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Genetics

Simultaneous deletion of D-alanine carboxypeptidase IB-C and penicillin-binding component IV in a mutant of *Escherichia coli* K12

(brute-force powerful mutagen selection/soluble and particulate enzymes/penicillin-binding protein)

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ABSTRACT **Mutants of *Escherichia coli* with much decreased activity of D-alanine carboxypeptidase (peptidyl-D alanine hydrolase, EC 3.4.12.11) were found among *E. coli* K12 extensively mutagenized with nitrosoguanidine treatment by assaying individual colonies for the enzyme activity. One such mutant with only 10–12% residual activity was characterized extensively. The soluble carboxypeptidase activity (corresponding to D-alanine carboxypeptidase IC of Tamura T., Imae, Y. & Strominger, J. L. [(1976) *J. Biol. Chem.* 251, 414–423] was deleted. This enzyme activity in the particulate fraction was markedly reduced but transpeptidase activity was normal. However, penicillin-binding component IV was deleted from the particulate fraction. Both the physiology and penicillin sensitivity of the organism were relatively normal, except that mutant cells were markedly more stable to penicillin-induced lysis, suggesting the possibility that carboxypeptidase IC really functions as an endopeptidase. The possible relationship of the deleted carboxypeptidase activity and the deleted penicillin binding component are discussed.**

D-Alanine carboxypeptidase (peptidyl-D alanine hydrolase, EC 3.4.12.11) (CPase) is one of a few enzymes known to be inhibited by penicillin and its derivatives (2–4). The enzyme activity *in vitro* is assayed (2) by the release of the terminal D-alanyl residue from uridine disphospho-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala), a precursor molecule in cell wall synthesis (5). The activity *in vivo* and function of CPase is not known. It has been speculated that it might be regulating the degree of crosslinkage between two peptidoglycan molecules by converting MurNAc-pentapeptide into MurNAc-tetrapeptide; the latter is not a substrate for the transpeptidation reaction (6). Recently it was reported that the CPase can function as a "pseudo" transpeptidase enzyme *in vitro* (3, 7–9) and it has been suggested (3, 7) that it functions in this way *in vivo* as well as *in vitro*. At least one of these carboxypeptidases is also able to catalyze the hydrolysis of cell walls, i.e., it has endopeptidase activity (8).

The primary objective in the present study was to use genetic studies to probe the physiology of the multiple penicillin binding components of *Escherichia coli*, and also in this way to identify which one(s) had transpeptidase activity *in vivo*, and which ones were lethal target sites for penicillin. The lethal target of penicillin action has long been believed to be an enzyme participating in cell wall synthesis, presumably the transpeptidase (6), and the inhibitory activity of penicillin on transpeptidation has been shown in *Staphylococcus aureus in vivo* (10), and in *E. coli* (5), *Bacillus stearothermophilus* (11), *Sporosarcina ureae* (12) and many other organisms *in vitro*. In view of the relative ease with which carboxypeptidase activity can be assayed and the speculation that carboxypeptidase activity *in vitro* might be a reflection of transpeptidase activity *in vivo* (3, 7) it was decided to attempt to isolate mutants with altered carboxypeptidase activity. Positive selection methods were not available, and thus colonies surviving after extensive mutagenesis with nitrosoguanidine were screened for carboxypeptidase activity. This selection method has previously been used to obtain a few other mutants for which no positive selection procedure was available (13–16); one of the best examples was the isolation of the *polA* mutant (14). Two mutants of *E. coli* with markedly decreased carboxypeptidase were found among 500 randomly-selected bacterial colonies. One of them has been extensively studied and is the subject of the present paper. A preliminary account of this work has previously been presented (17). While this manuscript was in preparation, a paper by Matsuhashi *et al.* (18) was received describing isolation of similar mutants.

Abbreviations: CPase, D-alanine carboxypeptidase; A_2pm, α, ϵ-diaminopimelate; DYABT, DYAB medium supplemented with thymine, 20 μg/ml (see ref. 1); PBC, penicillin-binding component.

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MATERIALS AND METHODS

Strains and Media. *E. coli* AT2538 was obtained from the *E. coli* Genetic Stock Center, Yale University (CGSC number 4518). Chromosomal markers of AT2538 are *thi-1, pyrE60, argE3, his-4, proA2, thr-L, leu-6, mtl-1, xyl-5, ara-14, galK2, lacY1, str-31,* λ^-, *supE44*. DYABT medium [DYAB medium (11) supplemented with thymine, 20 μg/ml] was used routinely for small cultures. For large scale preparation of cells (10–150 liters) YAB medium was used, because the strains used required neither diaminopimelic acid nor thymine. Minimal plate medium was M9 (19). Ampicillin was obtained from Squibb, and penicillin G from Sigma.

Mutagenesis. *E. coli* strain AT2538 was grown to a concentration of 10^9 cells per ml in DYABT medium at 30° with shaking, and treated with *N*-methyl *N'*-nitro-*N*-nitrosoguanidine (400 μg/ml) for 180 min at 30°, essentially according to Adelberg *et al.* (20). After washing the mutagenized cells, they were kept frozen in 50% glycerol at −80° until used. They were added to DYABT plates and incubated for 2 days at 30°. The frequency of cell survival was about 10^{-6}. To determine mutation frequency, individual colonies were streaked on various plates to check temperature sensitivity and nutritional requirements. Among 100 colonies tested, 36 had auxotrophic mutations in addition to the original ones and an additional 34 colonies showed temperature-sensitive growth on minimal medium plates supplemented with the original growth-requiring additions. About 10 colonies showed temperature-sensitive growth on rich medium (DYABT plates). Tryptophan-requiring auxotrophs were looked for specifically and one mutant was found among 324 colonies tested.

Treatment of Cells with Toluene. Individual colonies of

mutagen-treated cells were inoculated into 1 ml DYABT and grown overnight at 30° with shaking. Aliquots of 0.5 ml were inoculated into 5–10 ml of DYABT, and grown at 30° on a rotatory drum to mid-logarithmic phase (about 100 Klett units). Cells were centrifuged, washed with 0.05 M Tris·HCl, pH 7.4, and suspended in 0.5 ml of the same buffer at 0°. Toluene (15 μl) was added, and the cells were kept on ice for 20 min with occasional mixing on a vortex shaker. The cells were washed in the same buffer twice, suspended in 50 μl of buffer, and stored frozen until they were assayed for CPase activity.

Preparation of Cell Supernatant and Membrane Fraction. Cell paste (12–15 g) was suspended in an equal volume of 0.01 M Tris·maleate, pH 6.8, and centrifuged at 7500 rpm for 5 min in a Sorvall GS3 rotor. After resuspending the cells in the same buffer, they were broken in a French press at about 5700 lbs./inch^2. The broken cells were centrifuged twice at 3000 rpm for 20 min in the Sorvall SS34 rotor to remove debris and the supernatant was then centrifuged at 40,000 rpm (100,000 × *g*) for 2 hr in an A211 rotor of the IEC model B60 ultracentrifuge at 4°. The final supernatant was used as the source of the supernatant enzyme (protein, 30 mg/ml). The precipitate was suspended in 2 ml of Tris·maleate buffer and used as the source of the membrane enzyme. Glycerol (20%) was sometimes added to 20% (vol/vol) in the supernatant to stabilize the enzyme activity (8).

Assay of CPase. The assay mixture contained 250 μl of 1 M Tris·HCl buffer, pH 8.6, 100 μl of 0.5 M $MgCl_2$, and 100 μl of UDP-MurNAc-L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (containing 11,800 cpm of D-[^{14}C]Ala-D-[^{14}C]Ala per μl with a specific activity of 312 mCi/mmol) in a total volume of 2 ml. For the assay of toluene-treated cells, 15 μl of assay mixture and 10 μl of toluene-treated cells were mixed and incubated at 30° for 30 to 60 min. After incubation, the reaction mixture was boiled for 2 min, spotted on Whatman 3 MM paper (20 × 25 cm, eight samples on a single paper), and subjected to ascending chromatography in isobutyric acid/1 M NH_4OH (5/3) for about 6 hr (5). D-[^{14}C]Alanine was detected by autoradiography (several days of exposure on Kodak x-ray film RP Royal X-Omat); the corresponding area was cut and assayed for radioactivity in toluene-Omnifluor scintillation fluid (New England Nuclear). Alternatively, the reaction mixture was spotted on Whatman 3 MM paper (46 × 57 cm) and electrophoresed at 5000 V for 30 min at pH 3.5 (8). D-Alanine was coelectrophoresed as a marker and detected by ninhydrin color reaction; the corresponding area of [^{14}C]alanine was cut out and assayed in a scintillation counter with toluene-Omnifluor.

Transpeptidase Assay *In Vitro*. Preparation of crude enzyme by grinding with alumina, and the assay for transpeptidase, was performed essentially according to Izaki *et al.* (5). A typical assay mixture contained, in a total volume of 30 μl, 10 μmol of Tris·HCl buffer at pH 8.0, 1 μmol of $MgCl_2$, 4 nmol of UDP-GlcNAc, 0.18 nmol of UDP-MurNAc-L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (containing D-[^{14}C]Ala-D-[^{14}C]Ala, with a specific activity of 312 mCi/mmol).

Crude enzyme (20 μl) was incubated with 5 μl of various concentrations of penicillin G at room temperature for 15 min. Penicillinase (5 μl, 800 units/μl in water; Calbiochem) was added and the mixture was incubated for another 10 min at room temperature. Then 30 μl of assay mixture was added and incubation was continued at 30° for 30 min. At the end of incubation, the reaction mixture was boiled for 5 min, and 1 ml of CH_3COONH_4 (0.1 M)/EDTA (0.01 M) at pH 8 was added and the mixture was centrifuged at 10,000 rpm for 30 min in a Sorvall centrifuge with an SS34 rotor. The precipitate was washed twice more with CH_3COONH_4/EDTA buffer and finally was suspended in 100 μl of the same buffer. Trypsin (10 μl, 20 mg/ml; Sigma Chemical Co.) was added and the mixture was incubated at 37° for several hours, and then was boiled for 3 min. Freshly prepared lysozyme (10 μl, 20 mg/ml in CH_3COONH_4/EDTA; Sigma Chemical Co.) was added and the mixture was incubated at 37° for 13 hr; another 10 μl of lysozyme was then added and incubation was continued for another 13 hr. The sample was spotted on Whatman 3 MM paper and descending paper chromatography in isobutyric acid-1 M NH_4OH (5/3) for 19 hr was done according to Izaki *et al.* (5). The areas corresponding to the monomer and multimer were cut out and assayed with toluene-Omnifluor scintillation solution (21).

Table 1. D-Alanine carboxypeptidase activity in supernatant and particulate fractions of wild-type and mutant cells

Cells	cpm released × 10^{-6}/g wet weight cells: Supernatant	Particulate
Wild type	1.8	0.45
Mutant 172	0.17	0.057

The activity (the amount of [^{14}C]alanine released in 30 min at 30°) is expressed per g of wet cells. For the fractionation of supernatant and particulate, see the *text*.

RESULTS

Selection of a mutant with reduced CPase activity

About 500 colonies were randomly chosen from among the mutagen-treated cells and were assayed individually for CPase. Two mutants were found which had markedly reduced amounts of CPase activity in toluene-treated cells (10–12% of wild type). One of these, number 172, was studied extensively. It had the same auxotrophic requirements as the parent strain on M9 plates or in M9 medium, and grew normally at 30° or at 42°. It had almost the same morphology as the parent as determined by phase contrast microscopy, except that the distribution of cell length of mutant number 172 was slightly variable; some mutant cells were slightly longer than the parent strain.

CPase Activity in the Cell Supernatant and Particulate Fractions. After disruption of cells in a French press, the supernatant fraction was obtained by centrifugation at 100,000 × *g* for 2 hr. This fraction contained about 80% of the total CPase activity of whole cells (Table 1). The total activity in the mutant and wild-type particulate fractions are also shown in Table 1. The activity of the mutant supernatant fraction was about 10% of that of the wild type, and the activity of the particulate fraction was correspondingly reduced. Some properties of the residual, soluble carboxypeptidase were examined. Its ampicillin sensitivity (K_i of 10 μ M) was strikingly similar to that of the particulate carboxypeptidase IA, and it also had approximately the same K_m as CPase IA. It had a markedly different heat stability compared to the enzyme in the supernatant of the wild type, i.e., wild-type enzyme lost about 90% of its activity in 1 hr at 42°, while the mutant supernatant enzyme activity was completely stable under these conditions. The possibility of a CPase inhibitor in the mutant supernatant solution was excluded by appropriate mixing experiments with the wild-type enzyme. Kinetic properties of the wild-type su-

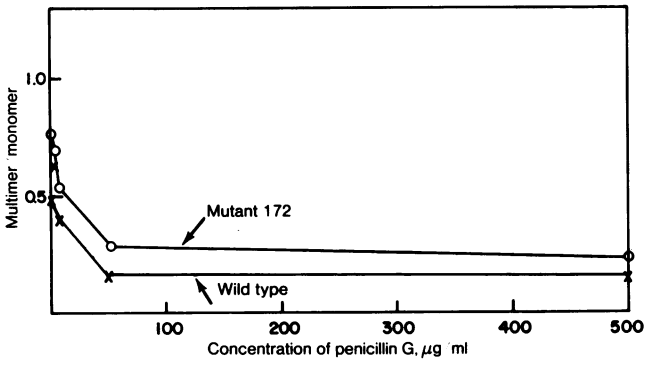

FIG. 1. Sensitivity of transpeptidase activity *in vitro* to penicillin G. Assay methods are described in *Materials and Methods*. The ratio of multimer to monomer units after lysozyme digestion was plotted versus penicillin G concentration. The total activity incorporated into the particulate fraction in the wild type was 10,000 cpm and in the mutant 11,770 cpm. Monomer plus dimer was 3400 cpm and 2640 cpm, and the lipid intermediate was 5070 cpm and 8420 cpm for wild-type and mutant 172 cells, respectively. (X—X) wild type; (O—O) mutant no. 172.

pernatant enzyme (K_m, 1×10^{-3}M; K_i for ampicillin, 2×10^{-8} M) were in good agreement with reported values (2).

Transpeptidase and penicillin binding activities in the particulate fraction

Transpeptidase Activity Measured *In Vitro* and *In Vivo*. Transpeptidase activity *in vitro* was measured according to Izaki *et al.* (5), by using alumina-ground membrane fraction as the source of enzyme. Total wild-type and mutant transpeptidase activities were the same and they were similarly sensitive to penicillin G (Fig. 1) and ampicillin. Transpeptidase activity was also assayed *in vivo* in both wild-type and mutant cells as described (1) in the presence or absence of ampicillin, 1 μg/ml. No difference in total activity or in ampicillin sensitivity was found.

Penicillin Binding Components. [^{14}C]Penicillin G binding components (PBCs) in the membrane were located by fluorography (21). *E. coli* cells contain six such binding components (21). PBC IV was missing in the mutant cells (Fig. 2).

Properties of wild-type and mutant cells

Ampicillin Sensitivity of Wild-Type and Mutant Cells on Agar Plates. Sensitivity of colony formation to ampicillin was measured on a rich nutrient agar plate containing various concentrations of ampicillin. No difference between wild-type and mutant bacteria was found (Fig. 3).

Ampicillin Sensitivity of Wild-Type and Mutant Cells in Nutrient Broth. Bacteria were grown in DYABT medium to mid-logarithmic phase and then diluted into DYABT medium containing ampicillin (0, 2, or 5 μg/ml). Subsequent growth was followed either by an increase of optical density (Fig. 4) expressed in Klett units or by an increase of viable counts measured on agar plates (Fig. 5). Wild-type cells showed earlier and more extensive lysis in liquid medium (Fig. 4*A*) than the mutant (Fig. 4*B*). Similarly, survival of mutant colony-forming units at 2 or 5 μg/ml of ampicillin was much greater than survival of wild type (Fig. 5 *A* and *B*). For example, at 2 μg/ml of ampicillin at 5.5 hr, the turbidity of wild-type cultures had fallen to 6 Klett units while that of mutant cultures was 30 Klett units; corresponding colony-forming units/ml were 6×10^4 and 170×10^4, respectively.

Deoxycholate Sensitivity of Wild-Type and Mutant Cells. Sensitivity to sodium deoxycholate was measured (23). Mutant cells were much more sensitive than wild-type cells (Fig. 6).

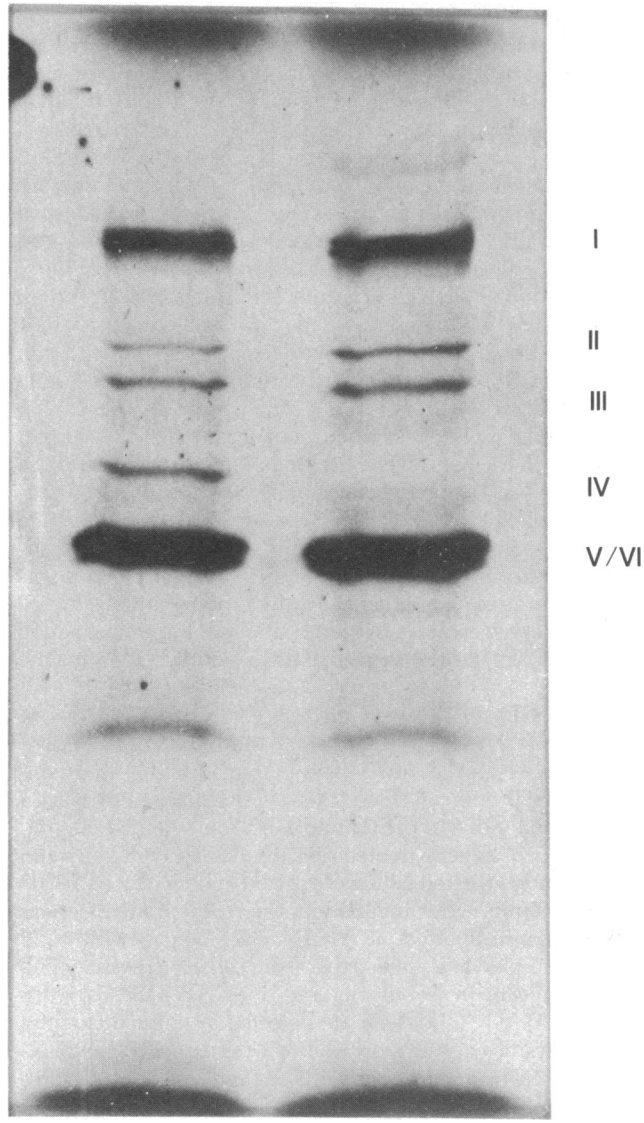

FIG. 2. [^{14}C]Penicillin G binding components. Membrane fraction was prepared by disintegration of cells with glass beads (22). [^{14}C]-Penicillin G binding and fluorography were done according to Spratt & Pardee (21). The exposure time was 21 days.

DISCUSSION

The isolation of a mutant in which the soluble carboxypeptidase is either deleted or drastically reduced in activity and in which the growth physiology of the cell, and in particular, its transpeptidase activity is unaltered suggests that a normal amount of this activity is not essential for the growth of the cell. The residual CPase activity in the supernatant fraction of the mutant cell was more heat resistant and less ampicillin sensitive than the carboxypeptidase activity in the wild-type cell. This result could be due to residual particulate carboxypeptidase in the supernatant fraction, to the presence of a second minor, previously undetected carboxypeptidase activity, or to an altered carboxypeptidase. Reduced or deleted activity clearly corresponds to D-alanine carboxypeptidase IC, the major soluble carboxypeptidase of *E. coli* which has been purified to homogeneity (8). A large fraction of this enzyme is also membrane bound (referred to as carboxypeptidase IB) and readily solubilized by LiCl (8). Deletion of carboxypeptidase IB must account for the reduction in activity in the particulate fraction of mutant number 172. Thus, these mutation data support

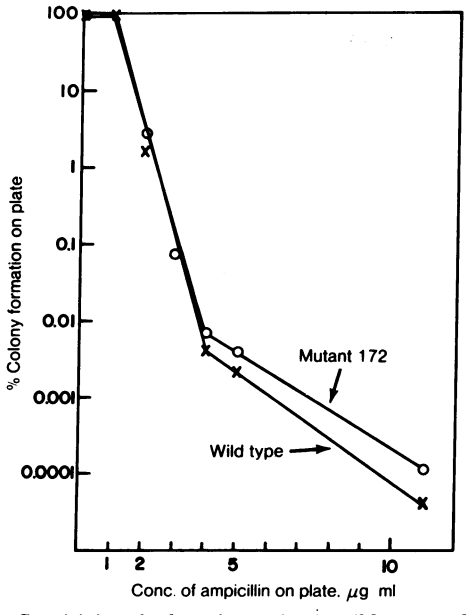

FIG. 3. Sensitivity of colony formation in wild-type and mutant cells to ampicillin. Cloned wild-type and mutant colonies were grown overnight in DYABT medium at 30°. They were added to DYABT plates, containing the appropriate concentration of ampicillin, and incubated at 30° for 3 days.

earlier data indicating that the soluble (IC) and particulate (IB) portions of this enzyme are the same protein. It is noteworthy that the purified enzyme has weak or negligent transpeptidase activity, but a strong endopeptidase activity (8), i.e., it catalyzes the hydrolysis of crosslinked dimers of cell wall. This fact may be related to the enhanced survival of mutant cells treated with ampicillin in liquid cultures. The enzyme could have some role in the opening of crosslinks in cell walls during normal growth and expansion of cell wall material, but if so, it is clearly not

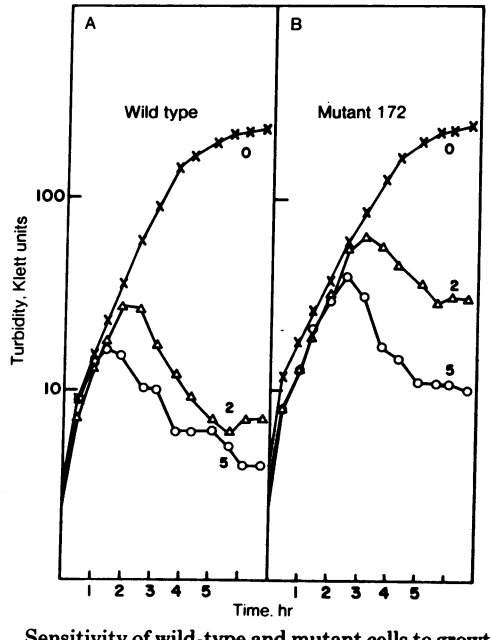

FIG. 4. Sensitivity of wild-type and mutant cells to growth with ampicillin in liquid medium. Late logarithmic phase cultures (0.1 ml) were transferred to 5 ml of fresh medium containing various concentrations of ampicillin, as indicated (μg/ml). Cultures were incubated at 30° with vigorous shaking and the growth was followed in a Klett-Summerson photoelectric colorimeter.

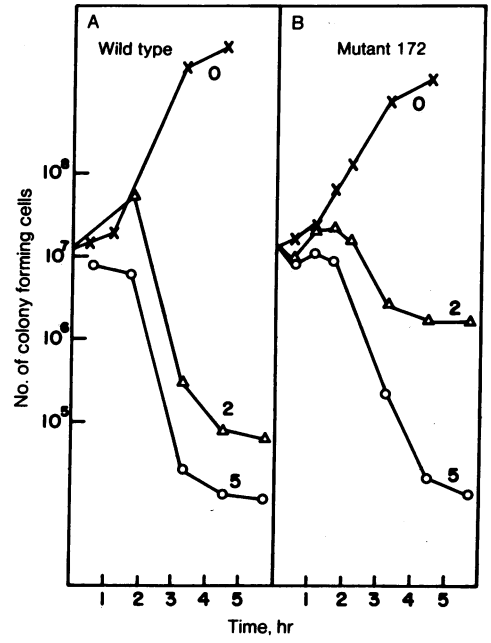

FIG. 5. Sensitivity of colony-forming ability to ampicillin in liquid medium. In the same experiment as in Fig. 4, an 0.05-ml aliquot was taken from cultures containing 2 or 5 μg/ml of ampicillin at the appropriate time, and was plated on DYABT plates after dilutions. The number of colonies on plates was counted after incubation at 30° for 2 days.

manifest in any major abnormality of growth or morphology in the conditions studied here.

The deletion of PBC IV activity is a second abnormality in the mutant cell. The simultaneous occurrence of this abnormality and the reduced or deleted soluble carboxypeptidase activity could be interpreted in several ways. Because the cells were heavily dosed with mutagens, and because nitrosoguanidine is known to produce many mutations in the same genetic region, it is possible that the genes for PBC IV and soluble carboxypeptidase are independent but closely linked. This in-

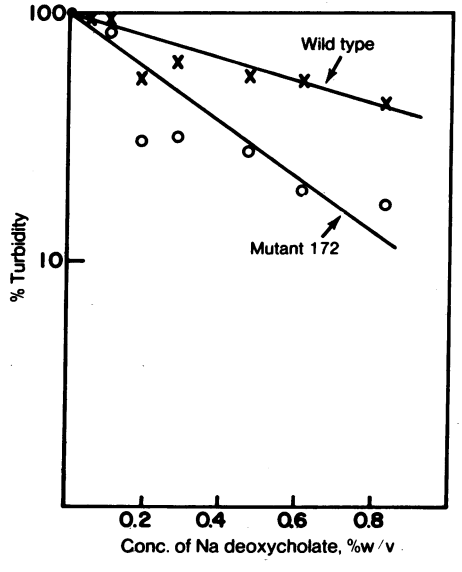

FIG. 6. Deoxycholate sensitivity of wild-type and mutant cells. Late logarithmic phase cultures were diluted into fresh medium containing various concentrations of sodium deoxycholate and the incubation was continued for 6 hr at 30°. Klett units were measured at the end of the incubation.

terrelationship is less likely because two virtually identical mutants were isolated independently by Matsuhashi *et al.* (18) although it is not excluded by this fact. Another possibility is that the genes for PBC IV and the soluble carboxypeptidase are in the same operon, and their simultaneous alteration is due to the fact that the mutation was a polar mutation or an operator mutation in this operon. Finally, it is possible that PBC IV and the soluble carboxypeptidase are directly related. For example, membrane-bound PBC IV may be a precursor of the soluble carboxypeptidase. The molecular weights of these two materials are in the same range and are compatible with this idea. However, the soluble carboxypeptidase does not bind [^{14}C]-penicillin G although it is sensitive to it and has a weak penicillinase activity (8). Therefore, if one is the precursor of the other, the transformation must also in some way affect the penicillin-binding activity. The portion of *E. coli* carboxypeptidase IC which is membrane bound and can be removed by salt extraction [called carboxypeptidase IB (8)] could actually be PBC IV, but this would leave unexplained the failure of this protein to bind [^{14}C]penicillin G in its soluble form (8). If PBC IV is a precursor of soluble carboxypeptidase IC and if this protein normally functions as an endopeptidase, then an attractive possibility is that this enzyme has a periplasmic location where it would have access to its cell wall substrate.

The present data also clearly establish that the major soluble carboxypeptidase IBC of *E. coli* is clearly not the physiological transpeptidase of *E. coli* as had been speculated for other organisms (3, 7), nor is it the lethal target site for penicillins. If it were a lethal target site, then at least one other lethal target site with identical sensitivity to penicillin would be necessary.

Nitrosoguanidine is an efficient mutagen which introduces multiple mutations in a small, clustered chromosomal region about 1.5 to 2 minutes in length (4). Thus, the selection method employed here could be useful in finding other cell wall mutants although it would be anticipated that many of these would be leaky mutants. Because nitrosoguanidine may introduce multiple mutations, some of the findings reported here (e.g., the deoxycholate sensitivity) must be interpreted with caution.

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