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# On the process of cellular division in *Escherichia coli*: A series of mutants of *E. coli* altered in the penicillin-binding proteins*

(murein synthesis/penicillin target/gene mapping)

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**ABSTRACT** **A series of mutants defective in penicillin-binding components (or proteins) (PBCs) was isolated from a collection of thermosensitive mutants of *Escherichia coli*. The mutants included mutations for each PBC (1 through 4) and a mutation in the activity of D-alanine carboxypeptidase 1a (PBC-5/6). PBC-1 was resolved into two components, PBC-1a and PBC-1b, which were the products of different genes referred to as *ponA* and *ponB*, respectively.**

**No mutation examined in *ponA*$^-$ or *ponB*$^-$, by itself, was associated with a thermosensitive defect in growth. The mutants having *ponB*$^-$ were hypersensitive to cephalosporins, which showed unusually high affinity to PBC-1a. Murein synthetic activity decreased markedly in cell-free preparations from the *ponB*$^-$ mutant. The double mutation *ponA*$^-$ *ponB*$^-$ was found to be lethal. A mutant having *ponA*$^{ts}$ and *ponB*$^-$ revealed thermosensitivity of growth and lysed at the restrictive temperature. It is concluded that PBC-1a and PBC-1b could share a biochemical reaction necessary for cell elongation so that the function of either one may bypass the loss of the function of the other.**

**With the series of mutants, the chromosomal locus of the gene for each PBC was mapped: *ponA* (PBC-1a) 73.5 min, *ponB* (PBC-1b) 3.3 min, *rodA* (PBC-2) 14.4 min, *ftsI* (PBC-3) 1.8 min, *dacB* (PBC-4) 68 min, and *dacA* (PBC-5/6) 13.7 min.**

Extensive studies on bacterial cell division have been carried out for many years and much information has accumulated (1–5), but we are still far from an ultimate understanding of cell division and cell elongation at the molecular level. It has been suspected that β-lactam antibiotics inhibit cell division and cell elongation by attacking penicillin-sensitive enzymes involved in murein biosynthesis during the bacterial cell cycle (6–10). Again, little evidence has been presented, so far, to substantiate this view. Penicillin-sensitive enzymes have been purified and the nature of the enzyme reactions has been extensively characterized (11–13); however, further clarification of their roles *in vivo* required the isolation of mutants altered in these enzymes. We isolated two classes of mutants (*dacB* and *dacA*) lacking the activity of penicillin-sensitive enzymes, D-alanine carboxypeptidase 1b or 1a, and constructed a double mutant defective in both (ref. 14; M. Matsuhashi, I. N. Maruyama, Y. Takagaki, Y. Nishimura, and Y. Hirota, unpublished data). These mutants grew normally, indicating that the enzymes are likely to be dispensable.

The discovery of penicillin-binding components (or proteins) (PBCs) in bacteria (15) and the development of a sensitive method for the detection of the PBCs (16, 17) opened a new approach to these problems. In *Escherichia coli*, six PBCs were described and PBC-1, PBC-2, and PBC-3 were inferred to be essential proteins for cell elongation, cell shape determination, and cell division, respectively (16, 18). The others, PBC-4 and PBC-5/6 (PBC-5 and PBC-6 are assumed to be the same enzyme), were thought not to be involved in the killing site of β-lactams (18), and were found to correspond to D-alanine carboxypeptidase 1b and 1a, respectively (14, 19, 20). Only a limited number of mutants defective in PBCs are available so far, and a more thorough investigation of PBC mutants of *E. coli* would be valuable. Thus, we carried out an extensive mutant isolation and characterization.

We document here a preliminary account of the isolation and characterization of a series of mutants in PBCs and the chromosomal location of their gene loci. Studies on the mutants defective in PBCs led us to the conclusion that PBC-1a and PBC-1b are involved in the final reaction step(s) of murein synthesis and are functionally interchangeable *in vivo*.

## MATERIALS AND METHODS

**Bacterial Strains.** An *E. coli* K-12 strain, PA3092 (F$^-$ *thr-1 leu-6 trp-1 his-1 thyA argH1 thi-1 lacY1 malA1 mtl-2 xyl-7 tonA2 supE44 str-9*) was heavily mutagenized with *N*-methyl-*N'*-nitrosoguanidine, as described (21). Thermosensitive mutants of independent origin that grew at 30° but not at 40° on L-agar plates (22) were isolated by a replica plating technique (23). Five thousand mutants were isolated and stocked; 500 of these served as the source of the PBC mutants. The main strains used are summarized in Table 1. The detailed nature of this collection will be described elsewhere. It should be noted that, because these mutants received heavy mutagenesis at the time of isolation, most of them are expected to carry multiple mutations both in selected indispensable genes and in unselected dispensable genes. In the collection, we found *lacZ*$^-$ (β-galactosidase$^-$) and *tna*$^-$ (tryptophanase$^-$) mutants at frequencies of about 7%, although these defective characters were not directly selected in this collection. If PBC mutants occur at comparable frequencies, we should expect to find mutants in each PBC among these 500 strains. As will be described in the text, mutants in each PBC were found. Because of the heavy mutagenesis, the thermosensitive defect of these mutants may or may not be associated with the defect of these PBCs.

**Assays for PBC.** The procedure of Spratt and Pardee (16) was followed with minor modifications: the ratio of acrylamide to *N,N'*-methylenebis(acrylamide) was raised to 60 and the concentration of acrylamide was 7.2% for the separation gel and 4.5% for the stacking gel (25). The stock solution of Tris·HCl buffer for the separation gel contained 18.2 g of reagent grade Trizma Base (Sigma) and 28 ml of 1 M HCl in 100 ml final volume and was used at 1:4 dilution in the gels. The buffer for the stacking gel was identical to that used previously (16).

**Bacterial Crosses and Phage P1 Transduction Experi-**

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Abbreviations: PBC, penicillin-binding component; MurNAc, N-acetylmuramyl.

* This is paper no. 11 of a series. Paper 10 of the series is ref. 31.

Table 1. Mutants affected in the penicillin-binding proteins

| Gene | PBC lost | Map location, min | Marker of joint transduction (%) | MIC,* μg/ml, for C | MIC,* μg/ml, for M | Remarks | Mutants in collection (JE no.) | Refs. |
|---|---|---|---|---|---|---|---|---|
| *ponA* | 1a | 73.5 | *aroB* (96-80) | 2 | 0.5 | Normal growth | 10151,† 10528, 10568, 10980,‡ 11022, 11099, 11104, 11179, 11262 | 14, 24 |
| *ponB* | 1b | 3.3 | *tonA* (82) | 0.05 | | Hypersensitive to cephalosporins | 10353, 10704, 10085, 10980‡ | 14, 24 |
| *rodA* | 2 | 14.4 | *lip* (81) | | 50 | Ovoid cells, thermosensitive growth, mecillinam-resistant | 11238, 10157, 11116, 10151† | 18 |
| *ftsI* | 3 | 1.8 | *leu-6* (82) | 4 | 2 | Thermosensitive growth, multi-nucleated filament at 42°, penicillin-resistant | 10730, MFT33 | 18, 24 |
| *dacB* | 4 | 68.0 | *argG* (87) | 2 | 0.5 | Normal growth, defective in D-alanine carboxy-peptidase 1b | 10012, 10064, 10555 | 14, 19 |
| *dacA* | 5/6 | 13.7 | *leuS* (87) | 2 | 0.5 | Normal growth, defective in D-alanine carboxy-peptidase 1a | 11191 | 20,§ |

* MIC, minimal inhibitory concentration; C, cephaloridine; M, mecillinam. MIC in the wild type was 2 for cephaloridine and 0.5 for mecillinam.
† Double mutant of PBC-1a and PBC-2.
‡ Double mutant of PBC-1a and PBC-1b shift.
§ M. Matsuhashi, I. N. Maruyama, Y. Takagaki, S. Tamaki, Y. Nishimura, and Y. Hirota, unpublished data.

**ments.** For the mapping experiments of the mutations, bacterial crosses and transductions with P1 were used. Because of the absence of an easily recognizable phenotypic character, in the mutants for PBC-1a and PBC-4, recombinants have to be tested directly for the penicillin-binding capacity of the PBC. A method of indirect mapping was applied for these mutations as described (26). First an approximate location on the chromosome map was determined by crosses. The recombinant classes that received small specific regions of the donor chromosome in the cross were selected and the PBCs were examined. The more detailed mapping was then carried out with P1 to find markers cotransducible with the mutations.

The mutation resulting in the loss of penicillin-binding ability in PBC-1b was directly associated with hypersensitivity of the mutant cell to cephaloridine, and was scored directly on the plates by this phenotypic character.

**Assay for Murein Synthesis *In Vitro*.** Murein synthesis and D-alanine carboxypeptidase activity were assayed in the cell-free system as described previously (9, 14), with 40 μl of the reaction mixture containing UDP-MurNAc-L-Ala-D-Glu-*meso*-$A_2$pm-D-[$^{14}$C]Ala-D-[$^{14}$C]Ala (14.7 μCi/μmol) as radioactive substrate ($A_2$pm = diaminopimelate).

## RESULTS

**Two Distinct Proteins in the PBC-1.** The strains in our thermosensitive mutant collection were derived from PA3092 (*E. coli* K-12). When the method of Spratt and Pardee (16) was applied to analyze PBCs in this parental strain, electrophoretic profiles of the PBCs were essentially the same as those in their report. However, it was noted that the PBC-1 did not give a single band (14, 24). By use of 7.2% polyacrylamide gel with low crosslinking, PBC-1 was separated definitely into four bands, three major and one minor (Fig. 1, h). This gel system was used to screen PBC mutants; mutants of PBC-1 were found to fall into two groups, one lacking in the uppermost band and the other lacking in all the lower bands. Furthermore, in the latter group, every mutant lost all the lower bands at the same time and recovered them simultaneously by P1 transduction of the wild-type gene. Therefore, we consider that the PBC-1


FIG. 1. Separation of the components of PBC-1 and a spectrum of PBCs in a series of the PBC mutants. Benzyl[$^{14}$C]penicillin (53 mCi/mmol, Radiochemical Centre, Amersham) was bound to cell envelopes prepared from various kinds of PBC mutants at 30° for 10 min, except that the envelope preparation in j was first incubated at 42° for 10 min and then benzyl[$^{14}$C]penicillin was bound at 42° for 5 min. The reaction was terminated, and the inner membrane proteins were solubilized, with sodium Sarkosyl. The proteins were separated on the modified gels described in *Materials and Methods*. The PBCs were detected by fluorography (27). Lanes a and h, wild type; b, *ponA*; c, *ponB*; d, *rodA*; e, *ftsI*; f, *dacB*; g, *ponB980* (modified type PBC-1b). Lanes i and j illustrate the temperature sensitivity of binding ability of PBC-1a in the quadruple mutant (*dacA dacB ponA*$^{ts}$*1104 ponB*): i, 30°; j, 42°. The *dacA* mutation causes loss of the D-alanine carboxypeptidase 1a activity of PBC-5/6 without loss of its penicillin-binding property.

reported previously contained two different components. The component giving the uppermost band was designated as 1a and that giving the lower bands as 1b (24). During preparation of this manuscript, Spratt *et al.* (28) reported the resolution of PBC-1 into two components, 1a and 1b, and demonstrated that PBC-1a was not essential. The defective mutations of 1a and 1b were mapped at separate loci on the chromosome as described in a later section. The reason 1b gives multiple bands is unknown.

**Mutants Defective in PBC-1a.** Eleven thermosensitive mutants defective in PBC-1a were found. Ten of these failed to bind penicillin to PBC-1a at 30° and 40°, and one of them carried a thermosensitive defect in penicillin-binding ability of 1a (1a-ts); i.e., PBC-1a did not bind penicillin at 42°, but did at 30°. Nine of these mutants, including this strain, were subjected to genetic investigations. Each of them was made thermoresistant by P1 transduction from a wild-type strain. All the thermoresistant transductants were still defective in PBC-1a, including 1a-ts. The mutation leading to the loss of penicillin-binding ability in PBC-1a was separated from thermosensitivity in growth and referred to as *ponA* (*p*enicillin-binding protein *one* A). Mating experiments showed that *ponA* was located close to *aroB*. Transduction with P1 was carried out from each of the nine mutants (*aroB*$^+$) to a strain carrying *aroB*$^-$, and *aroB*$^+$ transductants were selected. The frequency of joint transduction of *ponA* and *aroB*$^+$ was 80–96%, but no thermosensitive mutation in growth of the donors was transduced with *aroB*$^+$. Thus separation of the defect in PBC-1a from thermosensitive growth was confirmed. The chromosomal location of *ponA* was mapped at 73.5 min.

**Mutants Defective in PBC-1b.** Four mutants were found with respect to PBC-1b. The electrophoretic bands of PBC-1b were totally absent in three of them, and in one, the bands of PBC-1b migrated faster than those of the wild type. The multiple bands shifted *en masse* to a lower position in the electrophoretic pattern (Fig. 1, g). Sixteen thermoresistant transductants were obtained from one of the mutants, JE10353, by P1 transduction from the wild-type strain. Among them, 14 recovered band 1b but 2 did not. The defective mutation of PBC-1b was separated from the thermosensitive mutation in this strain, although they were closely linked. Thermoresistant revertants did not recover the band 1b. In a similar way, the mutation of PBC-1b in the other mutants, including the modified 1b-type mutant, was separated from that responsible for thermosensitive growth. The mutation resulting in the loss of penicillin binding ability of PBC-1b will be called *ponB* in conformity to *ponA*. The chromosomal location of *ponB* was mapped at 3.3 min. The frequency of cotransduction of *ponB* with *tonA* was about 82%. All three of the mutants that lost penicillin-binding ability in PBC-1b (*ponB*$^-$) were hypersensitive to cephalosporins and penicillins, and every transductant or recombinant that received the *ponB*$^-$ mutation became hypersensitive likewise. On the other hand, the mutant with the modified PBC-1b (*ponB980*) was not hypersensitive to these antibiotics. This mutation was mapped also at 3.3 min. All of these mutants grew well under a wide range of growth conditions.

**Mutants Defective in PBC-2 and PBC-3.** Four mutants defective in PBC-2 and two mutants defective in PBC-3 were isolated. The phenotypes of these mutant classes were similar to those of the previous report (18, 29). The chromosomal location of *rodA*, responsible for PBC-2 (30) was mapped at about 14.4 min with our three mutants and the frequency of cotransduction of *rodA* with *lip* was about 81%. The chromosomal location of both *ftsI* mutations, responsible for PBC-3 (24), was mapped at about 1.8 min and the frequency of cotransduction of *ftsI* with *leu-6* was about 82%.

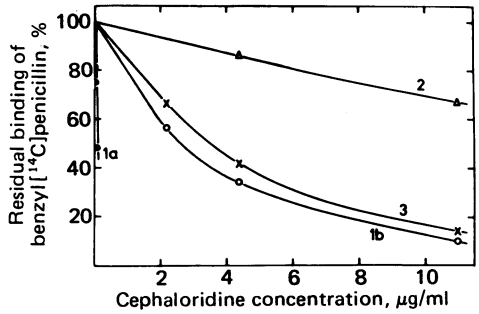


FIG. 2. Competition of cephaloridine for PBCs. Cell envelopes of the strain having modified PBC-1b were preincubated with a range of cephaloridine concentrations for 10 min at 30° and then benzyl[$^{14}$C]penicillin was added. At the end of an additional 10-min incubation, envelope proteins were solubilized by addition of sodium Sarkosyl at 1%. The PBCs were fractionated and detected as described in the legend to Fig. 1. The use of the strain with modified type PBC-1b allowed a better separation between PBC-1a and PBC-1b. Affinity of modified PBC-1b for cephaloridine was similar to that of wild-type PBC-1b. ●, PBC-1a; ○, PBC-1b; △, PBC-2; ×, PBC-3.

**Mutants Defective in PBC-4 and PBC-5/6.** By collaboration with Matsuhashi and his coworkers (14), two mutants (*dacB*) lacking in band 4 and activity of D-alanine carboxypeptidase 1b were found in our mutant collection. In addition, another mutant was found during the mutant screening. All of these thermosensitive mutant strains were made thermoresistant by P1 transduction from a wild-type strain. All the three thermoresistant strains grew normally at 40° as well as at 30°, whereas the defect of band 4 remained unaltered in these strains. All the three mutations of *dacB* were located at 68 min on the *E. coli* chromosome map, and the frequency of cotransduction of *dacB* with *argG* was about 87%. A mutant lacking activity of D-alanine carboxypeptidase 1a but still having the binding band of 5/6 was isolated and mapped by collaboration with Matsuhashi and his coworkers, and the gene controlling the enzyme activity was named *dacA* (14), and was located at 13.7 min on the *E. coli* chromosome map. The frequency of joint transduction of *dacA* with *leuS* was about 87%. (M. Matsuhashi, I. N. Maruyama, Y. Takagaki, S. Tamaki, Y. Nishimura, and Y. Hirota, unpublished data).

**Affinity of PBC-1a and PBC-1b for Cephalosporins.** PBC-1 has been inferred to participate in cell elongation, because cephaloridine, the only β-lactam that causes cell lysis at the lowest effective concentration, shows a higher affinity for PBC-1 than for PBC-2 or PBC-3 (18). PBC-1 is found to consist of two components. Therefore, the affinities of PBC-1a and PBC-1b were examined with a method similar to that of Spratt (17). Fig. 2 demonstrates the remarkable difference in affinity for cephaloridine between PBC-1a and the others. The concentration of cephaloridine required to inhibit [$^{14}$C]penicillin binding by 50% was 0.1 μg/ml for PBC-1a and 2.7 μg/ml for PBC-1b, and that of cephalothin was 0.04 μg/ml for PBC-1a and 20 μg/ml for PBC-1b, as determined under the conditions specified in the legend to Fig. 2. Cephalothin induced filamentation of cells at 5–10 μg/ml. At this concentration range, cephalothin binds to the PBC-1a and PBC-3, but does not to PBC-1b (compare ref. 17) *in vitro*. Furthermore, the cells defective in PBC-1b became hypersensitive to cephaloridine or cephalothin. Minimal inhibitory concentration of cephaloridine was in the range of 0.05–0.1 μg/ml, while it was 2 μg/ml in the isogenic wild type, and that of cephalothin was 0.2 μg/ml for the PBC-1b mutant and 3 μg/ml for the isogenic wild type. Cephalothin

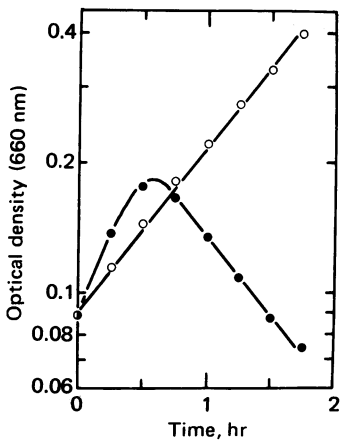


FIG. 3. Lysis of *ponA*$^{ts}$ *ponB* double mutant cells at the restrictive temperature. The mutant, JE5611 (*ponA*$^{ts}$*1104 ponB*) was grown at 30° in L broth containing 1% NaCl and half of the culture was transferred to 42° at time 0. Optical densities of the cultures (○, at 30°; ●, at 42°) were measured at 660 nm.

was effective at a concentration far below that required to bind with PBC-1b. Because the mutants lost penicillin-binding capacity in PBC-1b, one may expect that the loss confers an increased resistance to the β-lactam in this mutant. On the contrary, the loss of penicillin-binding ability in PBC-1b always accompanied the hypersensitivity to cephalosporins and penicillins. Considering the results described above, we assume that the biochemical reactions for cell elongation can proceed normally in the presence of either PBC-1a or PBC-1b, so that the loss of the function in either one, whether by a mutation or by action of a β-lactam, does not affect cell elongation. In the mutant defective in PBC-1b, cell elongation may be sustained by PBC-1a, which shows a very high affinity for cephalosporins and, therefore, cell elongation becomes hypersensitive to these antibiotics. The above assumption predicts lethality in the double mutant for PBC-1a and PBC-1b.

**Construction of Multiple Mutants in PBCs.** The mutants carrying the following combinations of mutations were constructed: (*i*) *ftsI* (PBC-3) *dacA dacB*, (*ii*) *ponA* (PBC-1a) *dacA dacB*, and (*iii*) *ponB* (PBC-1b) *dacA dacB*. These strains enabled us to eliminate D-alanine carboxypeptidase activities, which interfere with the measurement of D-alanine release coupled with the transpeptidation reaction, and to examine the possible relationship among the functions of different binding proteins. The multiple mutant (*i*) showed typical thermosensitive growth of *ftsI*, and (*ii*) and (*iii*) grew normally at 40° as well as at 30°. When we tried to introduce *ponA* into a strain carrying *dacA*, *dacB*, and *ponB* by mating or into a strain carrying *dacB* and *ponB* by P1 transduction, the trial was not successful. The experimental results suggested the lethality of the double mutant of *ponA* and *ponB*. An F$^-$ strain carrying *dacA*, *dacB*, *ponA*$^{ts}$*1104*, *str*, and *xyl* was crossed with a strain HfrC (O *tsx tonA ponB malA*) and recombinants were selected for ability to grow on xylose and streptomycin resistance. Although the parental strains were both thermoresistant, 19% of the recombinants became thermosensitive for growth at 40°. They lysed at the restrictive temperature (Fig. 3). Seven of them were subjected to further examinations and were found to have defects in both PBC-1a and PBC-1b (Fig. 1, i and j), while thermoresistant recombinants examined were shown to carry the wild-type PBC 1a or 1b, or both. The *ponA*$^{ts}$*1104 ponB* double mutant having wild-type alleles of *dacA* and *dacB* also showed thermosensitive growth. The results indicated that the double mutant defective in PBC-1a and PBC-1b was nonviable. It was concluded that the functions of PBC-1a and PBC-1b are interchangeable and take an essential part in murein synthesis required for cell elongation.

**Murein Synthetase Activity of the Mutants Defective in PBCs.** Synthesis of murein must be essential for cell elongation and cell division. Possible involvement of PBC-1a, PBC-1b, and PBC-3 in murein synthesis was examined *in vitro* by incorporation of radioactive UDP-MurNAc-pentapeptide into murein and release of D-alanine coupled with the transpeptidation reaction. As shown in Table 2, activity for murein synthesis *in vitro* was not changed in the mutant defective in PBC-3 and was slightly decreased in the mutant defective in PBC-1a. In the preparation of the mutant defective in PBC-1b, the activity was greatly decreased in the presence or absence of D-alanine carboxypeptidase 1a and 1b, although a considerable amount of the lipid-linked precursor was detected. Thus, PBC-1b is involved in the reaction at the final stage of murein synthesis. The residual activity manifested by the preparation of the *ponB* mutant may represent the activity of PBC-1a, which may be responsible for murein synthesis *in vivo* in the *ponB* mutant. An introduction of *dacA* and *dacB* mutation should reduce the release of the terminal D-alanine due to D-alanine carboxypeptidases and make it feasible to detect the D-alanine release coupled with transpeptidation. A considerable amount of release of D-alanine was observed in the preparation of the triple mutant *dacA dacB ponB*, irrespective of the sharp decline in

Table 2. Murein synthesis *in vitro* by membrane fractions from mutants for PBC

| Strain | Defect in PBC: 1a (*ponA*) | 1b (*ponB*) | 3 (*ftsI*) | 4 (*dacB*) | 5/6 (*dacA*) | Labeled reaction products, dpm/μg protein: Murein | D-Alanine | Lipid intermediate |
|---|---|---|---|---|---|---|---|---|
| JE5638 | + | + | + | + | + | 27.5 (100) | 31.3 (100) | 13.6 (100) |
| JE5630* | + | + | + | − | − | 26.4 (96) | 9.9 (32) | 13.1 (96) |
| JE5625* | + | − | + | − | − | 2.9 (11) | 14.9 (48) | 9.4 (69) |
| JE5600* | + | − | + | + | + | 4.6 (17) | 32.3 (103) | 8.5 (63) |
| JE5627† | + | + | + | − | − | 20.1 (73) | 10.6 (34) | 15.2 (112) |
| JE5629† | − | + | + | − | − | 14.0 (51) | 6.3 (20) | 11.3 (83) |
| JE5624† | + | + | + | − | − | 25.3 (92) | 17.4 (56) | 15.3 (113) |
| JE5623† | + | + | − | − | − | 25.7 (93) | 15.2 (49) | 12.1 (89) |

The values in parentheses are percentages of the wild type.
* JE5630 and JE5600 were parents of JE5625.
† Isogenic pairs.

the activity of murein synthesis. This result, however, must be interpreted with great caution, because the residual activity of D-alanine carboxypeptidases cannot be neglected.

## DISCUSSION

Mutants in a series defective in PBCs were isolated. They include the following: 11 mutants for PBC-1a, 4 mutants for PBC-1b, 4 mutants for PBC-2, two mutants for PBC-3, 3 mutants for PBC-4, and a mutant lacking in activity of D-alanine carboxypeptidase 1a (PBC-5/6). From the nature of these mutants, it is possible to discuss the roles of PBCs in cell growth and division. PBC-4 and PBC5/6 were identified as D-alanine carboxypeptidase 1b and 1a, respectively (14, 19, 20). They are likely to be dispensable for cell growth and division, although the possibility of their importance for growth and division cannot be excluded rigorously, as pointed out previously (14). PBC-1 was inferred to participate in cell elongation and its possible involvement in murein synthesis has been proposed (18). PBC-1 was resolved into PBC-1a and PBC-1b (24, 28). Furthermore, neither the *ponA* mutation (defect in PBC-1a) nor the *ponB* mutation (defect in PBC-1b), by itself, was associated with thermosensitive growth. One of the *ponA* mutants revealed a thermosensitive defect in PBC-1a (*ponA*$^{ts}$*1104*). This mutation by itself did not render the mutant thermosensitive in growth. However, the double mutant (*ponA*$^{ts}$*1104 ponB*) showed thermosensitive growth and lysed at the restrictive temperature. The function of PBC-1a and PBC-1b was explained as interchangeable in carrying out an essential reaction for cell growth.

The activity of murein synthesis was found to decrease sharply in the cell-free system of the *ponB* mutant. This mutant was able to propagate normally. Presumably PBC-1a took over the murein synthesis required for cell growth, as a substitute for PBC-1b. A major portion of the cell-free murein synthesis represented the activity contributed by PBC-1b. The residual part of the activity may be related to PBC-1a. β-Lactam antibiotics inhibit transpeptidation, but do not inhibit *in vitro* synthesis of glycan chain (7, 9). Therefore, it is surprising to find the decrease in the incorporation of MurNAc-pentapeptide in the PBC-1b mutant. An explanation for this phenomenon might be found in interactions between polymerase of glycan chain and transpeptidase acting in a complex. Mutations in PBC-1b might inactivate the total activity of the complex. Alternatively, PBC-1b might be an enzyme responsible for both transpeptidation and transglycosylation. The activities of PBC-1b for both reactions might have been lost by the mutation. Before understanding the precise functions of PBC-1a and PBC-1b, it would be necessary to analyze the *in vitro* reaction products and the murein synthesized *in vivo* by mutants with defects in PBC-1a or PBC-1b.

Extensive mapping studies of the mutations open a way to construct multiple mutants defective in the binding proteins, at will. It might be the right time to construct a series of relevant mutants by combining these mutations to dissect the roles of the binding proteins on cell growth, division, and penicillin-sensitive enzyme systems.

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