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β-Lactam Resistance in Clinical Isolates of *Escherichia coli* Caused by Elevated Production of the *ampC*-Mediated Chromosomal β-Lactamase

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Among cephalothin-resistant isolates from patients with urinary tract infections, six *Escherichia coli* strains were found to produce elevated amounts of a β-lactamase indistinguishable from that coded by the *ampC* gene of *E. coli* K-12. The resistance levels displayed by these isolates toward a number of β-lactams were, for five of them, considerably higher as compared with *E. coli* K-12 with the same amount of β-lactamase, implying the importance of intrinsic resistance in these isolates. Cefuroxime, and to a lesser extent cefamandole, were stable to hydrolysis by *E. coli* chromosomal β-lactamase but acted as inhibitors of the enzyme. Nevertheless, increased β-lactamase production mediated an increased resistance toward these drugs. No plasmids were found in the isolates, suggesting a chromosomal location for the respective *ampC* locus.

The tolerance level displayed by gram-negative bacteria toward a β-lactam is determined by (i) the amount and specificity of a possible β-lactamase, (ii) the diffusion rate through the outer membrane, and (iii) the degree of inhibition displayed by the β-lactam on a set of penicillin-binding proteins (2, 22, 23, 27).

β-Lactamases have been found in a large variety of enterobacteria. These enzymes are known to differ from each other in their substrate profiles, physical and biochemical properties, and in their reaction toward antisera (7, 24, 26). β-Lactamases may be coded for either by resistance plasmids or by chromosomal genes. Plasmid-mediated β-lactamases often hydrolyze penicillins at a higher rate than cephalosporins. The predominant plasmid-mediated β-lactamases are of TEM type (24). Chromosomal β-lactamases are species specific and differ markedly from each other. However, the major part of these enzymes hydrolyze cephalosporins at a high rate (24).

Escherichia coli K-12 produces a small amount of a type I, chromosomally mediated β-lactamase coded for by the *ampC* gene (3). The enzyme activity is so low that it does not contribute to the β-lactam tolerance level of these cells (10). However, β-lactam-resistant mutants of *E. coli* K-12 have been isolated that produce increased amounts of this β-lactamase (2, 3). The *ampA1* mutation leads to about a tenfold increase in β-lactamase production (10). This mutation has been mapped very close to the structural gene for chromosomal β-lactamase denoted *ampC* (17). The *ampA1* mutation is *cis* dominant in merodiploid strains and therefore likely occurs in a promotor-operator region determining the rate of transcription from the structural gene *ampC*. From *ampA1* mutants being resistant to about 10 μg of ampicillin per ml, second-step mutants resistant to at least 100 μg of ampicillin per ml (Amp^r-100) can be obtained. The majority of these Amp^r-100 mutants have acquired a second mutation very close to *ampA1* (unpublished data), whereas those of another group owe their high ampicillin resistance to an amplification of a deoxyribonucleic acid (DNA) segment carrying the *amp* region of the chromosome (4, 18). Mutants of this latter type are characteristically unstable with respect to their β-lactam resistance. They tend to segregate into intermediate resistance classes due to the loss of repetitive *amp* DNA (4, 18).

The clinical significance of the chromosomal β-lactamase of *E. coli* has not been assessed. We therefore screened, from urine samples of patients with overt urinary tract infections, for clinical isolates that produced elevated levels of the *ampC*-mediated β-lactamase.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains LA5 (*ampA*$^+$ *ampC*$^+$), LA51 (*ampA1 ampC*$^+$), and TE01 (Amp^r-100) are isogenic derivatives of *E. coli* K-12 strain PA2004 (obtained from R. Lavallé), producing various amounts of β-lactamase (18). Strain TE01 contains 10 identical *amp* repeats, each carrying the *ampA1* and *ampC*$^+$ alleles (4). Strain SN08, being *recA rpoB* and *ampC8* (4), was used in the conjugation

experiments. Strain H1t140301 produces a temperature-labile β-lactamase due to the *ampC12* allele (3). All clinical isolates were obtained from the urine of patients with urinary tract infections. The clinical isolates used in this study were denoted C11, C13, C14, C15, C16, and C17.

Media and growth conditions. The complete medium was Luria Broth (LB) medium of Bertani (1), supplemented with medium E (25) and 0.2% glucose. It was solidified with 1.5% agar (Luria Agar [LA] plate). The Casamino Acids medium contained basal minimal medium, 1.5% casein hydrolysate, 0.2% glucose, and 25 μg of uracil per ml when required. The bacteria were incubated at 37°C as shaken cultures. Growth was followed by optical density readings, using a Klett-Summerson colorimeter with a W66 filter.

Materials. α-Aminobenzylpenicillin (d-ampicillin) and benzylpenicillin were kindly provided by AB Astra, Södertälje, Sweden. Cefamandole and cephalothin were kindly donated by Eli Lilly & Co, Indianapolis, Ind. Cefuroxime, cephalosporin C, and cephaloridine were a gift from Glaxo Laboratories Ltd., Greenford, England. Lysozyme was from Sigma Chemical Co., St Louis, Mo. Zulkowsky starch (used in β-lactamase determination) was from E. Merck AG, Darmstadt, Germany. Agarose (ME grade) was obtained from Miles Laboratories, Ltd., Stoke Poges, England.

Determination of β-lactam resistance. β-Lactam resistance was tested by single cell test (15). The bacteria was grown in LB at 37°C on a rotary shaker and harvested in the logarithmic growth phase. About 100 to 400 cells were spread on LA plates containing different concentrations of the β-lactam. Resistance was defined as the concentration level permitting 50% of the cells to form colonies.

Mating procedure. Conjugation experiments using resistant clinical isolates as presumptive donors and rifampin-resistant *E. coli* K-12 strain SN08 as recipient was performed as previously described (15, 16).

Determination of β-lactamase activity. Cells growing exponentially in Casamino Acids medium were harvested and suspended in 0.05 M phosphate buffer (pH 7.4). Cells were lysed by the addition of ethylenediaminetetraacetic acid (EDTA; 10^{-3} M) and lysozyme (100 μg/ml), followed by an ultrasonic treatment using a Branson Sonifier B12. β-Lactamase activity in the lysate when using ampicillin and benzylpenicillin as substrate was assayed by the automated microiodometric method (11). β-Lactamase activity using cephalosporins as substrates was tested as described by Fu and Neu (6). Thus, the 2-ml reaction mixture contained 0.2 mM of the different cephalosporins in phosphate buffer (0.05 M, pH 7.4). The decrease in absorbance at 255 nm was recorded after the addition of crude extracts or purified β-lactamase. Specific activity in nanokatals is defined as the enzyme activity that hydrolyzed 1 nmole of β-lactam per s per mg of protein. Protein was determined by the method of Lowry et al. (12), with bovine serum albumin as the standard.

Isoelectric focusing. Isoelectric focusing of β-lactamase in crude extracts was performed as described (20). Ampholine (LKB-Beckman Instruments AB, Vollingby, Sweden) solution with the pH range of 4.5 to 11 was used.

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was performed by the discontinuous buffer system of Laemmli and Favre (8). An 11.5% acrylamide separation gel (1 mm thick) and a 4.5% acrylamide stacking gel were used. Samples were heated immediately before electrophoresis in sample buffer at 100°C for 5 min. Electrophoresis was run at a constant current of 20 mA. Gels were stained and destained by the method of Fairbanks et al. (5).

Immunological methods. The immunodiffusion analysis was performed by the method of Ouchterlony (19). The immunoelectrophoresis method of Laurell (9) was used. Agarose was dissolved in a barbital-glycin-tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.2) at a concentration of 1% (wt/vol). Ammonium sulfate-precipitated rabbit antiserum against purified *E. coli* K-12 β-lactamase was added at 45°C. Gels were prepared on glass plates (8 by 8 cm), and the electrophoresis was run overnight at 40 V per plate. The gels were washed, dried, stained, and destained. The area under the precipitation line was taken as a relative value of the amount of β-lactamase.

DNA preparation and DNA agarose gel electrophoresis. Clear lysates were performed from 200-ml cultures. The cells were harvested by centrifugation, and the pellets were suspended in 4 ml of cold 10% sucrose in 50 mM Tris-hydrochloride (pH 8.0) and kept on ice. Lysozyme (0.8 ml at 10 mg/ml) was added and incubated on ice for 5 min. A 0.8-ml amount of 0.25 M EDTA (pH 8.0) was added, and incubation was continued for 15 min. A 6.4-ml amount of 2% Triton X-100, 60 mM EDTA, 50 mM Tris-hydrochloride (pH 8.0) was added, and lysis was performed for 20 min on ice. The lysates were cleared by centrifugation at 26,000 × *g* for 30 min. Analysis of DNA was performed by electrophoresis in agarose slab gels (20 by 20 by 0.4 cm) with the electrophoresis buffer containing 20 mM sodium acetate- 2 mM EDTA in 33 mM Tris-HAc (pH 7.8). Staining was performed with ethidium bromide, and the gels were examined under a long-wave ultraviolet lamp.

RESULTS

Characterization of β-lactamase from cephalothin-resistant clinical *E. coli* isolates. The β-lactamase activities of 109 clinical isolates with different resistance levels toward cephalothin were analyzed by using cephalothin and benzylpenicillin as substrates. Among 55 isolates resistant to at least 16 μg of cephalothin per ml, six *E. coli* isolates were found with a higher specific β-lactamase activity toward cephalothin than toward benzylpenicillin. These six clinical isolates were further investigated with the purpose of comparing their respective β-lactamase with that of the *ampC*-mediated β-lactamase of *E. coli* K-12.

An immunodiffusion test using purified antibodies to *E. coli* chromosomal β-lactamase showed that crude extracts from these *E. coli* isolates contained protein with immunological

identity to purified chromosomal β-lactamase (Fig. 1). The control strain H1t140301 *E. coli* K-12 produces a heat-labile chromosomal β-lactamase. A precipitation line showing immunological identity with purified β-lactamase was found for an unheated cell extract of H1t140301. This was not the case using a heat-inactivated extract from the same strain (Fig. 1).

The six *E. coli* strains were compared with three laboratory strains of *E. coli* K-12 that were isogenic except for the β-lactamase region of the chromosome. The laboratory *E. coli* K-12 strain LA5 (Amp^r-1) is *ampA*$^+$ *ampC*$^+$ and produces low amounts of β-lactamase. In strain LA51 (Amp^r-10), due to the regulatory *ampA1* mutation, *ampC*-mediated β-lactamase production is increased about 12 times over that of its parental strain LA5 (2) (Table 1). Strain TE01 (Amp^r-100), being derived from LA51, contains in its chromosome about 10 tandem repeats of the *amp* region and therefore produces about 10 times more enzyme than does LA51 (18) (Table 1).

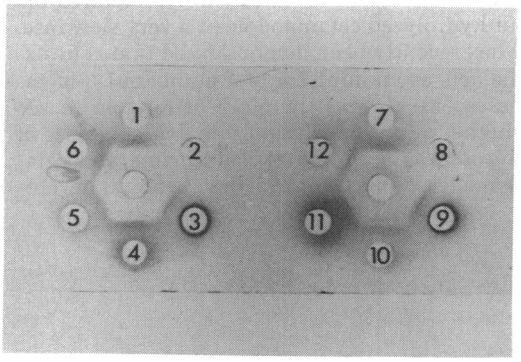

FIG. 1. *Immunodiffusion analysis of purified β-lactamase and of crude extracts of β-lactamase from six clinical E. coli isolates and E. coli K-12 strain H1t140301. The central wells contained partially purified rabbit antibodies against purified E. coli K-12 β-lactamase. Wells 1 and 7 contained purified E. coli K-12 chromosomal β-lactamase. Wells 2 and 8 contained heated extract (45°C for 15 min) from H1t140301, whereas wells 3 and 9 contained unheated extract from the same strain. The remaining wells contained extracts from the six clinical E. coli isolates as follows: 4, C11; 5, C13; 6, C14; 10, C15; 11, C16; and 12, C17.*

The β-lactamase substrate profile was similar in all six isolates and resembled that of the *E. coli* K-12 chromosomal β-lactamase (Table 1). Neither cefuroxime nor cefamandole was hydrolyzed by enzyme extracts from any of the strains tested. The relative amount of β-lactamase was analyzed by rocket immunoelectrophoresis with antibodies against purified *E. coli* K-12 β-lactamase. The six clinical isolates produced β-lactamase to a level about 24 to 48 times that of the low-β-lactamase-producing strain LA5 (Table 1). Interestingly, they all produced more enzyme than did the one-step *ampA1* mutant LA51 (Table 1). The amount of β-lactamase was plotted against β-lactamase activity by using ampicillin, benzylpenicillin, cephalosporin C, and cephalothin as substrates (Fig. 2). The relationship was the same for the clinical isolates as for the reference *E. coli* K-12 strains, suggesting that the β-lactamase monitored by the specific antiserum is the enzyme responsible for all β-lactamase activity in the respective strain. This view was strengthened by the fact that isoelectric focusing gave rise to only one fraction of β-lactamase activity. The isoelectric point was identical to that of purified chromosomal β-lactamase and was found to be 9.9. The molecular weight was determined by SDS–polyacrylamide gel electrophoresis after precipitation of the crude extracts with anti-β-lactamase antibodies

TABLE 1. *β-Lactamase substrate profile and amount of enzyme from clinical E. coli isolates and laboratory strains of E. coli K-12*

Strain	Relative rate of hydrolysis[a]						Relative amt of β-lactamase[b]
	Benzyl-penicillin	Ampicillin	Cephalosporin C	Cephalothin	Cefuroxime	Cefamandole	
LA5	100	15	300	200	0	0	1
LA51	100	12	260	185	0	0	12
TE01	100	7	225	180	0	0	100
C11	100	9	235	205	0	0	43
C13	100	13	300	265	0	0	24
C14	100	10	300	285	0	0	31
C15	100	10	375	260	0	0	30
C16	100	14	370	215	0	0	39
C17	100	10	235	165	0	0	48

[a] β-Lactamase activity against benzylpenicillin was taken as 100%.
[b] Determined by rocket immunoelectrophoresis as described in the text.

(Fig. 3). A protein identical in size to the purified chromosomal β-lactamase (36,000 daltons) was precipitated in all clinical isolates. In four of the clinical isolates, C11, C14, C16, and C17, but not in the laboratory *E. coli* K-12 strains LA51 and TE01, additional proteins with a molecular weight of about 12,000 were precipitated by the antiserum (Fig. 3). The nature of these proteins is not known at present.

The data suggested that the clinical isolates owed part of their β-lactam resistance to elevated production of the *ampC*-mediated chromosomal β-lactamase. The resistances toward several β-lactams are given for the six clinical isolates as well as for the *E. coli* K-12 reference strains (Table 2). All strains were significantly more resistant to the tested β-lactams as compared with the low-level β-lactamase-producer *E. coli* K-12 LA5 strain. Thus, all clinical isolates as well as strains LA51 and TE01 were also more resistant toward the β-lactamase-stable compounds cefuroxime and cefamandole. In Fig. 2, the resistance levels to benzylpenicillin, ampicillin, cephalosporin C, and cephalothin are given against the amount of β-lactamase produced. In five of the six clinical isolates, the β-lactam resistance levels were much higher than could be expected from their production of β-lactamase if the *E. coli* K-12 strains are used as references.

Inhibition of β-lactamase hydrolysis of cephalothin by cefuroxime and cefamandole. Both cefuroxime and cefamandole were stable to hydrolysis by crude extracts of β-lactamase. Purified chromosomal β-lactamase at a concentration of 50 μg/ml prepared from *E. coli* K-12 strain TE01 did not hydrolyze cefuroxime but hydrolyzed cefamandole at a very slow rate. It has recently been demonstrated that cefuroxime acts as an inhibitor of a number of β-lactamases (13). It was therefore of interest to see whether or not the chromosomal β-lactamase of *E. coli* was inhibited by cefuroxime and cefamandole. Table 3 shows that cefuroxime effi-

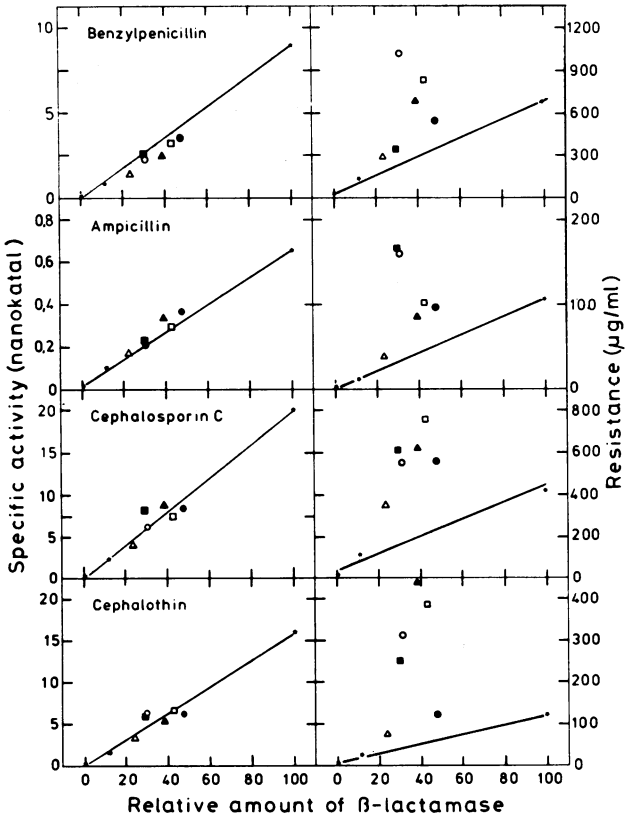

FIG. 2. *β-Lactamase activity and β-lactam resistance given against relative amount of β-lactamase. The E. coli K-12 strains LA5, LA51, and TE01 were used to obtain the functional relationships between activity and resistance versus β-lactamase level. Symbols: *, E. coli K-12 references; □, C11; △, C13; ○, C14; ■, C15; ▲, C16; and ●, C17.*

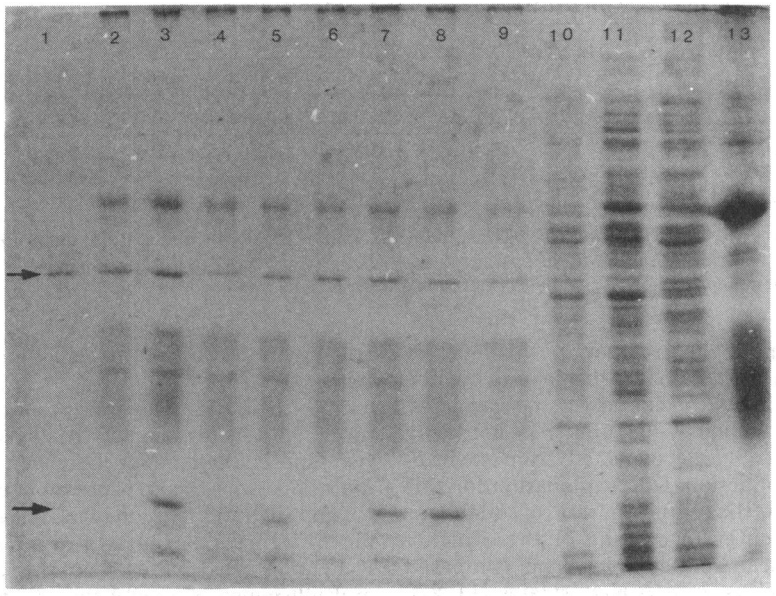

FIG. 3. *Slab gel polyacrylamide gel electrophoresis of antibody-precipitated material from cell extracts. Cell extracts of E. coli K-12 LA51 (slot 9), TE01 (slot 2), and the clinical isolates C11 (slot 3), C13 (slot 4), C14 (slot 5), C15 (slot 6), C16 (slot 7), and C17 (slot 8) were precipitated with partially purified rabbit antiserum against purified E. coli K-12 chromosomal β-lactamase, the location of which in slot 1 is indicated by the upper arrow. Slots 10 through 12 contained nonprecipitated crude extract material from TE01, C17, and C13, respectively. Slot 13 contained the rabbit antiserum used. The location of the additional proteins precipitated with rabbit antiserum is indicated by the lower arrow.*

TABLE 2. *β-Lactam resistance for clinical E. coli isolates and E. coli K-12 laboratory strains*

Strain	Resistance[a] (μg/ml)						
	Benzyl-penicillin	Ampicillin	Cephalosporin C	Cephalothin	Cephaloridine	Cefuroxime	Cefamandole
LA5	9	2.3	20	1.2	1.1	2.3	0.2
LA51	130	9	250	23	2.4	3.6	0.4
TE01	670	110	530	120	160	27	2.3
C11	800	100	750	390	58	20	2.9
C13	290	38	350	75	5	6.6	0.8
C14	1,100	160	550	310	31	23	3.9
C15	350	170	610	250	31	24	4.7
C16	680	90	620	460	17	27	2.5
C17	550	100	560	120	32	17	1.4

[a] Resistance was determined as the level of β-lactam permitting 50% of single cells to form colonies.

ciently inhibits the hydrolysis of cephalothin. Cefamandole did also act as an inhibitor to the enzyme, but the degree of inhibition was much less pronounced.

Genetic location of the β-lactamase gene. It was not possible to transfer resistance to ampicillin by conjugation from any of the six clinical isolates into *E. coli* K-12. Clear lysates were prepared from the isolates. Agarose gel electrophoresis did not reveal the presence of covalently closed circular DNA. It is therefore suggested that the β-lactamase in all six isolates is mediated by a chromosomally located gene.

DISCUSSION

The purpose of the present study was to demonstrate elevated production of the *ampC*-mediated chromosomal β-lactamse among β-lactam-resistant clinical isolates of *E. coli*.

The β-lactamases of six clinical cephalothin-resistant isolates showed immunological identity to the β-lactamase coded for by the *ampC* gene of *E. coli* K-12. Molecular weight, isoelectric point, and substrate profile were also similar to those of that enzyme. In *E. coli* K-12, we have earlier demonstrated that resistance toward β-

TABLE 3. *Inhibition of β-lactamase hydrolysis of cephalothin by cefuroxime and cefamandole*

β-Lactamase source	Hydrolysis of cephalothin[a]				
	No addition	Cefuroxime added		Cefamandole added	
		0.01 mM	0.1 mM	0.01 mM	0.1 mM
Cell extract C16	6.5	0.2	0.1	3.9	2.4
Cell extract TE01	20.1	0.5	0.2	18.5	12
Purified β-lactamase[b]	380	6.1	1.8	185	152

[a] The reaction mixture contained 0.2 mM cephalothin. Values represent nanokatals (nanomoles of substrate hydrolyzed per second per milligram of protein).

[b] A 7.5-μg/ml concentration of purified β-lactamase from *E. coli* was used.

lactams is linearly correlated to the level of chromosomal β-lactamase displayed by the strain (18). In five of the six *E. coli* isolates, resistance to various β-lactams was markedly higher than could be expected from their β-lactamase production. One explanation for this could be that the diffusion rate of β-lactams through the outer membrane of these highly resistant clinical isolates is lower as compared with the reference *E. coli* K-12 strains (14, 27). It is most unlikely that the clinical isolates owe their β-lactam resistance to the presence of additional β-lactamases, because isoelectric focusing revealed only one fraction containing β-lactamase activity. Moreover, β-lactamase activity and the amount of β-lactamase produced correlated in the same way in the clinical isolates as in the reference *E. coli* K-12 strains.

Cefuroxime and cefamandole are novel cephalosporins that have been shown to be stable to hydrolysis by the common gram-negative β-lactamases (13, 21). The chromosomal β-lactamase of *E. coli* was also unable to hydrolyze cefuroxime. Purified chromosomal β-lactamase hydrolyzed cefamandole but at a very slow rate. This activity of the enzyme was too low to be detected in crude extracts. It was interesting to note that elevated production of chromosomal β-lactamase was related to an increased resistance to both cefuroxime and cefamandole in all strains studied. Because the *E. coli* K-12 strains were isogenic except for the *ampC* region of the chromosome, it seems clear that the difference in the resistance to these drugs is solely due to the level of chromosomal β-lactamase produced. We have also demonstrated that mutation from low to high chromosomal β-lactamase production in clinical isolates of *E. coli* leads to enhanced resistance to both cefuroxime and cefamandole (data not shown). At least in the case of the former drug, it is hard to explain this increased resistance as due to hydrolysis. Cefuroxime binds strongly to the chromosomal β-lactamase and acts as an inhibitor of the enzyme. It is possible that pure binding of cefuroxime to the β-lactamase in the periplasmic space could result in the protection of penicillin-binding proteins.

In *E. coli* K-12 elevated production of chromosomal β-lactamase may be caused by at least two different genetic mechanisms: (i) regulatory mutations increasing expression of *ampC* (10, 17) and (ii) amplification of a DNA region coding for the structural gene *ampC* (4, 18). The *E. coli* K-12 mutant TE01 used in this study carries multiple copies of a DNA repeat 9,800 base pairs in size, each of which carries the structural gene *ampC* as well as the regulatory mutation *ampA1* (4, 18). Mutants amplified for the *amp* region of the chromosome are unstable and revert spontaneously to lower resistance (18). In the clinical isolates studied, revertants were found after prolonged incubation in the absence of selection. These revertants showed a β-lactamase production comparable to *E. coli* K-12 carrying the *ampA1* regulatory mutation (data not shown). Analogous to *E. coli* K-12, it may therefore be suggested that the enhanced levels of β-lactamase found in the clinical isolates are due to more than one genetic event. A combination of regulatory mutations similar to *ampA1* and possibly also amplification of the *amp* region are conceivable genetic mechanisms for enhanced β-lactamase production in the clinical *E. coli* species studied.

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