JOURNAL OF BACTERIOLOGY, June 1979, p. 1029-1032
0021-9193/79/06-1029/04$02.00/0

Vol. 138, No. 3

Behavior of Penicillin-Binding Proteins in *Escherichia coli* upon Heat and Detergent Treatments and Partial Purification of Penicillin-Binding Proteins 1A and 1B

JUN-ICHI NAKAGAWA,[1] HIROSHI MATSUZAWA,[2] AND MICHIO MATSUHASHI[1]*

Institute of Applied Microbiology[1] and Department of Agricultural Chemistry,[2] University of Tokyo, Bunkyo-ku, Tokyo, 113 Japan

Received for publication 20 March 1979

Penicillin-binding proteins differ greatly in heat sensitivity and sensitivity to detergents. The partial purification of penicillin-binding 1A and 1B proteins from *Escherichia coli* is described.

Penicillin binds to several proteins in the cytoplasmic membranes of bacteria. The nature and functions of these penicillin-binding proteins (PBPs) have been studied most extensively in *Escherichia coli*; at least seven PBPs in *E. coli* have been separated by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis (5, 6, 8-14), and mutants of *E. coli* defective in these PBPs have been isolated (1, 5-7, 10, 11, 13, 14). PBP-1A (and perhaps PBP-2) seems to function as a detour enzyme in compensating for lack of PBP-1Bs (14). The 1B PBP, which consists of at least three proteins (PBP-1B's), seems to be an essential enzyme involved in formation of the cross-linked peptidoglycan in vitro (2, 3, 14) and may function in the process of cell growth. There are reports of the functions of other PBPs, PBP-2 (8, 12), PBP-3 (8, 9, 13), PBP-4 (1, 6), and PBP-5 (6, 7).

In this study we examined the heat sensitivity of each PBP in *E. coli*. For the experiment shown in Fig. 1, membrane fractions were obtained from four strains of *E. coli*, K-12 strains JE1011, CD4, and PA3092, and strain B. The fractions were obtained as described previously (12) by sonicating the cells and suspending the preparation in 0.01 M sodium phosphate buffer (pH 7.0). Fractions were incubated for 10 min at the indicated temperatures, and then their binding of [^{14}C]penicillin G was measured. The [^{14}C]penicillin G-protein complexes were separated by SDS-acrylamide gel electrophoresis and located by fluorography (12, 14), and then they were measured quantitatively by counting the radioactivity in dried PPO (2,5-diphenyloxazole)-impregnated slab gel slices in toluene-PPO-POPOP [2,2'-*p*-phenylene-bis-(5-phenyloxazole)] with a liquid scintillation spectrometer at a counting efficiency (cpm/dpm) of 78%.

The results in Fig. 1 indicate that, in all four *E. coli* strains tested, PBP-1B's were very heat stable, their penicillin-binding activities being almost unimpaired by heating for 10 min at 60°C; PBP-1A and -2 were very sensitive to heat, their penicillin-binding activities being significantly reduced by heating for 10 min at 45°C; and other PBPs had intermediate heat stabilities. (In appropriate thermosensitive mutant strains they are much more sensitive [9, 10, 13; unpublished data].) Similar results were obtained when heat stability was examined in 0.05 M sodium phosphate (pH 7.0) or 0.05 M Tris-hydrochloride (pH 7.6) (data not shown) and also when PBPs, eluted with 1% (wt/vol) Triton X-100 in 0.01 M sodium phosphate buffer (pH 7.0), were heated in the presence of the detergent (Fig. 1).

Sometimes an eighth and ninth band of [^{14}C]penicillin G-protein complexes appeared, especially when the plate was kept at 10°C during electrophoresis. These proteins, referred to as PBP-7 and -8, were very heat labile in strains JE1011 and CD4, but appeared to be a little more stable in *E. coli* strain PA3092 and *E. coli* B (Fig. 1). Their functions are unknown (see also ref. 9).

An experiment using strain JST975srev6 (data not shown), which is defective in PBP-1B's (14), indicated that disappearance of radioactivity in the positions of PBP-1A and other PBPs during heating was not associated with the appearance of radioactivity in the position of PBP-1B's.

[^{14}C]penicillin G-PBP complexes could be solubilized to various extents by treatment of Triton X-100, sodium deoxycholate, and sodium dodecyl-*N*-sarcosinate (Sarkosyl) at 0°C (Fig. 2, experiment a). For the results in Fig. 2 the membrane fraction was prepared using 0.05 M Tris-hydrochloride buffer (pH 7.6). Loss of [^{14}C]penicillin G complexes during the extraction with sodium deoxycholate and further assay procedures could be observed with almost all

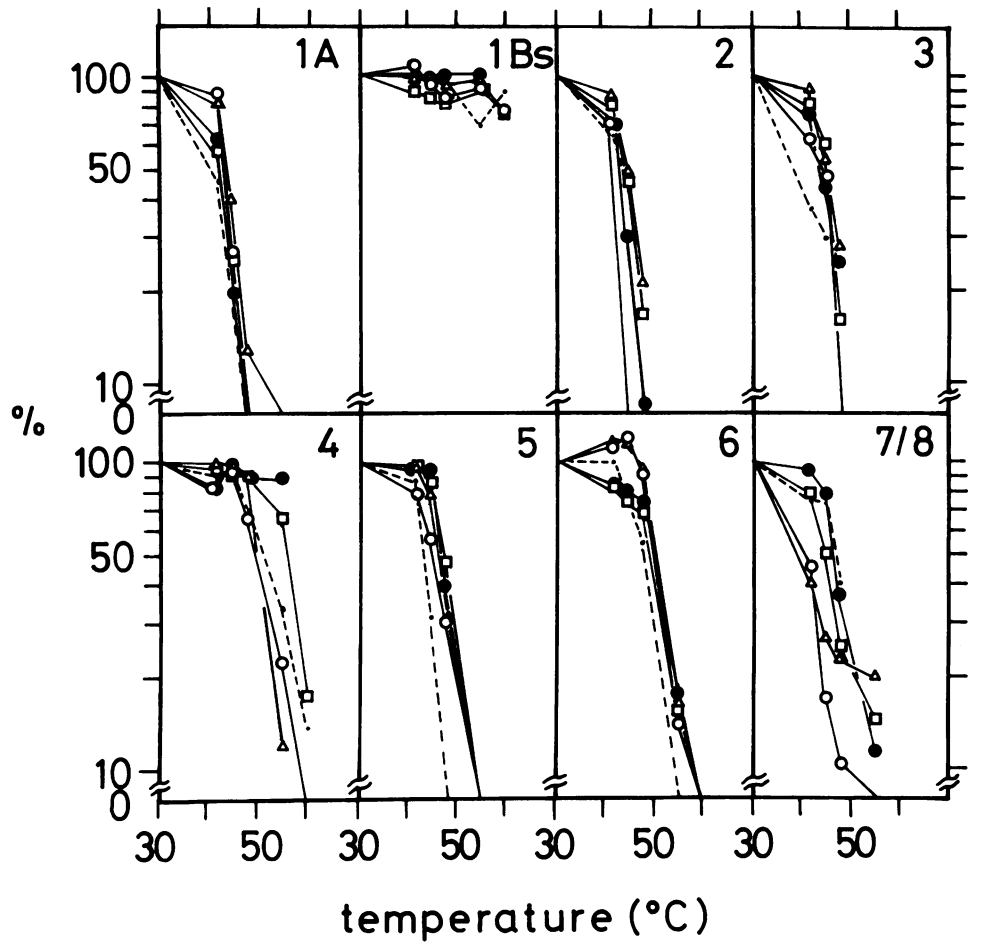

FIG. 1. *Heat sensitivities of penicillin-binding activities of PBPs in membranes of four strains of E. coli and of PBPs solubilized with Triton X-100. The membrane fraction was obtained essentially as described previously (12, 14). Samples (30 μl) of membrane fractions containing 600 μg of protein in 0.01 M sodium phosphate buffer (pH 7.0) were heated for 10 min at the indicated temperatures, and then 3 μl of 1 mM [^{14}C]penicillin G (50 Ci/mol, Radiochemical Centre, Amersham, England) was added and the mixture was incubated for a further 10 min at 30°C. The resulting [^{14}C]penicillin G-protein complexes were solubilized with Sarkosyl at 20°C in the presence of excess unlabeled penicillin G, treated with SDS-mercaptoethanol, separated by SDS-acrylamide slab gel electrophoresis at 10°C, and located by fluorography, essentially as described previously (12, 14). Radioactivities on the slab gel were measured as described in the text. Symbols: (○) membranes of strain JE1011; (△) membranes of strain CD4; (□) membranes of strain PA3092; (●) membranes of strain B; (---·---) extract of membranes of strain JE1011 with 1% (wt/vol) Triton X-100. For conditions of solubilization with Triton X-100 see legend to Fig. 2. PBP numbers (14) are shown in the figure. Ordinate: percent radioactivity; abscissa: temperature of preincubation.*

PBPs to a certain extent, for unknown reasons. Among them, however, loss of [^{14}C]penicillin G-PBP-5 complex could be partly due to the release of [^{14}C]penicillin G by β-lactamase activity of this protein (7, 15). The free PBPs on the membrane were probably solubilized by detergent in ways similar to penicillin G-PBP complexes, but their abilities to bind [^{14}C]penicillin G seemed to be lost to different extents. As a result of the extraction and inactivation processes, each PBP showed different penicillin-binding activities in the extracts with different detergents, depending on the detergent (and also the extraction conditions) (Fig. 2, experiment b). For instance, active PBP-1A and -4 could be extracted efficiently with 1% (wt/vol) Triton X-100 or sodium deoxycholate, and PBP-5 and -6 with 1% (wt/vol) Triton X-100, whereas active

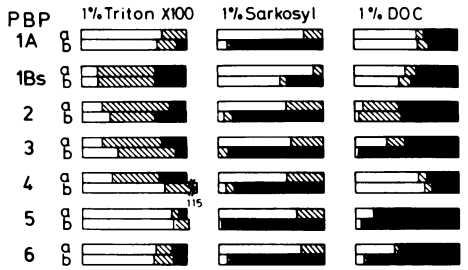

FIG. 2. *Solubilizations and stabilities of PBPs on treatment with detergents. The membrane fraction was prepared from E. coli strain JE1011 (12, 14) except that 0.05 M Tris-hydrochloride buffer (pH 7.6) was used instead of 0.01 M phosphate buffer (pH 7.0). In prelabeling experiments (experiments a, solubilization tests of complexes), 100 μl of the membrane preparation, containing 3 mg of protein, was labeled with 10 μl of [^{14}C]penicillin G (50 Ci/mol, 10 nmol) for 10 min at 30°C. After cooling in ice, 10 μl of 10% (wt/vol) solution of the indicated detergent in the same buffer was added, and the mixture was stirred for 10 min, let stand for 5 min at 0°C, and centrifuged for 30 min at 100,000 × g at 4°C. The resulting pellet was washed once with 40 μl of the above buffer containing 1% (wt/vol) of the same detergent and suspended in 100 μl of the same solution. The two supernatants were combined. Then, 4 μl of a solution of 45 mg of unlabeled penicillin G per ml containing 15% (wt/vol) Sarkosyl was added to both 60-μl samples of the supernatant and a suspension of the precipitate. The mixtures were kept at 20°C for 20 min, and then insoluble outer membranes were removed by centrifugation for 30 min at 10,000 × g at 20°C and the solution was subjected to SDS–mercaptoethanol treatment. Radioactivity was compared with that obtained by the standard PBP assay procedure (12, 14) in which [^{14}C]penicillin G–protein complexes on the membrane were directly solubilized with Sarkosyl at 20°C. When 1% (wt/vol) Sarkosyl was used for solubilization at 0°C, the second addition of Sarkosyl to the supernatant and treatment at 20°C were omitted and the radioactivity in unsolubilized fraction was calculated by the difference from the standard. In the postlabeling experiments (experiments b, solubilization and stability tests for free PBPs), incubation with [^{14}C]penicillin G was carried out on detergent extracts and residue suspensions (see above) in the presence of detergent. PBPs were then separated and detected in the standard PBP assay procedure. The radioactivity of each PBP was counted as described in the text. Data are expressed as percentages of the values obtained by the standard procedure. Symbols: a, prelabeled with [^{14}C]penicillin G; b, postlabeled with [^{14}C]penicillin G; white area, radioactivity in the detergent extracts; shadowed area, radioactivity in unsolubilized cytoplasmic membrane fractions; black area, radioactivity lost.*

PBP-1B's could be extracted most efficiently with 1% (wt/vol) Sarkosyl under the described conditions.

By use of the above solubilization and heat-

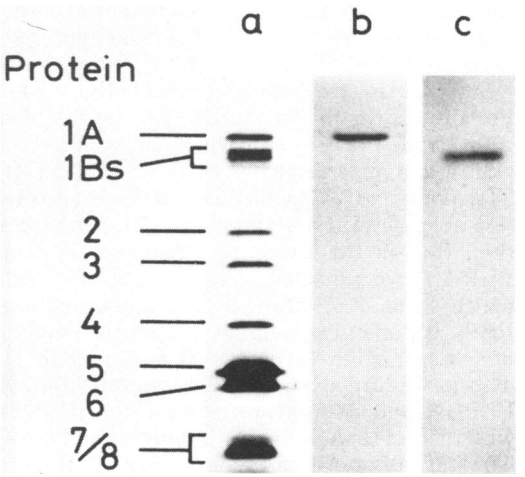

FIG. 3. *Partially purified proteins showing single bands of [^{14}C]penicillin G–protein complexes. The fluorogram after gel electrophoretic separation is shown. (a) membranes; (b) partially purified PBP-1A; (c) partially purified PBP-1B's. Numbering of PBPs is as in Fig. 1 and 2. The purification procedures, all carried out at 0 to 4°C, were as follows. PBP-1A: Membranes from E. coli strain JST975srev6 were prepared by a standard procedure (12), but were suspended in 0.05 M Tris-hydrochloride buffer (pH 7.6) instead of a phosphate buffer. To 2.4 ml of membrane suspension (22.9 mg of protein per ml, 7,830 arbitrary units) was added 240 μl of 10% (wt/vol) sodium deoxycholate (pH 8.5), and the mixture was vigorously mixed for 10 min and then let stand for 5 min. Insoluble material was removed by centrifugation at 100,000 × g for 30 min, and the supernatant (2.3 ml, 14.5 mg of protein per ml, 5,570 arbitrary units) was fractionated with ammonium sulfate (0 to 30% saturation). The protein was dissolved in 0.8 ml of 0.05 M Tris-hydrochloride buffer (pH 7.6) containing 1% (wt/vol) Triton X-100 (buffer A) and dialyzed for a total of 2 h against 100 volumes of buffer A with three changes of the buffer. The protein solution (0.8 ml, 9.0 mg of protein per ml, 3,100 arbitrary units) was applied to a hydroxyapatite column (10-mm diameter by 11-mm length) previously equilibrated with buffer A, and adsorbed protein was eluted with 0.05 M potassium phosphate buffer (pH 7.0) (0.8 ml, 1.44 mg of protein per ml, 1,245 arbitrary units). PBP-1B's: The membrane fraction was obtained from E. coli strain HAT293 (4) by a standard method (12) but was suspended in 0.05 M Tris-hydrochloride buffer (pH 7.6) instead of phosphate buffer (2.5 ml, 35.1 mg of protein per ml, 11,400 arbitrary units). The membrane fracton was heated for 10 min at 55°C, cooled in ice, and mixed with 125 μl of 20% (wt/vol) solution of Sarkosyl. Solubilization was carried out by vigorous mixing for 10 min and standing for a further 5 min. Insoluble material was removed by centrifugation at 100,000 × g for 30 min, and the supernatant was fractionated with ammonium sulfate (0 to 45% saturation). The precipitate was collected by centrifugation, dissolved in 0.8 ml of buffer A, and dialyzed*

inactivation techniques in combination with usual procedures for protein purification, fractions of PBP-1A and PBP-1B's, respectively, were obtained free from activities of other PBPs. For the results in Fig. 3, PBP-1A was purified from strain JST975srev6, which lacks PBP-1B's and -4 and superproduces PBP-1A and -2 (14). The binding of [^{14}C]penicillin G to PBP-1A fraction in this strain was about 2 to 10 times higher than that in the usual *E. coli* strain JE1011. PBP-1B's were purified from strain HAT293 (4), which lacks PBP-1A (14) and superproduces PBP-1B's (about three to four times the amount of [^{14}C]penicillin G bound in strain JE1011). This procedure achieved 7.6-fold purification of PBP-1A and 34-fold purification of PBP-1B's with respect to their penicillin-binding activities. The partially purified preparation of PBP-1B's (Fig. 3c) gave a slightly thinner band on SDS–acrylamide gel electrophoresis than PBP-1B's in the membrane fraction, but it is unknown if the radioactive band in Fig. 3c corresponds only to the slowest moving component of PBP-1B's. The final preparations of PBP-1A and -1B's obtained were not homogeneous, but gave many protein bands staining with Coomassie brilliant blue on slab gel electrophoresis. Further purification of these proteins and investigations of their enzymatic activities are in progress.

ADDENDUM IN PROOF

Recently we succeeded in demonstrating enzymatic activity for formation of crosslinked peptidoglycan from lipid(undecaprenol-PP)-linked precursor in highly purified preparations of PBP-1B's, which were obtained by ampicillin-Sepharose affinity column chromatography of the partially purified PBP-1B's as described in this report. The preparations seemed to have activities for both peptidoglycan polymerization and crosslinkage formation. Details of the results will be reported subsequently (J. Nakagawa, S. Tamaki, and M. Matsuhashi, Agric. Biol. Chem., vol. 43, 1979, in press).

for a total of 2.5 h against 100 volumes of buffer A with three changes of buffer. The protein solution (0.8 ml, 9.8 mg of protein per ml, 4,667 arbitrary units) was then subjected to chromatography on a DEAE-cellulose column (10-mm diameter by 13-mm length) previously equilibrated with buffer A. Unadsorbed PBP-5 and -6 were washed out with the same buffer, and then adsorbed protein was eluted with buffer A containing 0.3 M NaCl. The eluted protein fraction (1.0 ml, 3.4 mg of protein per ml, 1,330 arbitrary units) was applied to a column of hydroxyapatite (10-mm diameter by 10-mm length) previously equilibrated with buffer A. After other proteins were eluted with 3.2 ml of 0.2 M potassium phosphate buffer (pH 7.0) containing 1% (wt/vol) Triton X-100, PBP-1B's were eluted with 1.2 ml of 0.5 M potassium phosphate buffer (pH 7.0) containing 1% (wt/vol) Triton X-100. The fraction was dialyzed against buffer A (1.2 ml, 0.10 mg of protein per ml, 524 arbitrary units) and used directly for assay or was freeze-concentrated in vacuo. PBPs were assayed as described in the legend to Fig. 1. Activity is shown in arbitrary units based on the radioactivity (dpm) of bound [^{14}C]penicillin G.

LITERATURE CITED

1. **Iwaya, M., and J. L. Strominger.** 1977. Simultaneous deletion of *D*-alanine carboxypeptidase 1B-C and penicillin-binding component IV in a mutant of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **74**:2980–2984.
2. **Izaki, K., M. Matsuhashi, and J. L. Strominger.** 1966. Glycopeptide transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Proc. Natl. Acad. Sci. U.S.A. **55**:656–663.
3. **Izaki, K., M. Matsuhashi, and J. L. Strominger.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reaction in strains of *Escherichia coli*. J. Biol. Chem. **243**:3180–3192.
4. **Kamiryo, T., and J. L. Strominger.** 1974. Penicillin-resistant temperature-sensitive mutants of *Escherichia coli* which synthesize hypo- or hyper-cross-linked peptidoglycan. J. Bacteriol. **117**:568–577.
5. **Matsuhashi, M., I. N. Maruyama, Y. Takagaki, S. Tamaki, Y. Nishimura, and Y. Hirota.** 1978. Isolation of a mutant of *Escherichia coli* lacking penicillin-sensitive D-alanine carboxypeptidase IA. Proc. Natl. Acad. Sci. U.S.A. **75**:2631–2635.
6. **Matsuhashi, M., Y. Takagaki, I. N. Maruyama, S. Tamaki, Y. Nishimura, H. Suzuki, U. Ogino, and Y. Hirota.** 1977. Mutants of *Escherichia coli* lacking in highly penicillin-sensitive D-alanine carboxypeptidase activity. Proc. Natl. Acad. Sci. U.S.A. **74**:2976–2979.
7. **Matsuhashi, M., S. Tamaki, S. J. Curtis, and J. L. Strominger.** 1979. Mutational evidence for identity of penicillin-binding protein 5 in *Escherichia coli* with the major D-alanine carboxypeptidase 1A activity. J. Bacteriol. **137**:644–647.
8. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K12. Proc. Natl. Acad. Sci. U.S.A. **72**:2999–3003.
9. **Spratt, B. G.** 1977. Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. J. Bacteriol. **131**:293–305.
10. **Spratt, B. G.** 1978. *Escherichia coli* resistance to β-lactam antibiotics through a decrease in the affinity of a target for lethality. Nature (London) **274**:713–715.
11. **Spratt, B. G., V. Jobanputra, and U. Schwarz.** 1977. Mutants of *Escherichia coli* which lack a component of penicillin-binding protein 1 are viable. FEBS Lett. **79**: 374–378.
12. **Spratt, B. G., and A. B. Pardee.** 1975. Penicillin-binding proteins and cell shape in *E. coli*. Nature (London) **254**: 516–517.
13. **Suzuki, H., Y. Nishimura, and Y. Hirota.** 1978. On the process of cellular division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. Proc. Natl. Acad. Sci. U.S.A. **75**:664–668.
14. **Tamaki, S., S. Nakajima, and M. Matsuhashi.** 1977. Thermosensitive mutation in *Escherichia coli* simultaneously causing defects in penicillin-binding protein-1Bs and in enzyme activity for peptidoglycan synthesis *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **74**:5472–5476.
15. **Tamura, T., Y. Imae, and J. L. Strominger.** 1976. Purification to homogeneity and properties of two D-alanine carboxypeptidases I from *Escherichia coli*. J. Biol. Chem. **251**:414–423.