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Mutants of *Escherichia coli* lacking in highly penicillin-sensitive D-alanine carboxypeptidase activity

(*Escherichia coli* peptidoglycan/penicillin target/endopeptidase/transpeptidase)

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ABSTRACT **Mutants of *Escherichia coli* lacking in the highly penicillin-sensitive enzyme activities of D-carboxypeptidase, transpeptidase, and endopeptidase, and with the concomitant absence of penicillin-binding protein 4 of B. G. Spratt and A. B. Pardee [(1975) *Nature* 254, 516–517] were isolated. The defect of these mutants is ascribed to the lack of an enzyme, D-alanine carboxypeptidase Ib. Genetic mapping studies show the mutation (*dacB*) to be located at 68 min on the *E. coli* chromosome map. The *dacB* mutation results in the simultaneous loss of D-alanine carboxypeptidase and penicillin-binding protein 4.**

The mutants grew normally under a wide range of growth conditions. We conclude that the enzyme is not a necessary component for normal peptidoglycan biosynthesis in *E. coli*.

Bacterial envelopes contain a shape-maintaining element called the sacculus. The sacculus is a rigid, single molecule which encloses the cell completely (1). It is made of a polymer, called murein or peptidoglycan, which is a network of polysaccharide chains crosslinked by short peptide bridges (1–5). The formation of crosslinked peptidoglycan is an essential reaction for bacterial cell growth. Penicillin selectively attacks this reaction and kills bacterial cells (6–11). Two penicillin-sensitive enzyme activities, namely peptidoglycan transpeptidase and D-alanine carboxypeptidase, have been found (8–11). The peptidoglycan transpeptidase reaction displaces the terminal D-alanine residue from one of the two peptide chains to form crosslinked peptidoglycan. A possible explanation of the D-alanine carboxypeptidase activity is that it is an uncoupled transpeptidase reaction where water serves as the peptide acceptor (8). Because of the penicillin sensitivity of these reactions, they are considered to be responsible for crosslink formation in *Escherichia coli* (8–11). D-Alanine carboxypeptidase might be an enzyme that functions in displacing a D-alanine residue so as to limit the degree of crosslinking of the peptidoglycan (8). D-Alanine carboxypeptidase has been demonstrated in other bacteria (12–15) and in blue-green algae (13). These D-alanine carboxypeptidases were found to be highly sensitive to penicillin: the minimum inhibitory concentrations of penicillins with regard to these enzymes were less than those necessary for killing of growing cells. A killing site distinct from the carboxypeptidase has been postulated on the basis of inhibition experiments using penicillin and cephalosporin on *Bacillus subtilis, in vivo* (16).

In this report, we document the isolation of nonlethal mutants of *E. coli* lacking the highly penicillin-sensitive D-alanine carboxypeptidase. A preliminary account on the isolation of the mutants has appeared (17).

Abbreviations: MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; A_2pm, diaminopimelic acid.

MATERIALS AND METHODS

Isolation of Mutants. An *E. coli* K-12 strain, PA3092 (F^-, *thr, leu, trp, his, thyA, argH, thi, lacY, malA, mtl, mel, tonA, supE, str*) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, as described previously (18). Thermosensitive mutants of independent origin that grew at 30° but not at 40° on L-agar plates (19) were isolated by a replica-plating technique (20). Five thousand strains were isolated and stocked. This mutant collection served as a source of a mutant lacking a highly penicillin-sensitive D-alanine carboxypeptidase, as follows: One hundred and thirty mutants that lysed at 40° but grew normally at 30° were screened from 1000 thermosensitive mutants of this collection. The enzyme activities of these lysis-mutants were examined by the method described in the following section. The detailed nature of this collection will be described elsewhere.

Culture of Cells and Preparation of Enzyme. A 50-ml overnight culture of *E. coli* grown in 1.75% Difco antibiotic medium III at 30° was inoculated into 500 ml of the same medium. Exponentially growing cells at 30° under gentle shaking were harvested by centrifugation at 0–4°. The cells were washed with buffer and disrupted by grinding with alumina (21). Crude extract was prepared with ice-cold 0.05 M Tris·HCl at pH 7.6 containing 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, followed by a centrifugation at 6000 × *g* for 10 min. Then the particulate and the supernatant fractions were fractionated from the crude extract by differential centrifugation. The particulate fraction was sedimented at 100,000 × *g* for 1 hr, washed once, and suspended in the Tris buffer supplementd with 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol.

Separation of Enzyme Activity on a DEAE-Cellulose Column. The method was essentially the same as described previously (22), except that enzymes were extracted with 1% (wt/vol) Triton X-100, applied to the column without ammonium sulfate fractionation, and eluted stepwise.

Enzyme Assays. The methods for measuring activities of D-carboxypeptidase–transpeptidase–endopeptidase (23), peptidoglycan synthetase (8), and crosslinking reaction (8, 24) were as described previously.

Sodium Dodecyl Sulfate/Acrylamide Gel Electrophoresis of Penicillin-Binding Protein. An apparatus for electrophoresis in acrylamide slab gels was prepared according to the description of Studier (25). The procedures of Spratt and Pardee (26) for penicillin-binding proteins were followed.

Transduction with Bacteriophage P1. The method used for transduction experiments was described previously (19).

Reagents. Nonradioactive UDP-MurNAc-L-Ala-D-Glu-

Table 1. Assay of three activities of D-alanine carboxypeptidases

Strain	Fraction	Carboxy-peptidase	Trans-peptidase	Endo-peptidase
PA3092 (parent)	Supernatant	1798	92.5	2513
	Particulate	550	102.1	2064
JE10012 (mutant)	Supernatant	144	5.7	944*
	Particulate	330	48.5	492*
JE10064 (mutant)	Supernatant	72	2.8	1209*
	Particulate	446	57.8	341*

Carboxypeptidase activity was estimated by measuring the amount of D-[^{14}C]alanine liberated from the UDP-MurNAc-L-Ala-D-Glu-*meso*-A$_2$pm-D-[^{14}C]Ala-D-[^{14}C]Ala (8). Transpeptidase activity was estimated by measuring the amount of [^{14}C]glycine exchanged for the terminal D-alanine of UDP-MurNAc-pentapeptide by the enzyme fractions in the presence of 0.1% Triton X-100 (23). Endopeptidase activity was estimated by measuring the formation of [^{14}C]A$_2$pm-labeled disaccharide-tetrapeptide from bis(disaccharide-tetrapeptide) of *E. coli* in the presence of 1% Triton X-100 (23). Substrates and products were separated by paper chromatography with a solvent system of isobutyric acid/1 M ammonia (5:3, vol/vol) (8). All the enzyme activities were expressed as pmol/mg of protein per hr at 30°.
* Endopeptidase activity of the mutant preparations was not inhibited by penicillin G (1 mg/ml).

meso-A$_2$pm-D-Ala-D-Ala (27), radioactive UDP-MurNAc-L-Ala-D-Glu-*meso*-A$_2$pm-D-[^{14}C]Ala-D-[^{14}C]Ala (specific activity, 20 mCi/mmol) (28, 29), and radioactive bis(GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-[^{14}C]A$_2$pm-D-Ala) [called bis-(disaccharide-tetrapeptide)] (specific activity, 4.0 mCi/mmol) (23, 30) were prepared as previously described. [^{14}C]Glycine uniformly labeled (specific activity, 116 mCi/mmol) was purchased from New England Nuclear Co. [^{14}C]Penicillin G (specific activity, 53 mCi/mmol) was purchased from the Radiochemical Centre.

Protein Assay. The protein content of the extract was assayed according to the method of Lowry *et al.* (31), and extracts containing Triton X-100 were measured by the method of Wang and Smith (32).

RESULTS

Identification of D-Alanine Carboxypeptidase Mutants. Crude extracts prepared from two thermosensitive mutants of independent origin, JE10012 and JE10064, were found to have significantly less D-alanine carboxypeptidase activity than the extracts of the parental strain. The crude extracts of the parent and the mutants were then fractionated into particulate and supernatant fractions. Three enzyme assays for carboxypeptidase, transpeptidase, and endopeptidase activities were applied to each fraction. As shown in Table 1, the two mutants simultaneously lost almost all the activities of carboxypeptidase and transpeptidase and the greater part of the endopeptidase activity in the supernatant fraction. The residual activity observed in the supernatant fractions of the mutants was insensitive to penicillin, and may be attributed to some other endopeptidase. In the particulate fractions of the mutants, 60–80% of carboxypeptidase activity, 50–60% of transpeptidase activity, and 15–25% of endopeptidase activity of the parent were found under the assay conditions employed. Fig. 1 demonstrates the lack of transpeptidase activity of the supernatant fraction of mutant and the difference between the mutant and the parent in the penicillin sensitivity of the enzyme activities of the particulate fraction. The enzyme activity of the supernatant fraction of the parent was highly sensitive to penicillin, the

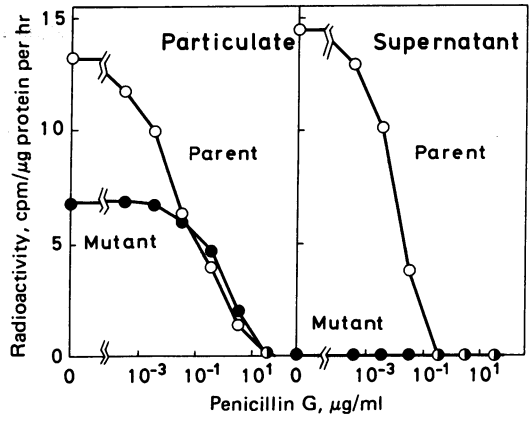

FIG. 1. Penicillin sensitivity of transpeptidase activities of particulate and supernatant fractions. See the legend to Table 1 for description on measurement of enzyme activity. ○, Parent (PA3092); ●, mutant (JE10064). Results were similar if the enzyme activities obtained from mutant JE10012 were tested.

half-inhibitory concentration being *ca* 10 ng/ml, whereas the activity of particulate fractions of the mutant and the parent was more resistant to penicillin. The enzyme activity in the particulate fraction of the parent was twice as high as the corresponding activity of the mutant. One-half of the former activity was completely suppressed at the same low concentration of penicillin as the enzyme activity of the supernatant. Thus, enzyme activity in the particulate fraction of the parent is explained to be the sum of two different enzyme activities, one being highly sensitive and the other less sensitive to penicillin, and the former is missing in the mutant. From these results, it is concluded that the three enzyme activities in the supernatant fraction and a part of the activities in the particulate fraction are manifested by the identical D-alanine carboxypeptidase.

Fractionation of Penicillin-Sensitive Enzymes on DEAE-Cellulose Column. D-Alanine carboxypeptidase activities in the particulate fraction were separated on a DEAE-cellulose column. Two enzyme fractions obtained were similar to those reported previously (22). Fig. 2 illustrates that the enzyme activity in the 0.2 M NaCl eluate was missing in the mutant. The two enzyme activities differed in their sensitivities toward penicillin, the first peak that ran through the column [fraction a (23, 33)] being moderately sensitive toward ampicillin and the second peak that was in the 0.2 M NaCl eluate of the parent [fraction b (23, 33)] being highly sensitive toward this antibiotic. The activity of the supernatant fraction from the parent was also highly sensitive (Fig. 1). High sensitivity of these fractions to penicillin has been reported (8, 12, 34). The enzyme activities of these fractions were completely deleted in the mutants.

D-Alanine Carboxypeptidase Mutation Is Not Lethal. Both JE10012 and JE10064 were isolated as thermosensitive mutants. These mutants were made thermoresistant by P1 transduction from a wild-type strain. The transductants grew normally under a wide range of growth conditions and were completely resistant to lysis at 40°, whereas the defects in enzyme activities as shown in the table and figures remained unaltered. Similar observations were obtained with a mutant of another D-alanine carboxypeptidase, fraction a (see ref. 17; unpublished data).

***In Vitro* Synthesis of Crosslinked Peptidoglycan.** The synthesis of peptidoglycan *in vitro* was measured by incorporation of MurNAc-L-Ala-D-Glu-*meso*-A$_2$pm-D-[^{14}C]Ala-D-[^{14}C]Ala as described in *Materials and Methods*. The thermosensitive mutants, the thermoresistant transductants, and

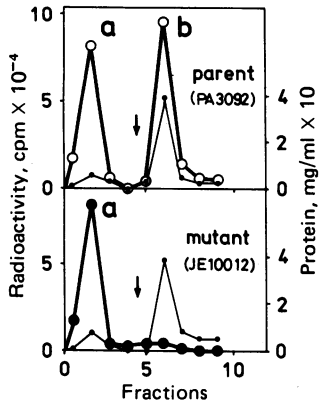

FIG. 2. Fractionation of transpeptidase activity on DEAE-cellulose column. At 0 (abscissa) samples were applied to the column (0.9 cm × 2.4 cm) of DEAE-cellulose (23) and washed with a buffer containing 50 mM Tris·HCl, pH 8.0/1.0% (wt/vol) Triton X-100/1 mM 2-mercaptoethanol. At the points indicated by the arrows the enzyme fraction b was eluted with the same buffer containing 0.2 M NaCl. ○ Or ●, transpeptidase activity; •, protein. Experiments using the enzymes obtained from mutant JE10064 and thermoresistant transductants of the mutant JE10012 or JE10064 (recipient strains) gave similar results.

the parent actively synthesized peptidoglycan, *in vitro*. The degree of crosslinking of peptidoglycan, synthesized *in vitro* as well as *in vivo*, of mutants and the parent was measured as described in *Materials and Methods*. No appreciable difference in the degree of crosslinking of peptidoglycan was observed.

Mapping of Gene for the D-Alanine Carboxypeptidase Fraction b. The abbreviation *dac* will be used for the gene loci corresponding to the D-alanine carboxypeptidases, as *dacA* and *dacB* for the enzyme fraction a and b, respectively. Mapping of the *dacB* mutation was carried out by the method employed in the study of other mutations that are lacking in an easily recognizable phenotypic character (35). Both *dacB* mutations in the mutants JE10012 and JE10064 were thus located at 68 min on the *E. coli* map (36). Details of the genetic studies will be reported subsequently.

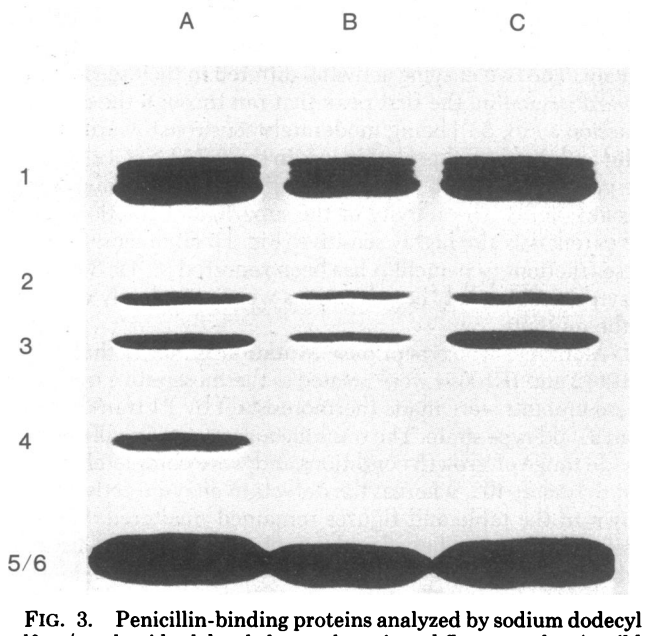

FIG. 3. Penicillin-binding proteins analyzed by sodium dodecyl sulfate/acrylamide slab gel electrophoresis and fluorography. A, wild type; B, mutant (JE10012); C, mutant (JE10064). The numbers indicate those given by Spratt and Pardee (26). In the present analysis three components were discernible in band 1.

Loss of a Penicillin-Binding Protein in the Mutants. Fig. 3 shows a pattern of penicillin-binding proteins separated by sodium dodecyl sulfate/acrylamide slab gel electrophoresis. In both mutants JE10012 and JE10064, a band corresponding to band 4 of Spratt and Pardee (26) was lost. Defect of the fraction b of D-alanine carboxypeptidase was associated with the loss of the penicillin-binding protein band 4. A number of transductants that received either *dacB* or its linked markers, or both, were obtained by transduction with P1 and examined with respect to penicillin-binding proteins. The loss of band 4 was not separable from the defect of D-alanine carboxypeptidase activity, as examined in the 36 transductants including four isogenic pairs of *dacB* and its wild type. The absence of band 4 was independent of the thermosensitivity of the mutants, persisting in their thermoresistant transductants obtained by P1 transduction. The missing enzyme in our mutants is most probably D-alanine carboxypeptidase IB described by Tamura *et al.* (34). The isolation of a mutant defective in this enzyme has been reported (37). All the three mutants isolated independently grew normally and lacked penicillin-binding band 4 (38). From these results, we conclude that the *dacB* mutation results in the loss of both D-alanine carboxypeptidase and penicillin-binding protein 4. The enzyme and the band 4 protein are either identical or distinct. In the latter case, *dacB* may act as a regulatory gene for the production of both proteins.

DISCUSSION

The *dacB* mutants grew normally under a wide range of growth conditions. These mutants synthesized normally crosslinked peptidoglycan *in vivo*, as well as *in vitro*. The following possibilities can be considered to explain the role of this enzyme in bacterial growth: the enzyme is essential but (*a*) the low residual activity of the mutant is enough for the cell to carry out the enzyme reaction, (*b*) the mutant enzyme becomes unstable and is inactive *in vitro* but is active *in vivo*, (*c*) there is an alternative reaction to bypass the lack of this enzyme; alternatively (*d*) the enzyme is not essential for the cell growth and division. D-Alanine carboxypeptidase in *B. subtilis* has been shown to be not essential (16, 39) and several lines of the experiments described in this report strongly support the last possibility, although the other alternatives were not excluded rigorously.

If possibility *d* could be proved to be true, then studies on the nonlethal *dacB* mutants would not reveal significant information relating to the nature of the real crosslinking enzyme system. It demonstrates, however, the existence of some indispensable components other than these enzymes. The availability of such mutants as described here offers a new possibility for the elucidation of the real enzyme system involved in crosslinking. Because the mutations suppress a significant part of D-alanine carboxypeptidase and endopeptidase reactions, they offer an invaluable tool for the analysis of the real enzyme system(s).

The sacculus is thought to constitute an apparatus by which the bacterial cell elongates and septates (40, 41). The nascent peptidoglycan molecules are formed from nucleotide precursors, UDP-MurNAc-pentapeptide and UDP-GlcNAc through the lipid cycle reaction (21, 42), and this formation should be localized at the growth zone of the sacculus (40, 43). These reactions have to be coordinated with regulatory circuits coupled with cell growth and division (40). Penicillin inhibition experiments suggest that the enzymes involved in elongation and septation of the sacculus are sensitive to penicillin (44–46).

Where can such a system be sought? We have a collection

of thermosensitive mutants of independent origin (see *Materials and Methods*). The number of mutants of this collection has reached the total number of genes of *E. coli*. Because these mutants received heavy mutagenesis at the time of the mutant isolation, most of them are expected to carry multiple mutations, both indispensable gene mutation(s) selected and dispensable gene mutation(s) unselected. It would be possible to find a relevant mutant by measuring the enzyme activities in question, for every mutant in the collection. Successful mutant isolations via indiscriminative examination of heavily mutagenized *E. coli* have already been reported (47, 48). Once the needed mutants are collected, mutations can be introduced into an appropriate strain by genetic means to construct an ideal strain for the enzymological study.

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