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Naturally Occurring Extended-Spectrum Cephalosporinases in *Escherichia coli*

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Genetic and functional characterization of the cephalosporinases produced by 65 clonally unrelated clinical *Escherichia coli* isolates revealed genetic diversity of the *ampC* genes and showed that Gln287, Cys287, Pro296, Leu298, and Phe350 substitutions were involved in extension of the hydrolysis spectrum to include ceftazidime and cefepime.

AmpC β-lactamases (cephalosporinases) are naturally produced by a variety of enterobacterial species (2, 17). Their hydrolytic properties are similar regardless of their origin (17). AmpC overproduction confers resistance to expanded-spectrum cephalosporins (2), except to cefepime and cefpirome, which are weakly hydrolyzed by these β-lactamases (1, 12). Since 1995, new variants deriving from cephalosporinases have been described in several enterobacterial isolates (1, 6, 7, 9, 12, 13, 14, 19). These enzymes, termed extended-spectrum AmpC β-lactamases (ESAC), are characterized by increased catalytic efficiency against oxyiminocephalosporins, including cefepime and cefpirome (8).

Repeated isolation of AmpC-producing *Escherichia coli* isolates with decreased susceptibility to extended-spectrum cephalosporins prompted us to investigate the prevalence of ESAC β-lactamases in a collection of *E. coli* strains recovered at the Bicêtre hospital from January 2002 to February 2005. Each isolate that was resistant to amoxicillin and to amoxicillin plus clavulanic acid and that had reduced susceptibility to ceftazidime and cefepime (MICs greater than or equal to 16 μg/ml and 0.5 μg/ml, respectively) without a positive synergy test (10) was retained for this study. Seven cephalosporinase-producing isolates, designated *E. coli* EC13 to *E. coli* EC19, were selected together with 56 *E. coli* isolates that did not produce AmpC at a significant level and two reference strains, *E. coli* KL (producing an ESAC) and *E. coli* 154792 (producing a typical cephalosporinase) (12).

The 65 isolates were compared by enterobacterial repetitive intergenic consensus PCR (22), whereas ESAC-producing isolates were also compared by pulsed-field gel electrophoresis analysis (16, 21). All of the isolates were genotypically unrelated (data not shown).

PCR amplifications of *ampC* genes were performed (13) with primers Int-B2 (5′-TTCCTGATGATCGTTCTGCC-3′) and Int-HN (5′-AAAAGCGGAGAAAAGGTCCG-3′), yielding a 1,315-bp amplification product that contained the entire *ampC* gene, including its own promoter sequence. Sequence analyses were performed with PAUP version 3.1.1 and software available at the internet websites www.ncbi.nlm.nih.gov and http://www.ebi.ac.uk/clustalw/. It revealed that *ampC* genes of *E. coli* may be divided into several clusters (Fig. 1). Since the species *E. coli* is divided into four main phylogroups (A, B1, B2, and D) (4), a PCR-based phylotyping analysis was applied to the 65 strains as previously described (4). It revealed that the *ampC* clusters described above are related to phylogroups A and B1, B2, and D (Fig. 1). ESAC-producing strains *E. coli* EC13 to *E. coli* EC19 and *E. coli* KL belonged to phylogroup A or B1. This common origin can be attributed to the high prevalence of *E. coli* strains of these phylogroups in the digestive flora (4).

Sequence analysis of the *ampC* genes of *E. coli* isolates EC13 to EC19 revealed mutations at position −42 or −32 or insertion of 1 bp between positions −15 and −16 in their own promoter region, which has been shown to account for AmpC expression at different levels (3, 15). Plasmids of these AmpC-producing strains were extracted (11) and transferred onto a nylon membrane (20). Hybridization of the membrane with a fluorescein-labeled probe that was made of the PCR product of the *ampC*-KL gene (12) failed to detect the β-lactamase gene in the plasmid DNA contents (data not shown). In addition, transformation experiments performed as previously described (12), with plasmid DNA of AmpC-producing isolates, failed to obtain AmpC-producing transformants. All of these results argued for a chromosomal location of those *ampC* genes.

Amplification with primers Int-B1 (5′-TTTTGTATGGAAC CAGACC-3′) and Int-HN of *ampC* genes from *E. coli* isolates EC1, EC2, EC13 to EC20, EC23, EC24, EC27, EC30, EC31, EC34, EC37, EC41, EC43, EC55, EC58, *E. coli* KL, and *E. coli* 154297 gave PCR products of 1,120 bp containing only the coding regions without their own promoters. These PCR products were cloned into pCR-BluntII-Topo (Invitrogen), and the recombinant plasmids were subsequently transformed into *E. coli* strain TOP10 as described previously (12), giving rise to clones harboring recombinant plasmids pEC1, pEC2, pEC13 to pEC20, pEC23, pEC24, pEC27, pEC30, pEC31, pEC34, pEC37, pEC41, pEC43, pEC55, pEC58, pKL, and pS4, respectively. In all of the recombinant plasmids, the orientation of the cloned insert was the same, with the *ampC* gene under the

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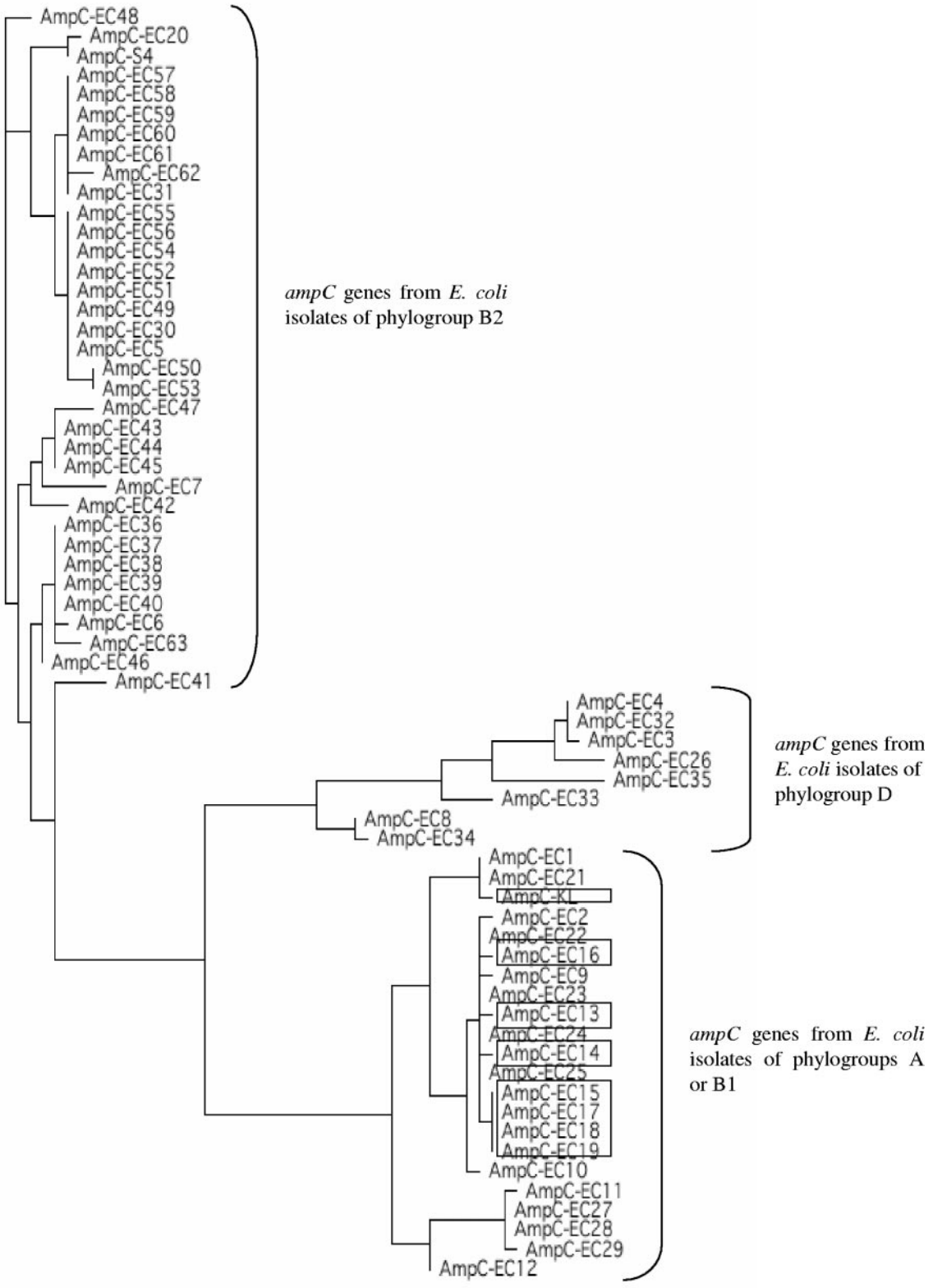

FIG. 1. Phylogeny of the chromosomal *ampC* gene of the 65 *E. coli* isolates studied. The tree was obtained by the parsimony method. Three major groups are shown. *ampC* genes coding for ESAC β-lactamases are boxed. The phylogroups of the *E. coli* strains are indicated by brackets.

TABLE 1. β-Lactamase activities of *E. coli* TOP10 strains harboring recombinant plasmids

Recombinant *E. coli* strain	β-Lactamase activity (μU/mg of protein)[a]	
	Cephalothin	Cefepime
TOP10(pEC1)	110,000	7
TOP10(pEC2)	110,000	7
TOP10(pEC13)	35,000	500
TOP10(pEC14)	60,000	200
TOP10(pEC15)	7,000	30
TOP10(pEC16)	110,000	50
TOP10(pEC17)	14,000	30
TOP10(pEC18)	35,000	500
TOP10(pEC19)	7,000	30
TOP10(pKL)	9,000	70

[a] One unit of β-lactamase activity is defined as 1 μmol of cephalothin or cefepime hydrolyzed per min. The β-lactamase activities shown are geometric mean determinations for three independent cultures. The standard deviations were within 10%. Recombinant *E. coli* strains TOP10(pEC13) to TOP10(pEC19) and TOP10(pKL) produced ESAC β-lactamases, whereas TOP10(pEC1) and TOP10(pEC2) produced typical cephalosporinases.

transcriptional control of the *lacZ* promoter flanking the cloning site.

The β-lactamase activity against cephalothin and cefepime and the MICs of several β-lactams were determined for recombinant strains as described previously (18). Results are shown in Tables 1 and 2. Recombinant strains *E. coli* TOP10(pEC1) and *E. coli* TOP10(pEC2) had β-lactamase activities and MICs similar to those of strains *E. coli* TOP10(pEC20) to *E. coli* TOP10(pEC58).

Comparison of amino acid sequences (Table 3), MICs (Table 2), and β-lactamase activities (Table 1) showed that the enhancement of the hydrolysis activity against ceftazidime and cefepime was related to Ser→Gln, Ser→Cys, His→Pro, Val→Leu, and Val→Phe substitutions at positions 287, 287, 296, 298, and 350, respectively (Table 1). The effects of the S287N and V298L substitutions on the resistance levels and β-lactamase activities are greater than those related to the S287C, H296P, and V350F substitutions (Tables 1 to 3). AmpC-EC13, AmpC-EC18, and AmpC-EC14, which have an S287N or V298L substitution, had reduced susceptibility to cefepime and cefpirome (MICs equal to 8 or 16 μg/ml), whereas AmpC-EC15, AmpC-EC16, AmpC-EC17, AmpC-EC19, and AmpC-KL, which presented an S287C, H296P, or V350F substitution, did not confer resistance to cefepime, although the MICs for the strains producing these proteins were 30- to 60-fold higher than those for wild-type *E. coli*.

The region containing residues 287, 296, and 298 is located inside or in close proximity to helix H-10 (7, 12). This region is probably a hot spot where amino acid deletions leading to extension of the hydrolysis spectrum were already described in AmpC^{D} from *E. coli* HKY28 (7), AmpC-HD from *Serratia marcescens* HD (13), and AmpC-CHE from *Enterobacter cloacae* MHN1 (1).

Interestingly, a V350F substitution is responsible for extended-spectrum hydrolysis in AmpC from *E. coli* belonging to phylogroup B1 but not in AmpC from *E. coli* belonging to phylogroup B2 (Table 2 and Fig. 1), suggesting that other residues may contribute to modify the hydrolysis spectrum in combination with a Phe350 substitution.

This study indicates that isolation of ESAC-producing *E. coli*

TABLE 2. MICs of β-lactams for *E. coli* clinical isolates EC13 to EC19 and for recombinant *E. coli* clones TOP10(pEC1), TOP10(pEC13) to TOP10(pEC19), TOP10(pKL), and TOP10(pCR-BluntII-Topo) containing the empty vector

β-Lactam(s)	MIC (μg/ml) for:																
	EC13	EC14	EC15	EC16	EC17	EC18	EC19	KL	TOP10 (pEC13)	TOP10 (pEC14)	TOP10 (pEC15)	TOP10 (pEC16)	TOP10 (pEC17)	TOP10 (pEC18)	TOP10 (pEC19)	TOP10 (pKL)	TOP10 (pEC1)	TOP10(pCR-BluntII-Topo)
Amoxicillin	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	2
Ticarcillin	>512	>512	>512	>512	>512	>512	>512	>512	256	256	8	64	8	256	8	8	2	2
Ticarcillin-CLA[a]	32	32	32	64	32	32	64	64	256	256	8	64	8	256	8	8	2	2
Piperacillin	64	64	64	64	64	64	64	32	8	128	16	32	16	8	16	16	8	2
Piperacillin-TZB[b]	8	8	8	8	8	8	8	4	8	128	8	16	8	8	8	8	8	2
Cephalothin	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512	1
Cefoxitin	64	128	128	64	32	64	64	32	32	256	8	64	8	32	8	32	128	1
Cefuroxime	64	128	32	32	32	128	32	32	128	256	8	128	8	128	8	32	128	0.06
Ceftriaxone	4	16	2	2	2	16	2	1	16	16	4	4	4	16	4	2	0.25	0.06
Cefotaxime	4	16	2	2	2	16	2	1	16	16	4	4	4	16	4	2	0.5	0.06
Ceftazidime	256	128	64	32	64	256	64	64	512	512	512	512	512	512	512	128	1	0.06
Aztreonam	4	2	1	0.5	1	4	1	0.125	16	16	1	1	0.5	16	0.5	0.125	0.06	0.06
Cefepime	16	8	2	0.5	2	16	2	2	16	16	2	2	2	16	2	4	0.06	0.06
Cefpirome	16	8	2	2	2	16	2	2	16	16	2	2	2	16	2	4	0.06	0.06
Imipenem	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06

[a] CLA, clavulanic acid (2 mg/ml).
[b] TZB, tazobactam (2 mg/ml).

TABLE 3. Comparison of amino acid sequences of ESAC β-lactamases and those of representative narrow-spectrum cephalosporinases

Strain	Phylogroup	AmpC name	Hydrolysis spectrum	Amino acid[a] at position:																				
				9	124	141	175	85	194	214	235	238	239	241	244	278	282	287	288	296	298	300	309	350	351
EC1	B1	AmpC-EC1	Narrow	T	E	T	Q	N	P	G	R	M	N	R	N	V	S	S	G	H	V	A	R	V	A
EC2	A	AmpC-EC2	Narrow	A	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
EC3	D	AmpC-EC3	Narrow	A	D	A	—	T	S	—	Q	L	K	L	T	L	I	—	D	R	—	—	C	—	T
EC4	D	AmpC-EC4	Narrow	A	D	A	—	T	S	—	Q	L	K	L	T	—	I	—	D	R	—	—	C	—	T
EC5	B2	AmpC-EC5	Narrow	—	—	A	K	—	S	—	Q	L	K	L	—	—	I	—	D	R	—	P	—	—	—
154297	B2	AmpC-S4	Narrow	—	—	A	K	—	S	—	Q	L	K	F	—	—	I	—	D	R	—	P	—	F	—
EC13	A1	AmpC-EC13	Extended	A	—	A	—	—	—	R	—	—	—	—	—	—	—	N	—	—	—	—	—	—	—
EC14	A	AmpC-EC14	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	L	—	—	—	—
EC15	A	AmpC-EC15	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	P	—	—	—	—	—
EC16	A	AmpC-EC16	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	C	—	—	—	—	—	—	—
EC17	A	AmpC-EC17	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	P	—	—	—	—	—
EC18	B1	AmpC-EC18	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	N	—	—	—	—	—	—	—
EC19	A	AmpC-EC19	Extended	A	—	A	—	—	—	—	—	—	—	—	—	—	—	—	—	P	—	—	—	—	—
KL	B1	AmpC-KL	Extended	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	F	—

[a] Dashes indicate residues identical to those of the AmpC-EC1 sequence. The residues involved in resistance to extended-spectrum cephalosporins are underlined.

strains, which are resistant to ceftazidime according to the CLSI criteria (5), occurred in clinical isolates and could be underestimated because of the slight reduction of susceptibility to cefepime and cefpirome.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *ampC* sequences reported here are DQ092424 (EC5), DQ092425 (EC6), DQ92426 (EC7), DQ092427 (EC8), DQ092428 (EC9), DQ092429 (EC10), DQ092430 (EC11), DQ092431 (EC12), DQ092432 (EC13), DQ092433 (EC14), DQ091198 (EC15), DQ092434 (EC16), DQ091197 (EC17), AY533244 (EC18), AY533245 (EC19) SQ092420 (EC20), DQ092421 (EC26), DQ092422 (EC30), and DQ092423 (EC31).

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