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Isolation of a mutant of *Escherichia coli* lacking penicillin-sensitive D-alanine carboxypeptidase IA

(cell wall synthesis/crosslinking of peptidoglycan/action of penicillin/transpeptidase/carboxypeptidase)

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ABSTRACT **A mutant of *Escherichia coli* that is deficient in D-alanine carboxypeptidase IA has been isolated. The enzyme is membrane bound and moderately sensitive to penicillin. It catalyzes *in vitro* both D-alanine carboxypeptidase and transpeptidase reactions. Being able to synthesize crosslinked peptidoglycan both *in vivo* and *in vitro* despite the absence of enzyme activity, the newly isolated mutant grew normally under a wide range of growth conditions. Therefore, this enzyme, like D-alanine carboxypeptidase IB, is not required for normal peptidoglycan synthesis in *E. coli*. The defect in the activity of D-alanine carboxypeptidase IA in the mutant however was not associated with disappearance of penicillin-binding proteins 5 and 6 (which have been shown to be D-alanine carboxypeptidase IA) or any of the other protein bands that bind [^{14}C]penicillin G. Genetic mapping studies showed that the mutation (*dacA*) is located close to *leuS* (13.7 min) on the *E. coli* chromosome map. Double mutants (*dacA dacB*) that are deficient in both D-alanine carboxypeptidases IA and IB were obtained. These double mutants also were found to grow normally and to catalyze normal formation of crosslinked peptidoglycan.**

The mode of action of penicillin has long been a subject of many enthusiastic investigations. Since its discovery by Fleming (1), this antibiotic was found to kill bacteria by inhibiting cell wall synthesis, causing cell lysis. Furthermore, many physiological and biochemical studies have shown that the key reactions in the synthesis of the cell wall peptidoglycan sacculus are those catalyzed by a penicillin-sensitive transpeptidase (2, 3) that causes crosslinking of peptidoglycan chains to form a rigid network covering the whole cell. In rod-shaped bacteria the cylindrical part of the sacculus is formed during cell growth (elongation) and the cap part is formed during cell division (septation). It is assumed that, in these two steps, different types of enzyme reactions are involved; studies reported by Gardner (4, 5) and Lederberg (6) indicated that elongation is only moderately sensitive to penicillin, whereas septation is very sensitive to it. Thus, at appropriately low concentrations of penicillin, filaments of *Escherichia coli* are formed.

The penicillin-sensitive enzymatic reactions in peptidoglycan synthesis were first observed *in vitro* in *E. coli* by Izaki *et al.* (7, 8) and by Araki *et al.* (9). Izaki *et al.* (7, 8) concluded that two enzymes, which they named peptidoglycan transpeptidase and D-alanine carboxypeptidase, were involved in the reactions; the former catalyzes the crosslinking of peptidoglycan with removal of the terminal D-alanine residue from one of the two peptide chains, and the latter catalyzes displacement of the terminal D-alanine residue without forming new crosslinkages. The penicillin-sensitive D-alanine carboxypeptidase activity was also observed when UDP-MurNAc-pentapeptide(L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala), a precursor of peptidoglycan synthesis, was used as the sole substrate in the absence of UDP-*N*-acetylglucosamine.

Several investigators (10–14) solubilized *E. coli* membranes with detergents and separated two D-alanine carboxypeptidases by DEAE-cellulose or CM-cellulose chromatography. Subsequently, the enzymes were purified to homogeneity (14). One enzyme, named D-alanine carboxypeptidase IA (14), had activities of both D-alanine carboxypeptidase and transpeptidase (assayed *in vitro* by an exchange reaction of the terminal D-alanine of UDP-MurNAc-pentapeptide with [^{14}C]glycine) and was moderately sensitive to penicillins. The other enzyme, named D-alanine carboxypeptidase IB (14), catalyzed D-alanine carboxypeptidase, transpeptidase, and endopeptidase activities, and it was highly sensitive to the antibiotics. Based on these findings, it was suggested that the enzyme that was very sensitive to penicillins causes crosslinking in septum formation and the moderately sensitive enzyme participates in crosslinking during cell elongation (12). However, it may be extremely difficult to infer the nature of reactions occurring *in vivo* from those catalyzed *in vitro*, especially when more than one type of reaction is catalyzed *in vitro*. Moreover, recent findings (15, 16) showed that, despite the lack of D-alanine carboxypeptidase IB, the cell could proliferate at a normal rate.

The present report describes the isolation of an *E. coli* mutant deficient in the moderately penicillin-sensitive D-alanine carboxypeptidase IA and shows that the lack of this enzyme also exerts no lethal effect on the cells. Moreover a double mutant (*dacA dacB*) was constructed and similarly showed no physiological impairment. If neither D-alanine carboxypeptidase IA nor IB is involved in the synthesis of peptidoglycan, and neither is essential for the growth of bacterial cells, then some other penicillin-sensitive enzymes must actually be responsible for the process of crosslinking.

A preliminary account of the isolation of the mutant has appeared (17).

MATERIALS AND METHODS

Isolation of Mutants. The mutant deficient in D-alanine carboxypeptidase IA (*dacA*) was isolated after assaying the appropriate enzyme activities in cell homogenates from a stock

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Abbreviations: *dacA*, mutant of *Escherichia coli* K-12, deficient in D-alanine carboxypeptidase IA; *dacB*, mutant deficient in D-alanine carboxypeptidase IB; *dacA dacB*, double mutant deficient in D-alanine carboxypeptidases IA and IB; *meso*-A_2pm, *meso*-2,2′-diaminopimelic acid; MurNAc, *N*-acetylmuramyl; GlcNAc, *N*-acetylglucosaminyl; disaccharide-tetrapeptide, GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-[^{14}C]A_2pm-D-Ala; pentapeptide, L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala.

Table 1. Activities of D-alanine carboxypeptidases contained in different cellular fractions obtained from the parent and the *dacA* mutant strains of *E. coli*

Strain	Fraction	Activity* Carboxy-peptidase	Transpep-tidase	Endopep-tidase
PA3092 (parent)	Supernatant	1466	63.1	2530
	Particulate	921	62.3	2410
JE11191 (mutant)	Supernatant	1410	53.2	3510
	Particulate	348	35.3	2860

* All enzyme activities were expressed in terms of pmol of products formed per mg protein per hr at 30°.

of thermosensitive mutants obtained from *E. coli* K-12, strain PA3092 (F⁻ *thr, leu, trp, his, thyA, argH, thi, lacY, malA, mtl, mel, tonA, supE, str*) treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (15). Double mutants (*dacA dacB*) were constructed by a genetic cross [Hfr(P4X) *dacA* × F⁻ *dacB*].

Isolation and Assay of Enzymes. Cells were ground with alumina and enzymes were separated on DEAE-cellulose as described (15). Transpeptidase activity was measured by following the exchange reaction of UDP-MurNAc-pentapeptide and [^{14}C]glycine in the presence of 0.1% Triton X-100 (11, 14). D-Alanine carboxypeptidase activity was assayed by measuring the release of D-alanine from UDP-MurNAc-pentapeptide labeled with D-[^{14}C]alanyl-D-[^{14}C]alanine (7, 8) and the endopeptidase reaction was followed by determining the quantity of radioactive GlcNAc-MurNAc-L-Ala-D-Glu-meso-[^{14}C]-A_2pm-D-Ala (called disaccharide–tetrapeptide) formed from radioactive bis(disaccharide–tetrapeptide) (11, 14). *In vitro* synthesis of peptidoglycan was measured with radioactive UDP-MurNAc-pentapeptide labeled either with *meso*-[^{14}C]-A_2pm or with D-[^{14}C]Ala-D-[^{14}C]Ala and UDP-GlcNAc as substrates (7, 8). Substrates and products were separated by paper chromatography with a solvent system of isobutyric acid/1 M ammonia, 5:3, (vol/vol) (7).

Other Procedures. Transduction with bacteriophage P1 (18), detection of penicillin-binding proteins (19), and all other procedures (15) were performed as described.

Reagents. Nonradioactive UDP-MurNAc-pentapeptide (20), radioactive UDP-MurNAc-pentapeptide labeled with *meso*-[^{14}C]A_2pm (40mCi/mmol) (21) or labeled with D-[^{14}C]Ala-D-[^{14}C]Ala (20 mCi/mmol) (21, 22), and radioactive bis(disaccharide-tetrapeptide) labeled with *meso*-[^{14}C]A_2pm (4.0 mCi/mmol) (11, 15) were prepared as described. Uniformly labeled [^{14}C]glycine (116 mCi/mmol) was purchased from New England Nuclear Company. (D,L + *meso*)-2,6-Diamino-[*1,7*-^{14}C]pimelic acid (30 mCi/mmol) was the product of The Radiochemical Centre, Amersham.

RESULTS

Identification of the Mutant Deficient in D-Alanine Carboxypeptidase IA. As reported (15, 16), the mutational loss of the highly penicillin-sensitive D-alanine carboxypeptidase IB was accompanied by the total disappearance of activity in the supernatant fraction of the alumina-ground cells, whereas in the particulate fraction there remained a certain amount of the same enzyme activity showing only moderate sensitivity toward penicillin. Carboxypeptidase and transpeptidase activities in the particulate fraction were reduced in the new mutant, strain JE11191 (corresponding to the levels of activities remaining in the particulate fraction from D-alanine carboxypeptidase IB mutants), but there was no loss of any activity in the supernatant fraction (Table 1; Fig. 1). The decrease of transpeptidase and carboxypeptidase activities in the particulate fraction was 44% and 62% respectively. The residual activity in the particulate fraction (like the activity in the supernatant fraction) functioned as penicillin-sensitive transpeptidase, carboxypeptidase, and endopeptidase, and these activities in both fractions of mutant JE11191 showed the same high degree of sensitivity toward penicillin G as those of D-alanine carboxypeptidase IB (Fig. 1). Therefore, it may be inferred that the enzyme activities that were missing in the particulate fraction of mutant JE11191 must have been those exhibiting moderate sensitivity toward penicillin G.

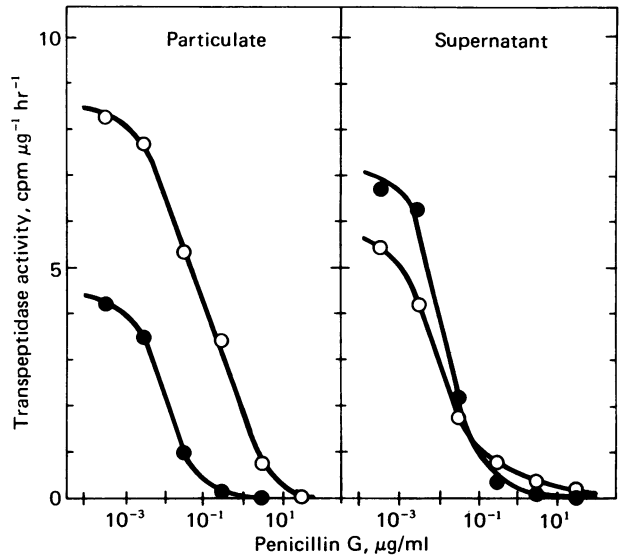

FIG. 1. Penicillin sensitivity of transpeptidase activities in particulate and supernatant fractions. O, Parent (PA3092); ●, mutant (JE11191). Similar results were obtained in the assay of D-alanine carboxypeptidase activity; the 50% inhibitory concentration of remaining D-alanine carboxypeptidase activities in both fractions from the mutant strain was also 10 ng/ml penicillin G. Endopeptidase activity existed in both fractions as two kinds of activities, penicillin-sensitive (due to D-alanine carboxypeptidase IB) and penicillin-insensitive. The latter, as mentioned in ref. 15, may be due to a new *DD*-endopeptidase.

The particulate fraction was subjected to DEAE-cellulose column chromatography (11, 14). No activity (measured by transpeptidase assay) was found in the column position corresponding to D-alanine carboxypeptidase IA in mutant JE11191 (Fig. 2*B*, compare to parental strain in Fig. 2*A*). The remaining activity in the mutant was eluted from the column in the position of D-alanine carboxypeptidase IB and the enzyme activity in this fraction was very sensitive to penicillin G, indicating that the enzyme activity is due to D-alanine carboxypeptidase IB. Because this mutant was found among thermosensitive mutants, the cell carries a thermosensitivity gene. However, its thermoresistant transductant also showed the absence of the same enzyme activities (Fig. 2*C*), indicating that the mutation causing the loss of D-alanine carboxypeptidase IA was an event not related to the thermosensitive mutation. Both peaks of enzyme activity were absent in the double mutant (*dacA dacB*) constructed from the strains carrying the individual mutations (Fig. 2*D*). Figs. 1 and 2 show the results obtained by the assay for the *in vitro* transpeptidase activity; similar results were obtained for the carboxypeptidase activity.

The mutant *dacA* grew normally, performing *in vivo* syn-

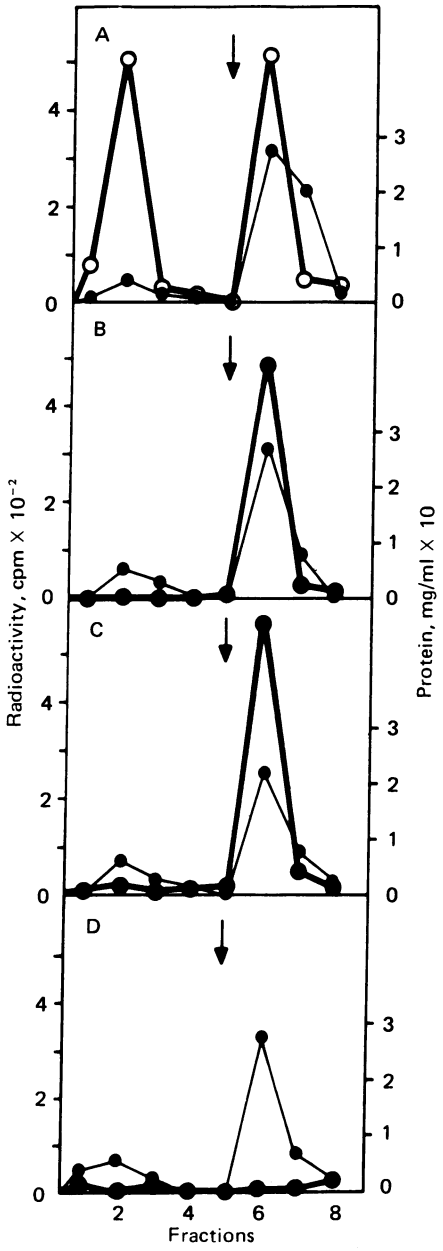

FIG. 2. Fractionation of transpeptidase activity on DEAE-cellulose. At 0 (abscissa), particulate enzyme preparations solubilized with Triton X-100 (14) were applied to the column (0.9 × 2.4 cm) of DEAE-cellulose (Whatman DE32), and D-alanine carboxypeptidase IA was washed out with 50 mM Tris·HCl, pH 8.0/1.0% (wt/vol) Triton X-100/1 mM 2-mercaptoethanol. At the points indicated by the arrows, D-alanine carboxypeptidase 1B was eluted with the same buffer containing 0.2 M NaCl. ○ or ●, Transpeptidase activity; •, protein. Experiments were performed with enzymes from: (*A*) original strain PA3092; (*B*) mutant JE11191 (*dacA*); (*C*) thermoresistant transductant of the mutant JE11191; and (*D*) the double mutant *dacA dacB*. The flow-through activity obtained from wild-type extracts was moderately sensitive to penicillin G (50% inhibitory concentration, 2 μg/ml), whereas the activity in the fraction after elution with 0.2 M NaCl showed very high sensitivity toward penicillin G (50% inhibitory concentration, 10 ng/ml) for all strains tested here. Thus it may be concluded that the activity in the 0.2 M NaCl eluate is due to D-alanine carboxypeptidase IB and not the contamination of D-alanine carboxypeptidase IA, which appears in the flow-through fraction.

Table 2. Degree of crosslinkage of peptidoglycan formed *in vivo* and *in vitro* by parent and mutant *E. coli* strains

Condition	Degree of crosslinkage, %*			
	PA3092 (parent)	JE11191 (*dacA*)	JE10012 (*dacB*)	JE5710 (*dacA dacB*)
In vivo, 30°	21.7	22.6	20.5	21.1
In vitro, 30°	19.5	24.7	23.8	30.0
42°	16.4	26.4	21.5	30.9

Experimental procedures were as described (8, 24).

* Degree of crosslinkage of peptidoglycan was calculated from the ratio (radioactivity) of bis(disaccharide-tetrapeptide) to [bis(disaccharide-tetrapeptide)+disaccharide-tetrapeptide], measured after separation of the lysozyme digests by paper chromatography. The ratio, 1, corresponds to 50% crosslinkage (19).

thesis of peptidoglycan that was crosslinked at a normal level, as in the *dacB* mutant (Table 2). Moreover, *in vitro*, the particulate enzyme fraction of both the *dacA* and *dacB* mutants synthesized crosslinked peptidoglycan either at 42° or at 30° with greater degrees of crosslinkage than in their parent strain, PA3092. Products with the greatest degree of crosslinkage were obtained with particulate enzyme from the double mutant lacking both D-alanine carboxypeptidases (*dacA dacB*). This may be because removal of D-alanine carboxypeptidase activities prevents the degradation of UDP-MurNAc-pentapeptide which is necessary for the crosslinking reaction *in vitro*.

Thus, it may be concluded that D-alanine carboxypeptidase IA, as was reported earlier for D-alanine carboxypeptidase IB (15, 16), is not required for normal peptidoglycan synthesis.

Mapping of Gene for the D-Alanine Carboxypeptidase IA. The gene *dacB* corresponding to D-alanine carboxypeptidase IB has previously been mapped at 68 min (15) on the *E. coli* map (23). Mapping of the *dacA* mutation was carried out by the method previously employed (15). Thus, *dacA* in the mutant JE11191 was located close to *leuS* [13.7 min of the *E. coli* map (23)]. Details of the genetic studies of *dacA* and *dacB* strains as well as of the double mutant (*dacA dacB*) will be reported subsequently.

Penicillin-Binding Proteins. Mutant strain JE11191 (*dacA*) did not show disappearance of any definite binding [^{14}C]penicillin G-proteins that were present in its parent strain PA3092 (Fig. 3). On careful examination of fluorograms, however, an increase in the amount of protein 6 and a slight decrease in the amount of protein 5 in the mutant (Fig. 3) became discernible. Because the relationship among the amounts of penicillin-binding proteins varies widely depending on the physiological conditions of the cells, the meaning of the changes in amount of penicillin-binding proteins in the *dacA* mutant strain remains unknown.

DISCUSSION

In the present studies on the enzymatic mechanism of synthesis of cell walls during growth and division of *E. coli*, mutants that are deficient in penicillin-sensitive D-alanine carboxypeptidases IA and IB (termed *dacA* and *dacB*) have been isolated. One of these enzymes, to which several names have been applied relating to its supposed function and mode of action and which was termed D-alanine carboxypeptidase IB by Tamura *et al.* (14), was lacking in two mutants that could grow normally under a wide range of growth conditions and could synthesize

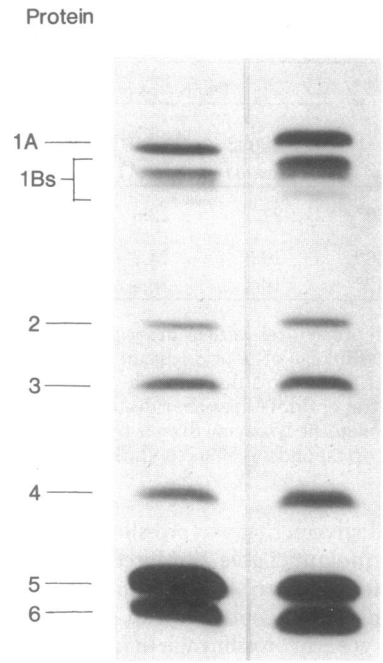

FIG. 3 Penicillin-binding proteins analyzed by sodium dodecyl sulfate/acrylamide slab gel electrophoresis and fluorography. (*Left*) Parent (PA3092); (*Right*) *dacA* mutant (JE11191).

crossbridged peptidoglycan (15, 16). In the present study, another mutant that was found to be deficient in similar enzyme, called D-alanine carboxypeptidase IA(14), has been obtained. Both these enzymes are Triton X-100-soluble and penicillin-sensitive. Despite the lack of either or both of these enzymes, the mutants were capable of normal growth during which they synthesized a normally crossbridged peptidoglycan.

These facts may indicate that the enzymes in question are not required for the normal crosslinking reaction of peptidoglycan biosynthesis. The real enzyme(s) involved in the crosslinking of the peptidoglycan is(are) that(those) observed in the *in vitro* coupled crosslinking reaction with concomitant release of D-alanine (7, 8, 19). These reactions (in contrast to those of carboxypeptidases IA and IB) are inhibited by Triton X-100, either because the enzyme proteins themselves are detergent-sensitive or because the tight coupling of enzyme and acceptor is disrupted by detergent. They are moderately sensitive to penicillin. It is generally assumed that the penicillin-sensitive enzymes have a specific site(s) to which the penicillin molecule(s) attaches to block the enzyme activity (3, 25, 26). Based upon this property of the enzyme, techniques for separation of the enzyme–penicillin complex by affinity chromatography (27) or by sodium dodecyl sulfate/acrylamide gel electrophoresis (27, 28) have recently been developed and characterization of each separated penicillin-binding protein is under investigation in many laboratories.

The enzymes involved in the crosslinking reaction of peptidoglycan synthesis in *E. coli* are supposedly the higher molecular weight penicillin-binding proteins 1 to 3 (19, 29, 30), whereas D-alanine carboxypeptidase IA is identical to penicillin-binding proteins 5 and 6 (31) and D-alanine carboxypeptidase IB, to penicillin-binding protein 4 (15, 16), both with lower molecular weights. However, the *dacA* mutation reported here that caused the loss of D-alanine carboxypeptidase activity in the membrane fraction did not cause the loss of penicillin-binding proteins 5 and 6, separated by sodium dodecyl sulfate/acrylamide gel electrophoresis. The *dacA* mutation, therefore, results in the production of an enzyme protein defective in the activity of D-alanine carboxypeptidase IA but active in binding penicillin. A similar effect has been reported by Tamura *et al.* (14) who observed that treatment of carboxypeptidase IA with sulfhydryl reagents inhibited carboxypeptidase activity of the purified enzyme but not its penicillin-binding activity. Carboxypeptidase IA also releases bound penicillin G as penicilloic acid with a half-time of 5 min at 37° (penicillinase activity) (14). Curtis and Strominger (32) have recently found that the release of penicillin G, like the carboxypeptidase activity, is inhibited by sulfhydryl reagents and that an acyl–enzyme intermediate formed from a normal substrate accumulates in the presence of these reagents. They also suggested that the present mutation may result from a genetically induced defect in the protein similar to the alteration produced by sulfhydryl reagents. If so, the *dacA* mutant will be a very useful tool for investigating the mechanism of catalysis by this interesting enzyme.

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