

## Sensitivity to Ampicillin and Cephalothin of Enzymes Involved in Wall Peptide Crosslinking in *Escherichia coli* K12, Strain 44

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After extraction of the membranes of *Escherichia coli* K12 strain 44 by Brij-36T, each of the four enzyme activities (natural transpeptidase, unnatural transpeptidase, carboxypeptidase and endopeptidase) of the wall peptide crosslinking system, occurs in two forms characterized by large differences in their sensitivity to ampicillin (but much smaller differences in their sensitivity to cephalothin). The fractionation of the enzyme activities into two groups of low and high sensitivity to ampicillin is achieved essentially by chromatography of the membrane extract on DEAE-cellulose.

In *Escherichia coli* K12, strain 44, the DD-carboxypeptidase, transpeptidase and endopeptidase activities are membrane bound [1,2]. Fractionation of the enzyme complex revealed the existence of two systems: a DD-carboxypeptidase-transpeptidase system for which the glutamate-amidated tetrapeptide L-alanyl-D-isoglutaminyl-(L)-meso-diaminopimelyl-(L)-D-alanine was a substrate and/or an inhibitor, and a DD-carboxypeptidase-endopeptidase system on which the glutamate-amidated tetrapeptide had no effect [2]. The use of  $\beta$ -lactam antibiotics that are known to interfere with the wall peptide crosslinking reaction in *E. coli* [3] was likely to provide further information on the properties and the functioning of the enzymes involved. The results of these investigations are presented in this paper. *E. coli* K12, strain 44, a  $\beta$ -lactamase-negative mutant and the fractions derived from its membrane [2], were of particular interest in this type of research. Indeed, the study of the action of  $\beta$ -lactam antibiotics on the peptide crosslinking system of wild strains of *E. coli* is complicated by the presence of cell bound  $\beta$ -lactamases [4–6] which hydrolyze the penicillin molecule into penicilloic acid [7]. The isolation of the  $\beta$ -lactamase-negative mutant, strain 44, is also described in the present paper.

Abbreviation. Brij-36T, polyoxyethylated ( $\bar{n}$  = 10) laurylether.

### MATERIALS AND METHODS

#### Strain

*E. coli* K12 F<sup>-</sup>, strain PA601 (originally from Dr E. Wollmann, Institut Pasteur, Paris) was selected because it exhibited a wide number of markers (*thr*<sup>-</sup>, *leu*<sup>-</sup>, *his*<sup>-</sup>, *arg*<sup>-</sup>, *pro*<sup>-</sup>, *B*<sub>1</sub><sup>-</sup>, *ade*<sup>-</sup>, *lac*<sup>-</sup>, *gal*<sup>-</sup>, *xyl*<sup>-</sup>, *mal*<sup>-</sup>,  $\lambda$ , ( $\lambda$ )<sup>-</sup>, *str*) and had no episomal  $\beta$ -lactamase. It had, however, a chromosomal  $\beta$ -lactamase.

#### Culture Media

(A) Broth medium contained 8 g broth Difco, 3 g yeast extract Difco, water, 1 l (final volume). (B) Broth agar medium was the above broth medium supplemented with agar, 18 g/l. (C) Soft broth agar medium was the above broth medium supplemented with agar, 9 g/l. (D) Minimum medium contained 40 g glucose, 450 ml phosphate-citrate buffer pH 7, water to 1 l, final volume. The phosphate-citrate buffer contained per l: 7 g K<sub>2</sub>HPO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g disodium citrate · 5.5 H<sub>2</sub>O, 0.1 g MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. (E) Minimum agar medium was the, above medium supplemented with agar: 18 g/l. (F) EMB agar medium was made from the following solutions: solution no 1 (100 ml) containing 0.4 g eosine and 0.065 g methylene blue; solution no 2 (100 ml) containing 5 g NaCl, 2 g K<sub>2</sub>HPO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>. The pH value was 6.5–7. Solution no 3

(700 ml) containing 10 g proteic peptone Difco, 1 g yeast extract Difco, 18 g agar; solution no. 4 (100 ml) containing 10 g of either lactose, galactose, xylose or maltose. (G) Difco antibiotic medium no 3.

#### *Determination of Auxotrophic Characters*

Minimum agar medium E was supplemented with the following growth factors: vitamin B<sub>1</sub> (20 µg/ml), the amino acids threonine, leucine, histidine, arginine, proline (34 µg/ml of each) and adenine (27 µg/ml). Determination of auxotrophic characters was made by replica plating on the above medium from which one of the growth factors was omitted in turn. For carbohydrate requirements, replicas were made on EMB media F supplemented with one of the selected sugars. Fermenting colonies were dark black while non-fermenting colonies were white.

#### *Tris-Maleate Buffer*

This contained per liter of final volume: 11.85 g Trizma-maleate, 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> · 7 H<sub>2</sub>O, 0.1 g MgSO<sub>4</sub> · 7 H<sub>2</sub>O, 5 mg Ca(NO<sub>3</sub>)<sub>2</sub> · 4 H<sub>2</sub>O, 0.25 mg FeSO<sub>4</sub> · 7 H<sub>2</sub>O. The pH was brought to 5.5 with HCl.

#### *Mutagen*

*N*-Methyl-*N*-nitroso-*N'*-nitroguanidine was purchased from Koch-Light Lab. (England, Batch no 39620).

#### *Single Cell Susceptibility to Rifampicin, Benzylpenicillin, Ampicillin and Cephalothin: LD<sub>50</sub> Values*

*E. coli* was grown at 37 °C in broth medium A to a density of 5 × 10<sup>8</sup> cells/ml and the cultures were diluted with a 70-fold dilution of the phosphate-citrate buffer (see medium D). Viable counts were made by suspending 100–200 cells in the soft broth agar medium C supplemented with various concentrations of antibiotics and spreading the suspensions on broth agar medium B containing the same concentrations of antibiotics. LD<sub>50</sub> values were determined graphically as the concentrations of antibiotic allowing 50% cell survival after overnight incubation at 37 °C.

#### *Effect of Ampicillin and Cephalothin on Cellular Morphology*

A log-phase culture of *E. coli* in medium G (absorbance of 0.4 at 550 nm) was supplemented with MgCl<sub>2</sub> (0.2% w/v) and sucrose (12% w/v). Aliquots (10 ml in 100-ml flasks) were then incubated for 90 min on a shaker at 37 °C in the presence of various concentrations of antibiotics. Changes in cell morphology were followed by phase-contrast microscopy.

#### *β-Lactamase Activity of Colonies of E. coli*

This was estimated by using the starch-iodide test of Perret [8] as described in [9]. The indicator plates were buffered at pH 7.4 with 10 mM phosphate and contained per l: 18 g agar, 0.127 g iodine, 0.254 g KI, 3 g soluble starch, 0.2 g benzylpenicillin.

#### *β-Lactamase Activity of Disrupted Cells of E. coli*

In many strains of *E. coli*, the β-lactamase is cell bound and is located in the periplasmic region [5]. Two procedures were used to release it. (a) The cells from an overnight culture grown at 37 °C in broth media (A or G) were washed with 0.3 M phosphate buffer pH 7.4, resuspended in the same buffer (10 g wet weight of cells per 100 ml) and disrupted with a French press (Aminco Amer. Inst. Co Inc. Silver Spring Maryland no. 4–3348) at 6000 kg; the β-lactamase activity of the disrupted cell suspension was measured with benzylpenicillin by a technique modified from Novick and Dubnau [10]. (b) β-Lactamase activity was also released by converting the cells to spheroplasts with lysozyme and sodium ethylenediaminetetraacetate (EDTA) according to the technique of Osborn *et al.* [11] (see also Materials and Methods in [1]). The β-lactamase activity was measured on the dialyzed supernatants obtained after removal of the spheroplasts by centrifugation. One β-lactamase unit is that amount of enzyme which hydrolyzes 1 µmol benzylpenicillin per hour at 37 °C and pH 7.

#### *Enzyme Preparations and Enzyme Activities*

The enzyme preparations (crude membranes of *E. coli* K12, strain 44, Brij-36 T membrane extract, membrane fractions A', A, B' and B) were those used previously (see flow sheet, Table 1 in [2]). The various enzyme activities and the corresponding assays (natural model transpeptidase, unnatural model transpeptidase, DD-carboxypeptidase and endopeptidase) were described previously (see Table 2 in [2]). All incubations were carried out at 37 °C in 0.06 M Tris-HCl buffer pH 8.5, containing 0.05 M MgCl<sub>2</sub>, 0.14 mM dithiothreitol and 0.36–8% (w/v) Brij-36T (final volume: 35 µl).

## RESULTS

#### *Isolation and Properties of β-Lactamase-Negative E. coli Strain 44*

*Mutagenesis.* *E. coli* K12 was grown at 37 °C in 150-ml flasks containing 10 ml broth medium on a New Brunswick shaker until each culture contained 5 × 10<sup>9</sup> cells. The cells from each culture were collected on millipore filters, washed with the Tris-maleate buffer and resuspended in 10 ml of the same buffer containing 1 mg *N*-methyl-*N*-nitroso-*N'*-nitroguani-

dine [12]. Each suspension was maintained at 37 °C for 15 min without shaking after which time the cells were collected by ultrafiltration, washed with the Tris-maleate buffer, resuspended in 10 ml broth medium and grown in 150-ml flasks for 2 h at 37 °C with shaking.

**Screening.** The cultures were diluted to about 30 cells/ml with a 70-fold dilution of the phosphate-citrate buffer (see medium D). Aliquots (5 ml) containing about 150 cells were membrane filtered, the filters deposited on broth agar medium and maintained at 37 °C overnight. Filters (with about 150 visible colonies on each of them) were then transferred to plates of starch-iodide indicator agar media and maintained at 37 °C for 15 min, after which time, those colonies that were not surrounded by a decolorized area were considered as possible  $\beta$ -lactamase-negative mutants. Contact of the colonies with the starch-iodine medium was restricted to 15 min in order to avoid killing of the cells. Examination of 10000 colonies by this procedure resulted in a yield of 54 possible  $\beta$ -lactamase-negative colonies. These colonies were transferred from the filters and streaked on plates of broth agar medium. After an overnight incubation at 37 °C, one colony was picked up from each of the 54 cultures and inoculated in broth medium. The same screening procedure as above was repeated and yielded 17 possible  $\beta$ -lactamase-negative mutants. Each of them was further examined by performing  $\beta$ -lactamase assays according to Novick's technique (a) on cells disrupted with the help of a French press, (b) on the dialyzed periplasmic fractions obtained by spheroplasting the cells and (c) on the lyzed spheroplasts. One strain, *i.e.* strain 44, was eventually classified as a  $\beta$ -lactamase-negative mutant.

Strain 44 had all the auxotrophic markers of the parental strain PA601 (Materials and Methods). It had an unaltered generation time (30 min by shaking in medium F at 37 °C) and an unaltered susceptibility to rifampicin ( $LD_{50}$  values: 1.7  $\mu$ g/ml). Strain 44 had no detectable  $\beta$ -lactamase activity (at least, less than 0.01 unit/g cells wet weight) while the parental strain contained 10 units of (periplasmic)  $\beta$ -lactamase per g cells (wet weight). Parallel to this, strain 44 was twice as sensitive to benzylpenicillin as the parental strain ( $LD_{50}$  values: 4.5 and 9  $\mu$ g/ml, respectively), as measured by determining the susceptibility of single cells on antibiotic-containing agar media.

#### *Effect of Ampicillin and Cephalothin on E. coli K12, Strain 44*

On solid media, the  $LD_{50}$  values of ampicillin and cephalothin for strain 44 were 0.7  $\mu$ M and 1.6  $\mu$ M, respectively (Fig. 1). In liquid F broth (supplemented with  $MgCl_2$  and sucrose see Materials and Methods),

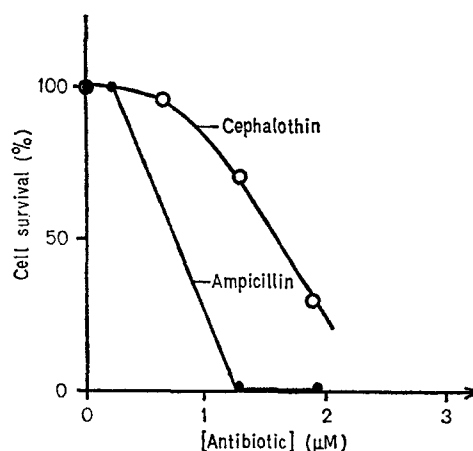


Fig. 1. Sensitivity of *E. coli* K12, strain 44, to ampicillin and cephalothin expressed as the ability of the cells to form colonies on plates with different concentrations of the antibiotics.  $LD_{50}$  values were determined graphically as the concentrations of antibiotics allowing 50% cell survival. For conditions, see text

ampicillin at 3–30  $\mu$ M concentrations inhibited septation and caused complete transformation of the strain into long rod-shaped cells. At higher concentrations of ampicillin (from 32 to 55  $\mu$ M), the filaments progressively disappeared and were replaced by oval-shaped forms exhibiting typical bulges. With cephalothin, the transitory transformation of the cells into filaments was not a clear-cut phenomenon. Partial filamentation was seen only within a narrow range of antibiotic concentrations (5–10  $\mu$ M) and complete transformation into oval-shaped forms was already complete at 25  $\mu$ M. Ampicillin and cephalothin also differ with regard to the sequence of the structural alterations they induce in *Proteus mirabilis* [13]. Marked cell elongation without division was caused by ampicillin (5.4–54  $\mu$ M) and not by cephalothin. The occurrence in *E. coli* strain B of two distinct systems involved in cell division and cell elongation respectively was also revealed by their different sensitivity to penicillin [14].

#### *Effects of Cephalothin and Ampicillin on the Unnatural Model Transpeptidase, DD-Carboxypeptidase and Endopeptidase Activities of the Brij-36T Membrane Extract*

The three activities were inhibited by 50% at the same 1  $\mu$ M concentration of cephalothin ( $ID_{50}$  values, Fig. 2). With ampicillin, the concentration dependence of the inhibition was much more complex (Fig. 2). It suggested the occurrence of two classes of enzymes of high and low sensitivity to ampicillin, respectively and distinguished the unnatural transpeptidase activity from the other two. About 50% of the total unnatural transpeptidase activity and

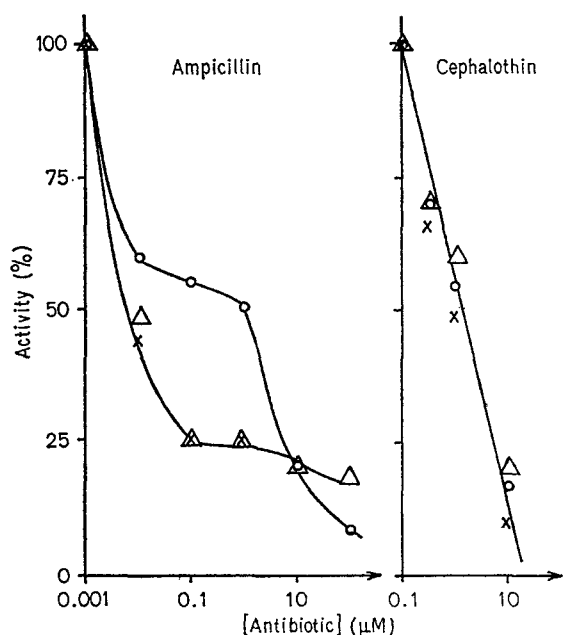


Fig. 2. Sensitivity to ampicillin and cephalothin of the unnatural transpeptidase (O), DD-carboxypeptidase (x) and endopeptidase (Δ) activities of a Brij-36T membrane extract of *E. coli* K12, strain 44. Radioactive UDP-*N*-acetylmuramyl-pentapeptide (0.3 mM, for carboxypeptidase assay), 1.2 mM non-radioactive UDP-*N*-acetylmuramyl-pentapeptide and 6 mM [ $^{14}$ C]glycine (for unnatural transpeptidase assay) or 0.3 mM radioactive bisdisaccharide peptide dimer (for endopeptidase assay) were incubated for 2 h at 37 °C with either 11 μg protein (for the carboxypeptidase) or 28 μg protein (for the unnatural transpeptidase and endopeptidase assays) of membrane extract. Reaction mixtures (35 μl, final volume) contained 0.06 M Tris-HCl buffer pH 8.5, 0.05 M MgCl<sub>2</sub>, 0.14 mM dithiothreitol and 0.36% Brij-36T. In the absence of antibiotics, 10% of the UDP-*N*-acetylmuramyl-pentapeptide was hydrolyzed to UDP-*N*-acetylmuramyl-tetrapeptide (release of the C-terminal D-[ $^{14}$ C]alanine residue; carboxypeptidase assay); 3% of the non-radioactive UDP-*N*-acetylmuramyl-pentapeptide was converted to UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-[ $^{14}$ C]glycine (unnatural transpeptidase assay) and 17% of the bisdisaccharide peptide dimer was hydrolyzed to disaccharide peptide monomer (endopeptidase assay). The data of Fig. 3 were calculated on the basis that, in the absence of antibiotic, the enzyme activities were equal to 100%.

75% of the total DD-carboxypeptidase and endopeptidase activities of the membrane extract appeared to be attributable to highly ampicillin-sensitive enzymes.

#### Effects of Cephalothin and Ampicillin on the Unnatural Model Transpeptidase, DD-Carboxypeptidase and Endopeptidase Activities of Fractions A and B

The sensitivity to the antibiotics (Fig. 2–4) was determined under conditions of initial velocities and with incubation times of 2–4 h. The ID<sub>50</sub> values of

cephalothin were 0.9 μM for the three activities of fraction B and 5 μM for those of fraction A (Fig. 3).

The ID<sub>50</sub> values of ampicillin were: 0.1 μM for the unnatural transpeptidase of fraction B and 0.7 mM for that of fraction A; 0.3 μM for the DD-carboxypeptidase of fraction B and 10 mM for that of fraction A; 4 μM for the endopeptidase of fraction B and 2 mM for that of fraction A (Fig. 3). Hence the activities of fraction B were about 1000 times more sensitive to ampicillin than those of fraction A.

The sensitivity to ampicillin of the enzyme activities of fractions A' and B' (see flow sheet, Table 1 in [2]) was virtually identical to that of the enzyme activities of fractions A and B, respectively (Fig. 4). It thus follows that the partition of the enzyme activities into two groups of low (fractions A' and A) and high (fractions B' and B) sensitivity to ampicillin was essentially achieved by chromatography on DEAE-cellulose. The enzyme fraction A' not adsorbed on DEAE-cellulose had a low sensitivity to ampicillin (ID<sub>50</sub> values: about 1 mM) and consequently, was not adsorbed on the ampicillin-affinose column (fraction A). The enzyme fraction B' adsorbed on and eluted from DEAE-cellulose had a high sensitivity to ampicillin (ID<sub>50</sub> values: about 1–0.1 μM) and consequently, was adsorbed on (and could be eluted from) the ampicillin-affinose column (fraction B). The chromatography on ampicillin-affinose, however, was a valuable means for further purification at least of fraction B' from which, as shown in Fig. 4, it appeared to remove some contaminating unnatural model transpeptidase of low sensitivity to ampicillin.

#### Effects of Ampicillin on Natural Model Transpeptidases

In a natural model transpeptidation, the pentapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-[ $^{14}$ C]alanyl-D-[ $^{14}$ C]alanine undergoes concomitant hydrolysis and transpeptidation so that tetrapeptide and peptide dimer are reaction products produced simultaneously. Within the limits of experimental error, it was observed that inhibition of the hydrolysis of the pentapeptide occurred at that concentration of ampicillin that inhibited the dimerisation of the same pentapeptide (Table 1). As observed with the other enzyme activities studied, the ID<sub>50</sub> value of ampicillin for the natural model transpeptidase of fraction A' (10 mM) was about 1000 times higher than that of fraction B' (10 μM). Moreover, there was no apparent close relationship between the sensitivity of the natural model transpeptidase and the sensitivity of the carboxypeptidase and unnatural model transpeptidase.

#### DISCUSSION

β-Lactamase-negative mutants have been obtained from *Bacilli* [15], *Staphylococci* [10] and, while

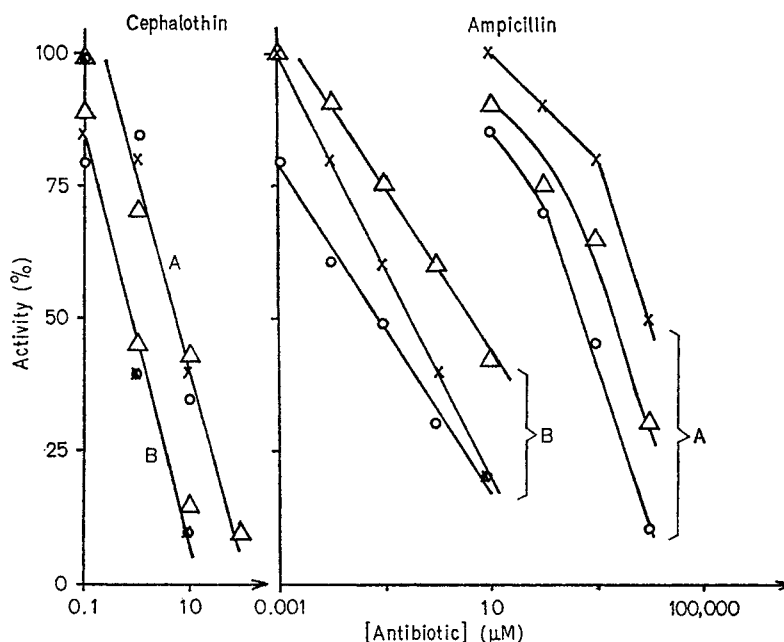


Fig. 3. Sensitivity to ampicillin and cephalothin of the unnatural transpeptidase (O), DD-carboxypeptidase (X) and endopeptidase ( $\Delta$ ) activities of fractions A and B. For the tests used in the assays, the concentrations of the substrates and the final composition of the reaction mixtures, see Fig. 2. Concentration of Brij-36T, however, was 3–8%. The incubation times at 37 °C were 2 h in all cases, except for the estimation of the endopeptidase activity of fraction A in which case the incubation time was 4 h. In the absence of antibiotics, the yields of DD-carboxypeptidase activity were 28% with fraction A and 20% with fraction B (expressed in terms of UDP-

*N*-acetylmuramyl-pentapeptide hydrolyzed), the yields of unnatural transpeptidation were 3% with fraction A and 1% with fraction B (expressed in terms of non-radioactive UDP-*N*-acetylmuramyl-pentapeptide converted into [ $^{14}$ C]-glycine containing UDP-*N*-acetylmuramyl-pentapeptide), and the yields of endopeptidase activity were 14% with fraction A and 12% with fraction B (expressed in terms of radioactive bisdisaccharide peptide dimer hydrolyzed). The data were calculated on the basis that, in the absence of antibiotics, the enzyme activities were equal to 100%.

Table 1.  $ID_{50}$  values of ampicillin for (a) the hydrolytic and transfer activities of the natural model transpeptidase (b) the unnatural model transpeptidase and (c) the DD-carboxypeptidase of fraction A' and B'

$ID_{50}$  values were determined graphically as the concentrations of ampicillin which inhibited the activity by 50%. For conditions and composition of the reaction mixtures, see Fig. 2 and 3. The natural model transpeptidase was tested as follows. Pentapeptide L-alanyl- $\gamma$ -D-glutamyl-(L)-meso-diaminopimelyl-(L)-D-[ $^{14}$ C]alanyl-D-[ $^{14}$ C]alanine (1.5 mM) was incubated for 2 h with fraction B' and for 4 h with fraction A'. In the absence of ampicillin, 37% and 25% of the pentapeptide were converted into tetrapeptide (hydrolysis) by fractions A' and B' respectively and 2.7% and 6% of the pentapeptide was converted into peptide dimer by fractions A' and B', respectively.

Fraction	Natural model transpeptidase		Unnatural trans-peptidase	DD-Carb-oxypeptidase
	hydrolysis	transpeptidation		
	mM		$\mu$ M	mM
A'	10	10	500	10
B'	0.010	0.010	0.5	0.0001

this work was in progress, from *E. coli* K12 [16]. Mutations in *E. coli* K12 occurred in the structural gene of the chromosomal  $\beta$ -lactamase. The new locus was designated *ampC* [16]. It is closely linked to the regulatory gene *ampA* at 82 min on the chromosomal map.

The peptide crosslinking enzyme complex, as it is extracted by Brij-36T from the membranes of *E. coli* K12 strain 44, occurs in two forms which can be separated from each other essentially by chromatography on DEAE-cellulose. One of them has a high sensitivity to ampicillin and cephalothin ( $ID_{50}$  values: about 1  $\mu$ M). The other is about 10 times less sensitive to cephalothin but 1000 to 10 000 times more resistant to ampicillin. A comparison between the  $ID_{50}$  values of ampicillin and cephalothin for the two isolated complexes and their  $LD_{50}$  values *in vivo* show that the highly sensitive enzyme complex is probably the one which contains the enzyme(s) responsible for the killing effect of these antibiotics. Even if one assumes that the enzyme complex of low sensitivity to ampicillin is not an artefact created either by the isolation and/or the extraction of the

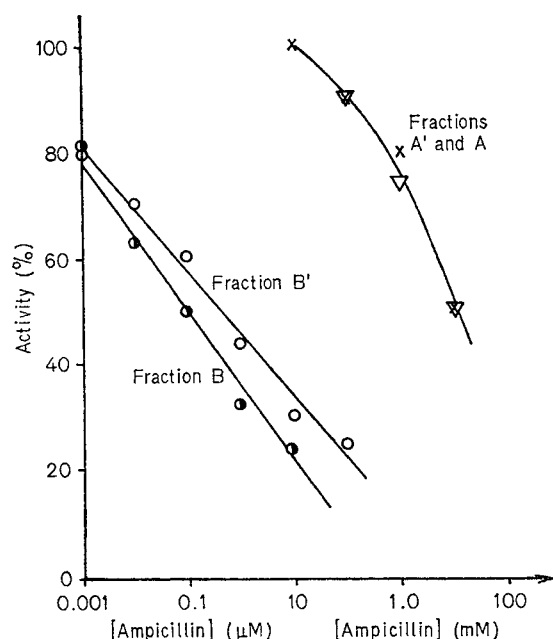


Fig. 4. Sensitivity to ampicillin of the DD-carboxypeptidase not retained on DEAE-cellulose, before (fraction A'; ×) and after (fraction A; ▽) filtration on ampicillin-affinose. Sensitivity to ampicillin of the unnatural transpeptidase adsorbed on and eluted from DEAE-cellulose, before (fraction B'; ○) and after (fraction B; ●) chromatography on ampicillin-affinose. For conditions of fractionation, see flow sheet, Table 1 in [2]. For conditions of assay, see Fig. 2 and 3. Concentrations of Brij-36T were 3–8%. Note that the only effect of treatment with ampicillin-affinose was to increase slightly the sensitivity of the enzyme activity of fraction B'.

plasma membrane, its physiological meaning and importance are obscure. It may be the result of an "aging" process. Initially synthesized at the central area of the cell [17] in a form highly sensitive to ampicillin, the peptide crosslinking enzyme complex, as the cell cycle proceeds, would be distributed over the surface of the rod in an altered form especially affecting its ampicillin sensitivity. On the other hand, elongation of *E. coli* K12, strain 44, when grown in a protected liquid medium, is inhibited at a concentration of ampicillin higher than that required to inhibit cell septation, whereas both septation and elongation are sensitive to almost the same concentration of cephalothin. Since, parallel to this, the two isolated enzyme complexes have a similar sensitivity to cephalothin *in vitro* whereas they greatly differed from each other with respect to their sensitivity to ampicillin, one might hypothesize that the enzyme complex of low sensitivity to ampicillin would be the one actively involved in cell elongation. It has been suggested that a transglycosaminidase of low sensitivity to penicillin would also occur in *E. coli* [14,18,19]. Whatever the exact mechanism, the present studies show that the peptide

crosslinking system of *E. coli* can undergo desensitization to ampicillin (and to a less extent, to cephalothin), suggesting that the constituent enzymes have different binding sites for substrates and  $\beta$ -lactam antibiotics. A similar conclusion was reached for the DD-carboxypeptidase-transpeptidase single polypeptide enzymes from *Streptomyces* R61 and R39 [20,21] and for the DD-carboxypeptidase of *Bacillus stearothermophilus* [22]. The four activities of the highly sensitive enzyme complex (fractions B' and B) have relatively slight but distinct differences in their responses to ampicillin and the same is true for the four identical activities of the highly resistant enzyme complex (fractions A' and A). Hence, the DD-carboxypeptidase-transpeptidase system which was defined on the basis of its ability to recognize the glutamate-amidated tetrapeptide [2], would consist of four synthetases predominantly acting either as natural or unnatural model transpeptidases and exhibiting a high or a low sensitivity to ampicillin. Similarly, the DD-carboxypeptidase-endopeptidase system which was defined on the basis of its inability to recognize the glutamate-amidated tetrapeptide [2], would consist of four hydrolases predominantly acting either as carboxypeptidase or endopeptidase and exhibiting a low or a high sensitivity to ampicillin. Such a multiplicity of enzyme activities is in apparent agreement with the occurrence of many different penicillin-binding sites in the membranes of *E. coli* [23]. As already discussed [2], however, it is possible that a limited number (two?) of different enzyme proteins could be involved in this complex system and that several distinct activities are catalyzed by a same enzyme protein of which the functioning would be altered by micro-environmental conditions.

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