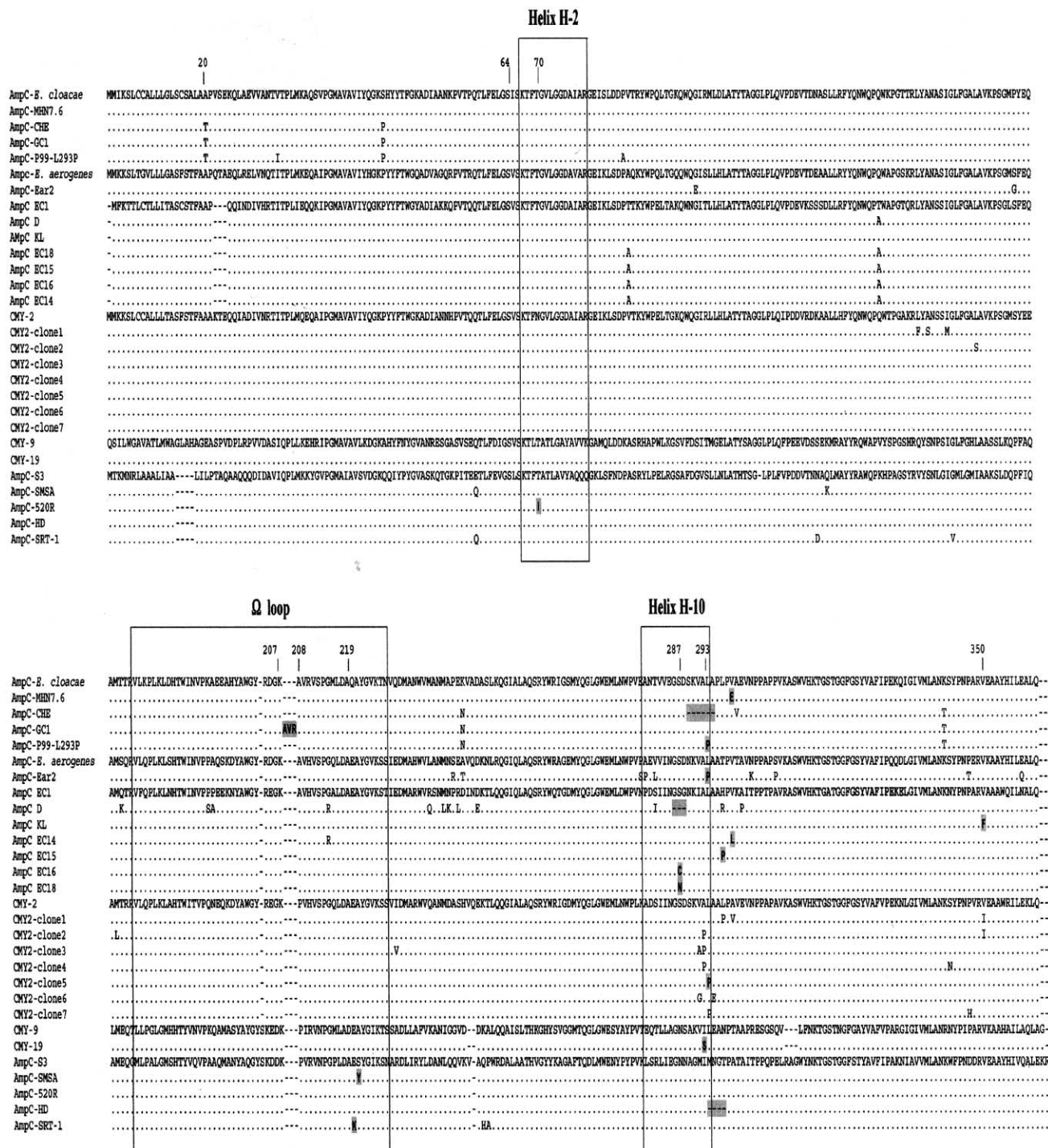


Fig. (3). Alignment of amino acid sequences of ESAC β -lactamases. AmpC from *E. cloacae* MHN1 [42], AmpC from *E. aerogenes* EarCOL [15], AmpC EC1 from *E. coli* EC1 (personal data), AmpC S3 from *S. marcescens* S3 [18], the plasmid-borne CMY-2 [25], are classical narrow spectrum cephalosporinases. The other β -lactamases presented in the figure are extended-spectrum AmpC enzymes. MHN7.6 derived from the cephalosporinase of *E. cloacae* MHN1 [42], AmpC CHE from *E. cloacae* CHE [14], AmpC GC1 from *E. cloacae* GC1 [22], AmpC P99-L293P from the cephalosporinase of *E. cloacae* [41], AmpC Ear2 from *E. aerogenes* Ear2 [15], AmpC^D from *E. coli* HKY28 [20], AmpC KL from *E. coli* KL [12], AmpC EC14, AmpC EC15, AmpC EC16, AmpC EC18 from *E. coli* EC14, EC15, EC16, and EC18 (personal data), respectively, CMY-2-clone 1 to CMY-2-clone 7 from CMY-2 [25], AmpC SMSA from *S. marcescens* SMSA [16], AmpC 520R from the cephalosporinase of *S. marcescens* [40], AmpC HD from *S. marcescens* HD [13], and AmpC SRT-1 from the cephalosporinase of *S. marcescens* GN16694 [18], CMY-9 [30], and CMY-19 [19]. The amino acid substitutions, which were demonstrated to expand the substrate specificity, were greyed.

overall flexibility of the enzyme, broadening its spectrum of action.

Recent personal results from ESACs produced by *E. coli* clinical isolates EC13, EC14, EC15, EC16, and EC18 allowed the identification of novel amino acid substitutions as a source of expanded-spectrum activity (personal data). Replacement of the Ser-287 by Asn or Cys, replacement of His-296 by proline, replacement of VAL298 by leucine and replacement of Val-350 by a phenylalanine, are responsible for the extension of the hydrolysis spectrum. In native AmpC, Ser287 forms hydrogen bonds with Asn346 and Arg349 [36]. Moreover, Asn346 and Arg349 are involved in the binding of the C3(4) carboxylate of β -lactams. Thus, it may be possible that, His286, Ser287 and Val350 replacements may modify the affinity of the enzyme for the cephalosporins. Modeling study are in progress to confirm these hypothesis.

Interestingly, several variant enzymes that derived from the AmpC β -lactamases of *C. freundii* [25], *E. cloacae* [41], and *E. aerogenes* [15], presented Leu293Pro replacement and shared the same kinetic characteristics. These data are clear evidence for the convergent evolution of ESAC enzymes because mutations have occurred independently on different gene frameworks, but all reduce the susceptibility to oxyiminocephalosporins likely in response to selective pressure imposed by the use of β -lactams [15,25,41].

6. BIOCHEMICAL DATA

Most of the ESAC enzymes have been purified to perform a detailed biochemical analysis. Study on the catalytic efficiencies or relative rates of hydrolysis of the variant cephalosporinases indicated that they displayed different hydrolytic profiles as compared to parental β -lactamases. ESAC β -lactamases show a higher level of hydrolysis of extended-spectrum cephalosporins, such as ceftazidime (**1**), cefotaxime (**2**), cefepime (**3**), and ceftiprome (**4**), whereas, parental narrow-spectrum cephalosporinases hydrolyzed these compounds on a weaker extent.

In the cases of ESAC produced by *S. marcescens* 520R [40], *S. marcescens* SRT-1 [18], *S. marcescens* SMSA [16], and *E. cloacae* GC1 [22], that have amino acid changes in the Ω loop structure or replacement at position 70, the mutant enzymes had increased K_{cat} values for extended-spectrum cephalosporins, together with increased K_m values (Table 2). These results agree with a modification of the enzyme structure leading to a better nucleophilic attack of the cephalosporins by the Ser-64 whereas the binding of the substrate is decreased.

The variant AmpC β -lactamase produced by the laboratory-obtained mutant *C. freundii* GN346, which presented the replacement of Glu219Lys in the Ω loop, exhibited unexpected kinetic parameters [43]. The K_{cat} value, for ceftazidime, was about 100-fold reduced as compared to the wild-type enzyme, whereas the K_m value, estimated by the K_i value, was ca. 300-fold decreased. Globally, the estimated catalytic efficiency was unchanged. The kinetic parameters for the other extended-spectrum cephalosporins, such as cefotaxime (**2**), cefepime (**3**), and ceftiprome (**4**), were not determined. These results were surprising since the kinetic pa-

rameters of the ESAC β -lactamase produced by *S. marcescens* GN16694, which also had Glu219 to lysine replacement, were the opposite, as described above. It is possible that the amino acid replacement at position 219 in the AmpC of *C. freundii* lead to different structural modification than in the AmpC of *S. marcescens*. Nevertheless, since the catalytic efficiency of the variant cephalosporinase of *C. freundii* GN346 for the extended-spectrum cephalosporin tested was unchanged, as well as the MIC value of this antibiotic, this variant enzyme was not considered as being an ESAC β -lactamase and was not retained in our review, as well as related articles [44,45].

ESAC β -lactamases that have amino acid changes inside the loop ranging from residues 287 to 298 amino acid, such as variant cephalosporinases produced by *E. cloacae* CHE [14], *E. aerogenes* Ear2 [15], *K. pneumoniae* HKY327 [19], *E. coli* HKY28 [20], *E. coli* EC16 [personal data], *E. coli* EC14 [personal data], *E. coli* EC15 [personal data], *E. coli* EC18 [personal data], *S. marcescens* HD [13], exhibited enzymatic parameters that were the opposite to those of ESAC β -lactamases with modifications in the Ω loop. These variant cephalosporinases showed a global increase of the catalytic efficiency for the cephalosporins, which is mainly due to an increase of the affinity (decreased K_m values) whereas the K_{cat} values are decreased.

The kinetic parameters for penicillins of ESACs were not significantly altered. It was noteworthy that imipenem was not hydrolyzed by these type of enzymes and that the weak hydrolysis rate of aztreonam was not strongly increased as compared to that of the parental β -lactamases.

The cephalosporinase of *E. coli* clinical strain 1740, which was selected for its unusual phenotype of resistance to oxyiminocephalosporins while susceptible to the combination of piperacillin plus tazobactam, had lower K_m value for cephaloridine as compared to the purified classical AmpC β -lactamase from *E. coli* D31 [21]. This result is consistent with ESAC β -lactamase with amino acid changes inside the loop laying from residues 286 to 298. Moreover, the AmpC produced by *E. coli* 1740 presented a 10- and 100-fold lower IC_{50} values for clavulanic acid and tazobactam, respectively (Table 3). Unfortunately, this cephalosporinase was not sequenced and their mutation remained to be identified. This higher susceptibility to inhibitors was also reported for AmpC^D, produced by the clinical *E. coli* HKY28 [20]. AmpC^D exhibited approximately 5- to 10-fold lower K_i values than classical cephalosporinase against all three inhibitors. Tazobactam was the best inhibitor and had the lowest IC_{50} for AmpC^D. The IC_{50} and K_i values of the other ESACs remained to be clarified to determine if the increase susceptibility to tazobactam is a common feature shared by all these variant enzymes.

7. SUSCEPTIBILITY TO β -LACTAMS

The susceptibility to β -lactams of ten ESAC-producing enterobacterial clinical isolates and their corresponding clones, and eleven in vitro-obtained ESAC-producing strains is presented in Table 4 and Table 5.

The clinical strains producing ESAC β -lactamases were all resistant to amoxicillin and narrow spectrum cephalosporins, such as cephalothin, cefoxitin, and cefuroxime (**6**),

Table 2. Kinetic Data of ESAC β -Lactamases and the Parental Wild-Type Cephalosporinases

AmpC CHE from *E. cloacae* CHE [14], AmpC GC1 from *E. cloacae* GC1 [22], AmpC Ear2 from *E. aerogenes* Ear2 [15], AmpC^D from *E. coli* HKY28 [20], AmpC KL from *E. coli* KL [12], AmpC 1740 from *E. coli* 1740 [21], AmpC HD from *S. marcescens* [13], AmpC SMSA from *S. marcescens* SMSA [16], AmpC SRT-1 from *S. marcescens* GN16694 [18], and AmpC 520R from *S. marcescens* 520R [40], were ESACs, whereas AmpC P99 from *E. cloacae* P99 [14], AmpC Ear1 from *E. aerogenes* Ear1 [15], AmpC S4 from *E. coli* 154792 [12], AmpC D31 from *E. coli* D31 [21], AmpC S3 from *S. marcescens* S3 [13], were narrow-spectrum cephalosporinases

	Benzypenicillin			Piperacillin			Cephalothin			Relative V_{max}^a	Cephaloridin			Cefoxitin			Cefuroxime			
	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)		k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	Relative V_{max}^a	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)
AmpC CHE	10	1	10,000	- ^b	-	-	23	1.5	15,300	-	600	185	3,200	-	2	-	-	-	-	-
AmpC GC1	5.66	3.1	1,800	-	-	-	74.8	31	2,400	-	-	-	-	-	-	-	24.1	26	920	
AmpC P99	10.1	3.1	3,300	-	-	-	50	40	1,250	-	300	600	500	-	5	-	-	-	-	-
AmpC Ear1	10	4.5	2,200	-	-	-	190	36	5,200	-	240	230	1,040	-	1.8	-	-	-	-	-
AmpC Ear2	5	5.3	940	-	-	-	50	7.0	7,100	-	255	96	2,650	-	4.2	-	-	-	-	-
AmpC _D	-	-	-	-	-	-	-	-	-	-	64	100	1,600	0.043	1.2	38	-	-	-	-
AmpC KL	4	15	270	5	6	830	250	20	12,500	-	400	65	6,000	0.1	0.1	1,000	-	0.2	0.05	4,000
AmpC S4	7	30	230	3	25	120	210	75	2,800	-	150	450	330	0.2	0.1	2,000	-	0.1	30	2,000
AmpC 1740	21	143	150	-	-	-	-	-	-	-	68	57	1,200	-	-	-	-	-	-	-
AmpC D31	91	18	5,000	-	-	-	-	-	-	-	376	450	835	-	-	-	-	-	-	-
AmpC HD	50	10	5,000	0.1	0.5	200	95	2	48,000	-	-	-	-	0.01	3	2	-	4.5	5	1,000
AmpC S3	35	9	4,000	0.01	0.1	110	1,200	125	9,600	-	-	-	-	0.002	1	2	-	2.5	7	350
AmpC SMSA	50	50	1,000	-	-	-	780	1,300	600	-	-	-	-	-	-	-	-	-	-	-
AmpC SRT-1	-	-	-	-	-	-	-	-	-	100	-	660	-	-	-	-	100	-	180	-
AmpC 520R	-	-	-	-	-	-	-	-	-	-	400	3,500	120	-	-	-	-	-	-	-
CMY-9	-	-	-	0.14	97	1.4	630	120	5,300	-	99	1200	83	50	60	830	-	-	-	-
CMY-19	-	-	-	0.031	16	3.5	380	230	1,700	-	240	1500	160	0.12	0.9	130	-	-	-	-

	Cefotaxime				Ceftazidime				Cefepime			Cefpirome			Aztreonam		
	Relative V_{max}^a	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	Relative V_{max}^a	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μ M)	k_{cat}/K_m (s ⁻¹ /mM)
AmpC CHE	-	0.5	0.05	10,000	-	<1	1	-	2	3.0	700	2	14	140	-	-	-
AmpC GC1	-	-	-	-	-	>0.9	>742	-	-	-	-	-	-	-	0.011	1.1	10

(Table 2) contd....

	Cefotaxime				Ceftazidime				Cefepime			Cefpirome			Aztreonam		
	Relative V_{max}^a	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ /mM)	Relative V_{max}^a	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ /mM)	k_{cat} (s ⁻¹)	K_m (μM)	k_{cat}/K_m (s ⁻¹ /mM)
AmpC P99	-	0.5	0.5	1,000	-	<1	20	-	1	15	60	1.25	67	18	-	-	-
AmpC Ear1	-	0.15	>500	-	-	-	16	-	0.4	126	3	0.3	25	10	-	-	-
AmpC Ear2	-	0.15	10	15	-	-	9.8	-	0.4	9.1	44	0.5	23	22	-	-	-
AmpC ^D	-	0.37	31	12	-	0.084	5.7	15	1	49	21	1.5	21	71	-	-	-
AmpC KL	-	0.1	0.05	2,000	-	0.05	1	50	0.2	5	40	0.2	15	15	-	-	-
AmpC S4	-	0.5	0.1	3,300	-	0.02	90	0.5	0.2	>1,000	<0.2	0.2	>1,000	<0.2	-	-	-
AmpC 1740	-	-	-	-	-	-	-	-	-	-	-	-	Nd	-	-	-	-
AmpC D31	-	-	-	-	-	-	-	-	-	-	-	-	Nd	-	-	-	-
AmpC HD	-	5	2	2,300	-	270	20	13,000	50	6	8,400	270	100	2,800	<0.001	-	-
AmpC S3	-	6	7	800	-	5	>1,000	<5	80	>1,000	<80	120	>1,000	<120	<0.001	-	-
AmpC SMSA	-	800	980	800	-	520	570	900	330	1,000	0.33	217	650	330	-	-	-
AmpC SRT-1	410	-	410	-	20	-	150	-	-	-	-	-	-	-	-	-	-
AmpC 520R	-	250	1,500	170	-	-	>5,000	12	-	-	-	-	-	-	-	-	-
CMY-9	-	0.27	0.28	960	-	1.8	560	3.2	NH ^c	950	-	3.6	390	0.009	-	-	-
CMY19	-	0.33	31	11	-	0.085	3.7	230	1.8	630	0.003	0.58	25	0.023	-	-	-

^a Values relative to that for cephaloridine, which was set at 100.^b -, not determined.^c NH, not hydrolyzed**Table 3. IC₅₀ and K_i Values of β-Lactamases Inhibitors**

The extended-spectrum AmpC^D is expressed by *E. coli* HKY28 and XL1-Blue (pBE28w) and AmpC^R is the narrow-spectrum cephalosporinase that is produced by the revertant clone [20]. AmpC 1740 is an extended-spectrum cephalosporinase produced by the clinical strain *E. coli* 1740 whereas AmpC D31 is a typical narrow-spectrum AmpC produced by *E. coli* D31 [21]

	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	320	3.9	9.2	1.4	8.7
AmpC ^R	140	4,100	24	780	25	1,100
AmpC 1740	30	Nd ^a	Nd	Nd	0.08	Nd
AmpC D31	330	Nd	Nd	Nd	9	Nd

^a Nd, not determined.

Table 4. MIC of Several β -Lactams for the Clinical Isolates and their Corresponding Recombinant Clones

E. coli HKY28 and *E. coli* XL1-Blue (pBE28w) produced Amp^C^D [20], whereas the revertant Amp^C^R is produced by *E. coli* XL1-Blue (pBE28R) [20]. Amp^C KL was produced by *E. coli* KL and *E. coli* DH10B (pBK-KL) [12], Amp^C 1740 by *E. coli* 1740 [21], Amp^C Ear2 by *E. aerogenes* Ear2 and its recombinant clone *E. coli* DH5 α /p-Ear2 [15], Amp^C SRT-1 by *S. marcescens* GN16694 and its clone *E. coli* JM53/pGFR5 (SRT-1) [18], Amp^C SMSA by *S. marcescens* SMSA and its clone *E. coli* JM101 pBK-Ser^R [16], whereas *E. coli* JM101 pBK-Ser^S produced the revertant Amp^C^R [16]. Amp^C HD is produced by *S. marcescens* HD and its clone *E. coli* DH10B (pBK-HD) [13], Amp^C CHE by *E. cloacae* CHE and its clone *E. coli* JM101/pBK-CHE [14], Amp^C GC1 by *E. cloacae* GC1 [22], Amp^C S4 by *E. coli* DH10B (pBK-S4) [12], *E. coli* DH579/p-EarCOL produced the Amp^C of the wild-type *E. aerogenes* EarCOL [15], *E. coli* DH10B (pBK-S3) produced Amp^C S3 of *S. marcescens* S3 [13], and the clone *E. coli* JM101/pBK-OUdhyp produced the Amp^C of the wild-type *E. cloacae* OUDhyp [14]

β -Lactams	<i>E. coli</i> KL	<i>E. coli</i> DH10B (pBK-KL)	<i>E. coli</i> HKY28	<i>E. coli</i> XL1-Blue (pBE28w)	<i>E. coli</i> 1730	<i>E. coli</i> DH10B (pBK-S4)	<i>E. coli</i> XL1-Blue (pBE28R)	<i>E. aerogenes</i> Ear2	<i>E. coli</i> DH5 α /p-Ear2	<i>E. coli</i> DH5 α /p-EarCOL	<i>K. pneumoniae</i> HKY327	<i>E. coli</i> DH5 α (pBC-CMY-9)	<i>E. coli</i> DH5 α (pBC-CMY-19)
Ticarcillin	>512	4	- ^b	-	-	8	-	-	-	-	-	-	-
Ticarcillin-Clavulanate ^a	64	4	-	-	-	8	-	-	-	-	-	-	-
Piperacillin	64	8	8	8	32	8	8	-	-	-	128	8	64
Piperacillin-Tazobactam ^a	8	8	4	2	2	8	4	-	-	-	-	4	32
Cefoxitin	64	128	16	32	32	128	>128	-	-	-	-	>128	128
Cefuroxime	64	128	-	-	-	32	Nd	-	-	-	-	-	-
Cefotaxime	8	8	16	32	4	0.5	8	32	6	4	128	>128	128
Ceftriaxone	8	8	-	-	-	0.25	-	-	-	-	-	-	-
Ceftazidime	64	128	32	128	32	1	16	512	64	6	>128	64	>128
Aztreonam	0.125	0.06	8	16	4	0.06	16	16	2	1	-	4	16
Cefepime	4	4	2	4	-	0.06	0.03	32	8	0.25	4	0.13	4
Cefpirome	4	4	2	4	-	0.06	0.03	12	4	0.25	0.25	8	16

β -Lactams	<i>S. marcescens</i> GN16694	<i>E. coli</i> JM53/pGFR5 (SRT-1)	<i>S. marcescens</i> HD	<i>E. coli</i> DH10B (pBK-HD)	<i>S. marcescens</i> SMSA	<i>E. coli</i> JM101 pBK-Ser ^R	<i>E. coli</i> JM101 pBK-Ser ^S	<i>E. coli</i> DH10B (pBK-S3)	<i>E. cloacae</i> GC1	<i>E. cloacae</i> CHE	<i>E. coli</i> JM101/pBK-CHE	<i>E. coli</i> JM101/pBK-OUdhyp
Ticarcillin	-	-	>512	>512	>256	>256	1	128	-	-	-	-
Ticarcillin-Clavulanate ^a	-	-	256	256	-	-	-	128	-	-	-	-
Piperacillin	-	-	512	256	2	2	1	256	-	-	-	-
Piperacillin-Tazobactam ^a	-	-	32	128	-	-	-	128	-	-	-	-
Cefoxitin	-	-	8	128	-	-	-	>512	-	512	64	32
Cefuroxime	100	200	128	512	-	-	-	128	-	-	-	-
Cefotaxime	-	3	2	16	16	16	4	8	-	32	4	0.5
Ceftriaxone	100	-	2	16	-	-	-	8	-	-	-	-
Ceftazidime	100	50	64	512	64	128	0.75	16	800	512	64	0.5
Aztreonam	100	-	0.5	0.25	-	-	-	0.06	400	-	-	-
Cefepime	-	-	32	512	1	1	0.125	2	-	256	8	0.03
Cefpirome	-	-	8	64	1	1	0.125	2	-	128	4	0.03

^a Clavulanic acid at fixed concentration 2 μ g/ml, tazobactam at fixed concentration 4 μ g/ml.

^b -, not determined.

Table 5. MICs of β -Lactams for Laboratory Mutant Strains and their Corresponding Clones

S. marcescens 520R and its clone *E. coli* DH5 α /pG520R extended-spectrum AmpC 520R, whereas recombinant clone *E. coli* DH5 α /PGS3 produced the narrow-spectrum AmpC from *S. marcescens* S3 [40]. Laboratory mutant *E. coli* pBGMHN-7.6 produced ESAC, whereas *E. coli* pBGMHN produced the parental narrow-spectrum β -lactamase [42]. *E. coli* JM83 (pUC19-L293P) produced the mutant AmpC that derived from the enzyme of *E. cloacae* P99 [41]. *E. coli* DH5 α (pACSE2-clone1) to *E. coli* DH5 α (pACSE2-clone7) produced ESACs that derived from the narrow-spectrum CMY-2 which was produced by *E. coli* DH5 α (pACSE2-CMY-2) [25].

β -Lactams	<i>S. marcescens</i> 520R	<i>E. coli</i> DH5 α /pG520R	<i>E. coli</i> DH5 α /PGS3	<i>E. coli</i> pBGMHN	<i>E. coli</i> pBGMHN-7.6	<i>E. coli</i> JM83 (pUC19-L293)	<i>E. coli</i> JM83 (pUC19-L293P)	<i>E. coli</i> DH5 α (pACSE2-CMY-2)	<i>E. coli</i> DH5 α (pACSE2-clone1)	<i>E. coli</i> DH5 α (pACSE2-clone2)	<i>E. coli</i> DH5 α (pACSE2-clone3)	<i>E. coli</i> DH5 α (pACSE2-clone4)	<i>E. coli</i> DH5 α (pACSE2-clone5)	<i>E. coli</i> DH5 α (pACSE2-clone6)	<i>E. coli</i> DH5 α (pACSE2-clone7)
Piperacillin	64	8	32	2	8	128	64	256	512	512	512	512	512	512	512
Piperacillin-Tazobactam ^a	- ^b	-	-	1	2	-	-	-	-	-	-	-	-	-	-
Cefoxitin	256	16	4	32	8	-	-	-	-	-	-	-	-	-	-
Cefuroxime	>1,024	256	256	-	-	-	-	128	512	1024	512	512	256	512	256
Cefotaxime	256	8	4	1	4	-	-	64	128	128	128	128	128	128	128
Ceftriaxone	512	8	1	Nd	-	128	32	-	-	-	-	-	-	-	-
Ceftazidime	512	16	1	0.5	64	64	256	256	2048	2048	2048	2048	1024	1024	2048
Aztreonam	128	4	2	-	-	64	8	64	128	128	128	256	64	256	128
Cefepime	-	-	-	0.015	4	8	8	2	64	32	32	32	32	32	32
Cefpirome	64	2	1	0.015	1	-	-	-	-	-	-	-	-	-	-

^a Clavulanic acid at fixed concentration 2 μ g/ml, tazobactam at fixed concentration 4 μ g/ml

^b -, not determined

except *E. coli* HKY28 and *S. marcescens* HD that had intermediate susceptibility or were susceptible to cefoxitin, respectively. Furthermore, these isolates were all resistant to ceftazidime (1). Most of the clinical isolates were susceptible to cefotaxime (2), ceftriaxone (5), cefepime (3), and cefpirome (4), except *E. coli* HKY28 and *S. marcescens* SMSA, which had intermediate susceptibility to cefotaxime (2) and ceftriaxone (5), *K. pneumoniae* HKY327, which was resistant to cefotaxime, *E. aerogenes* Ear2 and *S. marcescens* HD, which were resistant to cefepime (3), whereas *E. cloacae* CHE was resistant to these four compounds, according to the criteria of the CLSI [46]. It was noteworthy that AmpC production by *E. aerogenes* Ear2 and *E. cloacae* CHE was depressed, whereas it still remained inducible in *S. marcescens* HD and *S. marcescens* SMSA and thus expressed at a lower level in those species.

In order to eliminate the other mechanisms of resistance expressed by the clinical isolates, such as efflux system and loss of membrane permeability, the genes coding for the ESAC β -lactamases were cloned and subsequently expressed in *E. coli* strains. MICs of β -lactams for recombinant clones mirrored those of clinical isolates (Table 4). MICs of all β -lactams bearing an oxyimino group in the lateral side chain, such as ceftazidime (1), cefotaxime (2), cefepime (3), cefpirome (4), cefuroxime (6), and aztreonam were increased as compared to those obtained for recombinant strains expressing the parental wild-type AmpC β -lactamases. All the re-

combinant *E. coli* strains expressing ESAC were resistant to ceftazidime (1). Moreover, none of the ESAC-producing clones were resistant to cefotaxime (2), aztreonam, cefepime (3), and cefpirome (4), except *E. coli* DH10B (pBK-HD) that was resistant to cefepime and *E. coli* DH5 α (pBC-CMY-19) that was resistant to cefotaxime (2), and presented a reduced susceptibility to cefpirome (4). Thus, the major phenotypic modification resulting from the alteration of the hydrolytic activity leads to ceftazidime (1) resistance.

ESAC activity was not counteracted by clavulanic acid inhibition and no detectable synergy between extended-spectrum cephalosporins and clavulanic acid was observed on routine antibiogram [20,21]. In vitro synergy indicate that cloxacillin/oxacillin may still counteract the hydrolytic activity of ESACs as known for classical cephalosporinases (Mammeri, H.; Nordmann, P., Personal communication) It is noteworthy that ESAC seems to be more susceptible to tazobactam, in the combination piperacillin/tazobactam, than classical AmpC enzymes thus explaining the good susceptibility of ESAC-producing strains that contrasts with apparent resistance to ceftazidime (1) [20,21].

8. CONCLUSION

ESACs are cephalosporinases with broadened substrate activity. Eight ESACs produced by enterobacterial clinical isolates have been characterized so far at the biochemical and molecular levels.

Two groups of ESACs sharing similar kinetic characteristics were identified. One group of ESAC enzymes is characterized by an increase of the K_{cat} values. Modifications in the Ω loop or replacement of the Threonine at position 70 alter the enzymatic activity of these variant enzymes by an indirect effect on the active site [16,18,22-24]. The ESACs of the second group presented modifications in the loop laying from residue 286 to residue 298, just above the active site, and presented an increased affinity for extended-spectrum cephalosporins. Biochemically, the structural modifications of ESACs accounted for an increase of the catalytic efficiency against all oxyiminocephalosporins [12-15,20,25,41]. However, ESACs confer resistance to ceftazidime (**1**), whereas susceptibility to the other extended-spectrum cephalosporins, such as cefotaxime (**2**), cefepime (**3**), and ceftiofime (**4**) may vary from one enzyme to another.

Naturally occurring resistance to expanded-spectrum cephalosporins can be mediated by over-expression of the chromosomal cephalosporinase or by the expansion of the substrate specificity of the AmpC β -lactamases. Only few clinical isolates producing ESACs have been identified. However, no resistance pattern may allow reliable detection of ESAC-producing strains. By routine susceptibility tests, ESAC production could be misidentified for overexpression of typical cephalosporinase combined with loss of outer membrane permeability [9, 47], and therefore underestimated. Moreover, due to several efflux systems [48], the frequent decreased susceptibility to cefepime (**3**) of *Pseudomonas aeruginosa* should not allow an easy detection of ESACs. However, a recent communication described the first identification of an ESAC-producing *P. aeruginosa* clinical isolate. The *P. aeruginosa* TUH1529 strain has been selected because of its unusual phenotype of resistance with a decreased susceptibility to imipenem. The biochemical and molecular characterization of this β -lactamase has identified a Thr79Ala substitution inside the H-2 helix, as compared to the narrow-spectrum AmpC PAO1 β -lactamase, responsible for an increased catalytic activity. This variant enzyme conferred resistance to ceftazidime (**1**), aztreonam and reduced significantly the susceptibility to imipenem.

As most clavulanic acid inhibited extended-spectrum β -lactamases derived from the plasmid-borne TEM-type penicillinases [49, 50], the discovery of plasmid-mediated class C β -lactamases may be a prelude to isolation among clinical isolates of new variants of class C enzymes with extended-substrate activity. Plasmid-mediated cephalosporinases that are now reported worldwide in *Enterobacteriaceae* may enhance the probability to select for ESACs just by increasing the number of cephalosporinase-producing enterobacterial strains that usually do not produce AmpC enzymes (*E. coli*, *P. mirabilis*, *K. pneumoniae*, *Salmonella sp.*). This hypothesis may be suggested by the recent description of the plasmid-borne CMY-19 that exhibited a broadened substrate-specificity as compared to its progenitor CMY-9 [19].

The precise role of cephalosporins to select for ESAC remain to be determined as well as that of carbapenems to select for combined resistance mechanisms [51]. Spread of ESACs, which provide a large spectrum of resistance may lead to the potential compromise of the clinical utility of most β -lactams.

ACKNOWLEDGMENTS

Most of our data that have been obtained here are the results of work sponsored by a grant from the Ministère de l'Éducation Nationale et de la Recherche (UPRES-EA3539), Université Paris XI, Paris, France, and the European Community (6th PCRD, LSHM-CT-2003-503-335).

We thank L. Poirel, who is a researcher from INSERM France, for correction of the manuscript.

FOOTNOTES

¹ Ishii, Y.; Kimura, S.; Alba, J.; Gotoh, N.; Nishimo, T.; Yamaguchi, K.; 45th International Congress of Antimicrobial Agents and Chemotherapy, Washington D. C., United States, Abstract C1-93

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