Peptidoglycan Synthetic Activities in Membranes of *Escherichia coli* Caused by Overproduction of Penicillin-binding Protein 2 and RodA Protein*

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Penicillin-binding protein (PBP)-2 and the RodA protein are known to function in determining the rod shape of *Escherichia coli* cells. Peptidoglycan biosynthetic reactions that required these two proteins were demonstrated in the membrane fraction prepared from an *E. coli* strain that overproduced both of these two proteins and which lacked PBP-1B activity (the major peptidoglycan synthetase activity in the normal *E. coli* membranes). The cross-linked peptidoglycan was synthesized from UDP-N-acetylmuramylpentapeptide and UDP-N-acetylglucosamine in the presence of a high concentration of cefmetazole that inhibited all of PBP except PBP-2. The peptidoglycan was synthesized via a lipid intermediate and showed up to 30% cross-linking. The cross-linking reaction was strongly inhibited by the amidinopenicillin, mecinillin, and by other β-lactam antibiotics that have a high affinity for PBP-2, but not by β-lactams that had very low affinity for PBP-2. The formation of peptidoglycan required the presence of high levels of both PBP-2 and the RodA protein in the membranes, but it is unclear whether the two proteins was primarily responsible for the extension of the glycan chains (transglycosylation). However, the sensitivity of the cross-linking reaction to specific β-lactam antibiotics strongly suggested that it was catalyzed by PBP-2. The transglycosylase activity of the membranes was sensitive to enramycin and vancomycin and was unusual in being stimulated greatly by a high concentration of a chelating agent.

Peptidoglycan synthesis is an important part of the duplication cycle of bacterial cells. During the cell cycle peptidoglycan synthesis is required for the elongation of the lateral cell wall, for the formation of the septum, and for the determination of the bacterial cell shape. β-Lactam antibiotics interfere with peptidoglycan synthesis and have been shown to produce specific morphological effects in *Escherichia coli* and other related Gram-negative bacteria by binding to the high molecular weight penicillin-binding proteins (PBPs) (1–5). Four of the high molecular weight PBPs of *E. coli* have been proposed to be primarily involved in peptidoglycan synthesis during the cell cycle. PBP-1A and -1B (*M* approximately 90,000) are involved in cell elongation, PBP-2 (*M* 70,000) in the determination of cell shape, and PBP-3 (*M* 60,000) in septum formation. Recent work suggests that each of these PBPs is a peptidoglycan synthetase and that they act together within the cytoplasmic membrane to catalyze the formation of the bag-shaped peptidoglycan network (6–15).

In 1966 Izaki et al. (16) demonstrated a transglycosylase- and a penicillin-sensitive DD-transpeptidase activity in a crude cellulase membrane fraction of *E. coli* that together synthesized cross-linked peptidoglycan from lipid-linked precursors. Subsequently it was found (3) that membranes prepared from a mutant that lacked PBP-1B activity failed to catalyze either the transglycosylase or transpeptidase reactions, and this unexpected result was subsequently explained by the discovery that PBP-1B catalyzed both of these reactions (6–8). The very low level of peptidoglycan synthetase activity in cell membranes prepared from a mutant of *E. coli* that lacks PBP-1B has allowed us to search for the activities catalyzed by the other high molecular weight PBPs. In this report we describe our investigation on the enzymatic activity of PBP-2. We show that membranes prepared from a strain of *E. coli* that lacks PBP-1B and which greatly overproduces PBP-2 had very low levels of peptidoglycan synthetase activity. However, if these membranes also contained high levels of the RodA protein (the product of the cell shape gene, *mrdB* (or *rodA*)), they catalyzed the following reactions of peptidoglycan synthesis.

Transglycosylase reaction:

\[
\text{GlcNAc-MurNAc-(L-Ala-D-Glu-meso-Apmm-D-Ala-D-Ala)-PP-lipid}
\]

\[
+ \text{[-GlcNAc-MurNAc-(L-Ala-D-Glu-meso-Apmm-D-Ala-D-Ala)]_n, X} \rightarrow \text{[-GlcNAc-MurNAc-(L-Ala-D-Glu-meso-Apmm-D-Ala-D-Ala)]_n + lipid-PP}
\]

where X is an acceptor, probably PP-lipid;

*The abbreviations used are: PBP, penicillin-binding protein; Apmm, 2,6-diaminopimelic acid; MurNAc, N-acetylmuramic acid.*
Transpeptidase reaction (mecillinam sensitive):

\[
\text{GlcNAc-MurNAc-L-Ala-D-Glu-meso-Apm-D-Ala-D-Ala} \rightarrow \text{d-Ala-d-Ala}
\]

\[
\text{GlcNAc-MurNAc-L-Ala-D-Glu-meso-Apm-D-Ala-D-Ala} \rightarrow \text{d-Ala (2)}
\]

Preliminary accounts of this work have appeared (5, 12).

**EXPERIMENTAL PROCEDURES**

Radioactive Materials and Reagents—Radioactive UDP-MurNAc-pentapeptide labeled in meso-Apm (specific activity, 44 Ci/mol nucleotide) or in meso-\[^{1-14}C\]Apm (specific activity, 44 Ci/mol. Amerchol International, England, UDP-MurNAc-dipectide and DL-alanyl-DL-alanine or from \[^{1-14}C\]alanine (specific activity, 120-160 Ci/mol, New England Nuclear), and UDP-MurNAc-tripeptide (3). A mixture of partially purified alanine racemase, \[^{1-14}C\]alanine (specific activity, 113.8 mCi/mg, NEP salt) was a generous gift from Dr. P. Cassidy of Merck Sharp and Dohme. Mccellinam, enramycin (Takeda Chemical Industries, Osaka), cefmetazole (Sankyo Co., Tokyo), latamoxef (Shionogi & Co., Osaka), MT-141 (relinixin, Meiji Seika Co., Yokohama), and monemycin (Hoechst) were generous gifts from the respective companies. Benzylpenicillin potassium salt and vancomycin were commercial products. Tunicamycin was kindly provided by Dr. A. Takatsuki (University of Tokyo).

Escherichia coli Strains and Construction of Plasmids—Strain JST975 lacking PBP-1B (mrdB) was derived from strain JE1011 (F" thr leu trp his thy thi ara lac gal xyl mtl rpsL tonA) as described (3). Strain JST9793 (F" lip-9 mrcB proA purB his metB lac gal rpsL) was constructed by mating AB1325 (F" lip-9 proA purB his metB lac gal rpsL) with CD49751 (HfrC mrcB proA metB lac) (8) selecting for mtrA rpsL. Strain JST9753 was transduced to lip- at 30 °C using \(^{32}P\)labeled JST9753, and lysogens were identified by their immunity to \(^{32}P\)I and their thermosensitivity at 43 °C. \[^{32}P\]I is a defective transducing phase carrying the E. coli chromosomal genes from lip to leuS including dacA, mrdB(RodA), mrdA(pbpA), and several other genes (17). The plasmid pHS202, which carries the intact mrdB and mrdA genes and two open reading frames coding for 17- and 7.7-kDa proteins, was constructed by subcloning the 7.2-kilobase \(^{32}P\)SalI fragment from XAM dip24 (18), a defective transducing phase similar to \[^{32}P\]I, into the \(^{32}P\)SalI site of pACYC184. pH503 and pH504 are derivatives of pH202 that have the mrdB and mrdA genes, respectively, inactivated by the insertion of Tn5 (18). pH5502 is a derivative of pH502 that has an insertion of Tn5 which does not inactivate either the mrdB or mrdA genes (18), pTP75, which expresses wild type RodA protein and thermosensitive PBP-2, and pTP71, which expresses thermosensitive RodA protein and wild type PBP-2, were constructed as described below. First, strain TMM5 (lip mrdA3) (19), which produces a thermolabile form of the RodA protein, were transduced to lip- using \(^{32}P\)I for 10 min to remove unbroken cells, and grown for 30 min. The resulting membranes were washed once in buffer A and resuspended in the original volume of fresh medium (lacking spectinomycin) and grown for 2 h at 30 °C to allow expression of the cell shape genes on the amplified plasmids. Cells were harvested by centrifugation, washed once with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 mM MgCl₂ (buffer A), and stored frozen at -80 °C. Bacterial membranes (the particulate membrane fraction) were prepared by disrupting the cells by sonication in buffer A (100 mg, wet weight, of cells/ml) at 0 °C centrifuging at 5,000 x g for 10 min to remove unbroken cells, and pelleting the membrane fraction at 100,000 x g for 30 min. The membranes were washed once in buffer A and resuspended in the same buffer.

Detection of PBP-2—The levels of PBP-2 in cell membranes were

**Fig. 1. Construction of plasmids carrying mutant alleles of the mrdA(pbpA) and mrdB(RodA) genes.** See text for details.
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measured by labeling with [14C]- or [3H]benzylpenicillin, extracting the cytoplasmic membrane proteins with Sarkosyl (sodium dodecyl sarcosinate, Ciba Geigy), and separating the proteins on a 7.5% sodium dodecyl sulfate-polyacrylamide gel as described (1). A fluorogram was prepared using the 2,5-diphenyloxazole method and pre-fogged Fuji RX x-ray film as described (21).

Enzyme Assays—The assays of transglycosylase and transpeptidase activities were normally performed in 0.5-ml Pyrex test tubes. The standard reaction mixture contained (in a final volume of 37 μl) 56-60 mM Tris-HCl buffer, pH 8.5, 1-27 mM MgCl2, 13.5 mM potassium EDTA (pH 8.5), 100-400 μg (as protein) of membranes, and, as substrates, 0.36 nmol of UDP-MurNAc-pentapeptide (-L-Ala-D-Glu-L-Lys-D- Ala-D-Mur) and 10 nmol of UDP-GlcNAc. The reaction mixture was incubated at 37°C for the time indicated, and then the reaction was stopped by boiling for 1 min. The mixture was subjected to paper chromatography using Whatman No. 3 MM filter paper and isobutyric acid, 1:10 volume ratio. Each of the reaction products was quantitated using a liquid scintillator (toluene/2,5-diphenyloxazole/100-600 mg; counting efficiency of 75%). The determination of the extent of cross-linking was carried out by digestion of the radioactive peptidoglycan product with lysozyme, followed by separation of the products, and measuring the radioactivity in the uncross-linked monomer(s) and cross-linked dimers(s) of the repeating units of the peptidoglycan as described previously (8). The percentage of cross-linking was defined as 50% the ratio of (radioactivity of cross-linked dimer(s)) to (radioactivity of uncross-linked monomer(s) plus cross-linked dimers(s)). Thus, if everything in the lysozyme digest was cross-linked, the ratio was unity and the extent of cross-linking was defined to be 50%.

RESULTS

Transglycosylase-Transpeptidase Activities of the PBP-2-RodA Protein System in E. coli Membranes and Mecillinam Sensitivity of the Transpeptidase Activity—The enzymatic activity of PBP-2 has been the subject of much speculation for many years because of the unique function attributed to this protein in the determination of the rod shape of the cell (1, 2). It has finally been established that PBP-2 is a peptidoglycan synthetase, but at least the RodA protein, the product of another cell shape gene, mrdB (or rodA), is required for the expression of its activity. Ishino et al. (12) demonstrated a sequence of reactions for the formation of cross-linked peptidoglycan from the UDP-MurNAc-pentapeptide (L-alanyl-d-glutamyl-meso-14C-diaminopimelyl-d-alanyl-d-alanine) and UDP-GlcNAc in membranes from E. coli JST7553 (mrcB+) cells that had been induced for a defective λ prophage [λlipS5cl857Qam73 (17)] carrying the chromosomal region covering leuS to lip (17). As a result of the thermoinduction of the phage, the cells produced a large amount of PBP-2 (the product of the mrdA (pbpA) gene) and of those proteins encoded by the other genes of the transducing phage, including the RodA protein, PBP-5 (the dacA product) and a 54-kDa protein (22) encoded by a gene located between mrdB (rodA) and dacA. The membranes thus contained, in addition to the high levels of PBPs and normal levels of PBPs 1A, 3, 4, and 6, a very large amount of PBP-5 which, because of its α-alanine carboxypeptidase activity, made it difficult to assay the enzymatic reactions catalyzed by PBP-2. Curtis and Strominger (23) have previously purified a small amount of PBP-2 on an affinity column of 6-aminopenicillanic acid-BSA-Sepharose from a crude mixture of E. coli PBPs, which had been pretreated with cefoxitin, a β-lactamase-resistant antibiotic. Other compounds that have high affinity for PBP-2, for example, N-formimidoylthienamycin, also strongly inhibited this transpeptidation reaction (data not shown). However, β-lactams that fail to bind to PBP-2 had no effect, or only a slightly inhibitory effect, on the transpeptidation reaction (e.g. cefmetazole at concentrations up to 100 μg/ml; latamoxef and MT-141 (cefminox, Ref. 25) at concentrations up to 40 μg/ml).

The λ transducing phage used in the above experiments carried a large fragment of E. coli chromosomal DNA covering leuS to lip, and the membranes prepared from the induced lysogens are expected to contain elevated levels of several proteins in addition to PBP-2 and the RodA protein. The overproduction of these other proteins may influence the assay of the enzymatic activities of PBP-2 and the RodA protein. To minimize any possible effect of the products of adjacent genes we, therefore, used for the biosynthetic experiments a plasmid (pHS506) which carried only the mrdA (pbpA) and mrdB (rodA) genes (18) and two open reading frames that could encode proteins of M, 7,700 and M, 17,500 (2). The 7.2-kilobase Sau3A fragment of chromosomal DNA in the plasmids pH506 has been demonstrated to express

12 (cf. Fig. 2). Peptidoglycan was synthesized by the membranes of cells that overproduced PBP-2 and the other proteins expressed from the λ transducing phage, in a reaction mixture containing 20 μg/ml 7α-methoxycephalosporin, cefmetazole (24) (Table II, Experiment 1). The peptidoglycan formed under these conditions was cross-linked to about 20%, and the only PBP that should be capable of catalyzing the cross-linking reaction under the conditions used in these experiments is PBP-2. The cross-linking was almost completely inhibited by 6.4 μg/ml benzylpenicillin (50% inhibition by 1 μg/ml) and was completely inhibited by 1 μg/ml mecillinam (50% inhibition by 0.2 μg/ml), an aminopenicillin that binds only to PBP-2 and results in the growth of E. coli as spherical cells (1). The sensitivity of the cross-linking reaction to low concentrations of mecinillin is strong evidence for the involvement of PBP-2 in the reaction since this protein is the only known target of the antibiotic. Other compounds that have high affinity for PBP-2, for example, N-formimidoylthienamycin, also strongly inhibited this transpeptidation reaction (data not shown). However, β-lactams that fail to bind to PBP-2 had no effect, or only a slightly inhibitory effect, on the transpeptidation reaction (e.g. cefmetazole at concentrations up to 100 μg/ml; latamoxef and MT-141 (cefminox, Ref. 25) at concentrations up to 40 μg/ml).

2 S. Asoh, H. Matsuzawa, F. Ishino, J. L. Strominger, M. Matsuhashi, and T. Ohta, manuscript in preparation.
TABLE II
Peptidoglycan synthesis by membranes from cells overproducing PBP-2 and RodA protein and the effects of antibiotics

| Condition       | Peptidoglycan synthesis | Lipid-linked precursor | Release of D-alanine |
|-----------------|--------------------------|------------------------|----------------------|
|                 | pmol/mg protein          | % pmol/mg protein       | pmol/mg protein       |
| Experiment 1    |                          |                        |                      |
| Complete        | 56.3                     | 18.9                   | 182                  |
| +1.6 µg/ml benzylpenicillin | 48.0                    | 8.6                    | 184                  |
| +6.4 µg/ml benzylpenicillin | 57.7                    | 3.2                    | 180                  |
| +0.4 µg/ml mecillinam | 60.9                    | 4.9                    | 196                  |
| +1.0 µg/ml mecillinam | 64.5                    | 1.4                    | 189                  |
| +40 µg/ml cefmetazole | 58.4                    | 18.2                   | 195                  |
| +100 µg/ml cefmetazole | 48.4                    | 20.0                   | 200                  |
| +40 µg/ml latamoxef | 50.5                    | 13.7                   | 195                  |
| +40 µg/ml ceftimox | 63.4                    | 7.8                    | 187                  |
| Experiment 2    |                          |                        |                      |
| Complete        | 101                      | ND²                    | 243                  |
| +6.4 µg/ml benzylpenicillin | 128                    | ND²                    | 292                  |
| +1.0 µg/ml mecillinam | 198                    | ND²                    | 195                  |
| Experiment 3    |                          |                        |                      |
| Complete        | 60.6                     | ND²                    | 217                  |
| +5 µg/ml enramycin | 13.3                    | ND²                    | 276                  |
| +50 µg/ml enramycin | 0.9                     | ND²                    | 326                  |
| Experiment 4    |                          |                        |                      |
| Complete        | 127                      | ND²                    | 318                  |
| +3 µg/ml moenomycin | 122                    | ND²                    | 327                  |
| +30 µg/ml moenomycin | 104                    | ND²                    | 350                  |
| +50 µg/ml vancomycin | 60.1                    | ND²                    | 390                  |
| +50 µg/ml vancomycin | 12.1                    | ND²                    | 406                  |
| +2.8 µg/ml tunicamycin | 20.6                    | ND²                    | 134                  |
| +28 µg/ml tunicamycin | 4.3                     | ND²                    | 27.4                  |

¹ For the complete reaction system see "Experimental Procedures." Cell envelopes prepared from E. coli JST7953/Δlip5Δβ5754am73 were used. All samples contained 20 µg/ml cefmetazole. The reaction time was 70 min, the reaction temperature was 37 °C. Cell envelopes prepared from E. coli JST7953/Δlip5Δβ5754am73 which were not induced synthesized very low levels of peptidoglycan in the presence of cefmetazole (11 pmol/mg of protein/75 min).

² The radioactive substrate, UDP-MurNAc-pentapeptide, UDP-GlcNAc was used. Other conditions were similar to Experiment 1, but cell envelopes prepared from E. coli JST7953/pHS506 were used and 100 mM D-alanine was present in the reaction mixture to prevent further degradation of D-alanine. The reaction time was 90 min. Results are expressed as picomoles of D-alanine/mg of protein. D-Alanine that was released could not be separated completely from contaminating radioactive activity on a one-dimensional chromatogram. Therefore, some of the radioactivity at the position of alanine in the experiment with benzylpenicillin or mecillinam could be due to some contamination.

³ For the complete reaction system see "Experimental Procedures." Cell envelopes prepared from E. coli JST7953/Δlip5Δβ5754am73 which were not heat induced synthesized very low levels of peptidoglycan in the presence of cefmetazole (11 pmol/mg of protein/75 min).

⁴ The radioactive substrate, UDP-MurNAc-pentapeptide, UDP-GlcNAc was used. Other conditions were similar to Experiment 1, but cell envelopes prepared from E. coli JST7953/pHS506 were used and 100 mM D-alanine was present in the reaction mixture to prevent further degradation of D-alanine. The reaction time was 90 min. Results are expressed as picomoles of D-alanine/mg of protein. D-Alanine that was released could not be separated completely from contaminating radioactive activity on a one-dimensional chromatogram. Therefore, some of the radioactivity at the position of alanine in the experiment with benzylpenicillin or mecillinam could be due to some contamination.

⁵ ND, not determined.

⁶ Conditions were similar to Experiment 1, but the reaction time was 90 min.

⁷ Conditions were similar to Experiment 1, but cell envelopes prepared from E. coli JST7953/pHS506 were used, and the reaction time was 60 min.

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The presence of 20 µg/ml cefmetazole. The peptidoglycan that was formed was 25-30% cross-linked. The absence of D-alanine carboxypeptidase activity in these membranes allowed the transpeptidase reaction to be followed by the release of D-alanine. If UDP-MurNAc-pentapeptide labeled in D-[¹⁴C] Ala-D-[¹⁴C] Ala was used, release of D-[¹⁴C] Ala could be observed and was inhibited by appropriate amounts of mecillinam and other β-lactams (Table II, Experiment 2). The transglycosylase activity was inhibited significantly by 5 µg/ml enramycin (Table II, Experiment 3) and by 50 µg/ml vancomycin (50% inhibition by 5 µg/ml), but moenomycin (30 µg/ml) had almost no effect. Tunicamycin (2.8 µg/ml) was significantly inhibitory, indicating that the formation of the lipid intermediate was involved in the synthesis of the peptidoglycan (Table II, Experiment 4).

Both the cross-linked peptidoglycan product and the uncleaved product synthesized in the presence of mecillinam formed condensed spots at the origin of the paper chromatogram where the heat-inactivated reaction mixture was applied, indicating that the products were insoluble in water in both cases (Fig. 3). The insolubility may have been due to very long glycan chains in the peptidoglycan that was synthesized.

Effect of a High Concentration of Magnesium Ion and a Chelating Agent—As described under "Experimental Procedures," the standard reaction mixture contained 13.5 mM potassium EDTA (either at pH 6.0 or 8.5) and 28 mM magnesium chloride. It is interesting that, for unknown reasons, a high concentration of chelating agent enhanced the synthesis of peptidoglycan in the membrane system. As shown in Table III, the formation of peptidoglycan was strongly decreased.

1. I. Takase, M. Doi, F. Ishino, S. Asoh, W. Matsuzawa, T. Ohta, and M. Matsushashi, manuscript in preparation.
TABLE III

| Conditions were similar to Experiment 4 of Table II. |
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### Peptidoglycan Synthesis by PBP-2 and RodA Protein

The synthesis of peptidoglycan from UDP-linked precursors was inhibited by tunicamycin (the specific inhibitor of the formation of lipid-linked MurNAc-pentapeptide (26)) suggesting that it occurred through the formation of lipid-linked intermediates (Table II, Experiment 4). This was further shown by a chasing experiment (Fig. 4). Incubation of membranes for 16 min with labeled UDP-MurNAc-pentapeptide (meso-[3H]diaminopimelic acid) and unlabeled UDP-GlcNAc resulted in the synthesis of labeled lipid-linked intermediates, a part of which were incorporated into peptidoglycan over a 60-min period following the addition of an excess of unlabeled UDP-MurNAc-pentapeptide. There was also an appreciable increase in the radioactivity at the position of UDP-MurNAc-pentapeptide on the paper chromatogram during the chasing procedure which may have been due to the formation of the nucleotide from the monosaccharide-lipid intermediate by the reverse reaction. Alternatively, it could be a decomposition product of lipid intermediates with a similar chromatographic mobility of UDP-MurNAc-pentapeptide. In any case, only a minor part of the lipid intermediates formed in the first 16 min could be converted into peptidoglycan. If purified preparations of the lipid intermediates (labeled in amino acid or GlcNAc) were used as substrate, only very poor incorporation was obtained. It is probable that the conditions for peptidoglycan synthesis from lipid intermediates in this membrane system are far from optimal, but it is also possible that the PBP-2-RodA protein system only utilizes a special fraction of the lipid intermediates that was not present in the purified lipid preparation.
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TABLE IV

Requirement for PBP-2 and the RodA protein for mecillinam-sensitive peptidoglycan synthesis in E. coli membranes

For reaction conditions see "Experimental Procedures." The reaction time was 75 min.

| Membrane source (plasmid)* | Memecillinam (10 μg/ml) | Peptidoglycan (pmol/mg protein) | Cefmetazole (50 μg/ml) | Lipid intermediates % |
|---------------------------|------------------------|-------------------------------|-----------------------|-----------------------|
|                           | No cefmetazole         | Cross-linkage                 | Lipid intermediates   | Cefmetazole (pmol/mg protein) | Lipid intermediates % |
|                           |                        | %                             |                       |                        |                       |
| pH506 (PBP-2, RodA)       | -                      | 133                           | 13.4                  | 398                   | 195                           | 25.3                  | 448                |
|                           | +                      | 249                           | 2.9                   | 418                   | 320                           | 0.1                   | 388                |
| pH503 (PBP-2)             | -                      | 16.5                          | 9.0                   | 293                   | 20.5                          | 6.5                   | 396                |
|                           | +                      | 27.0                          | 8.9                   | 363                   | 25.3                          | 0.9                   | 395                |
| pH504 (RodA)              | -                      | 40.8                          | 7.2                   | 307                   | 43.1                          | 1.3                   | 372                |
|                           | +                      | 56.4                          | 7.6                   | 367                   | 45.6                          | 0.1                   | 372                |
| None                      | -                      | 19.0                          | 6.4                   | 340                   | 20.8                          | 3.9                   | 411                |
|                           | +                      | 24.8                          | 6.1                   | 384                   | 21.4                          | 0.7                   | 420                |

* Membranes (223 μg of protein) from E. coli strain JST9753 defective in mrcB (PBP-1B-), containing plasmids as listed were used.

RodA protein in peptidoglycan synthesis by using membranes prepared from strains that carry plasmids expressing thermostable forms of PBP-2 and the RodA protein (Fig. 1). Fig. 5A shows that the synthesis of peptidoglycan (transglycosylase) and the level of cross-linking (transpeptidase) were similar at 30 and 37 °C in membranes which contained elevated levels of wild type PBP-2 and RodA protein. However, when membranes were used that had elevated levels of wild type RodA protein, but thermosensitive PBP-2, there was a slight decrease in the transglycosylase activity at 37 °C compared to 30 °C, but a much more significant thermostability of the extent of cross-linking (Fig. 5B). When cell membranes were used that had elevated levels of wild type PBP-2, but thermostable RodA protein, thermostability of the transglycosylase activity was more significant than in the other membranes. There was also some effect on the level of cross-linking; probably this may be caused mainly by the inhibition of the transglycosylase activity (Fig. 5C).

Identification of the Reaction Product—The peptidoglycan formed in the presence of cefmetazole, using membranes prepared from cells expressing greatly elevated levels of PBP-2 and the RodA protein, was digested with lysozyme and the products were separated by two-dimensional thin layer chromatography. The autoradiogram shown in Fig. 6A shows that the main product of lysozyme digestion was the compound C6 or C6', the repeating unit of peptidoglycan GlcNAc-MurNAc-tetrapeptide or GlcNAc-MurNAc-pentapeptide respectively (27, 28), and the cross-linked dimer muropeptides C3 or C3', bis(GlcNAc-MurNAc-tetrapeptide) or bis(GlcNAc-MurNAc-tetrapeptide)-D-alanine, respectively (27, 28). A few minor products were also obtained but were not characterized. The peptidoglycan synthesized in the membrane system in the

![Fig. 5. Transglycosylase (TG) and transpeptidase (TP) activities in membranes from E. coli cells containing plasmids expressing wild type and thermostable mutant forms of PBP-2 and the RodA protein. Transpeptidase activity was determined by the extent of cross-linkage in the peptidoglycan synthesized. A, strain JST9753 (mrcB)/pHS506(mrdA' mrdB'); B, JST9753 (mrcB)/pTP51(mrdA3 mrdB'); C, JST9753 (mrcB)/pTP71(mrdA' mrdB4). The reaction was carried out at 36 °C (open symbols) and 37 °C (closed symbols) in the presence of cefmetazole as described in the legend to Fig. 3. Circles, transglycosylase activity; triangles, the extent of cross-linkage.](image-url)
The mrdB gene product has an enzymatic role in peptidoglycan synthesis. The protein is probably a bifunctional enzyme catalyzing both the transglycosylase and transpeptidase reactions and, therefore, appears to be similar to PBP-1A, PBP-1B, and PBP-3 which have also been shown to be bifunctional (5, 10, 11, 13–15). The results suggest that both PBP-2 and the RodA protein are required for peptidoglycan synthesis, but it is difficult to establish which of the two proteins is responsible for the transglycosylase activity. The transpeptidase activity that we detected was presumably catalyzed by PBP-2 since the reaction was strongly inhibited by low concentrations of mecillinam, which binds exclusively to PBP-2, and by other β-lactams that have high affinity for PBP-2 (e.g. benzylpenicillin and N-formimidoylthienamycin (36)), but was only poorly inhibited by those β-lactams that have very low affinity for PBP-2 (e.g. cefmetazole, latamoxef, and MT-141 cefminox (25)). The thermosensitivity of the transpeptidation reaction in membranes that contained high levels of thermosensitive PBP-2 also supports the view that this activity was catalyzed by PBP-2. The RodA protein may regulate the activity of PBP-2 or the two proteins may form a complex which together functions as a peptidoglycan synthetase with a special role in the determination of bacterial cell shape. The proteins may act together to form an initiation piece (or ring) of peptidoglycan at the center of the cell (5, 14) to ensure the formation of the correct rod shape of the cell. At present, however, we cannot completely eliminate the possibility that PBP-2 acts as the transpeptidase and that the RodA protein acts as the transglycosylase, but we believe this to be unlikely. Moreover, our membranes also contained two other smaller proteins (7.7- and 17-kDa proteins) presumably encoded by two open reading frames in the chromosomal insert of the plasmids that we used. The possibility remains that these proteins play some role in the biosynthetic process described above. The clone that contains only genes of PBP-2 and the RodA protein has to be isolated in order to exclude the activity of the smaller proteins. We are also trying to reconstitute a peptidoglycan synthetic system from purified protein components, including PBP-2, RodA, and other proteins such as the 7.7- and 17-kDa proteins (see above), or 54-kDa proteins (22) the function of which were so far not required for the peptidoglycan synthesis in vitro but could be important in the in vivo process. However, even attempts to reconstitute an active mecillinam-sensitive peptidoglycan synthetic system by sonication of a mixture of membrane vesicles containing high levels of PBP-2 (and smaller proteins but not RodA) and those containing high levels of the RodA protein have been unsuccessful. Furthermore, we have not been able to achieve peptidoglycan synthesis from purified lipid-linked intermediates using either membranes or purified PBP-2.

On the other hand, the amino acid sequence of PBP-2 has recently been obtained from the nucleotide sequence of the mrdA(pbpA) gene. The amino acid sequence shows several regions of substantial similarity to the sequences of other high molecular weight PBPs determined by others (37, 38). A putative penicillin-binding site containing the sequence Ser-Xaa-Xaa-Lys was found which has been found at the active site of all PBPs. The existence of this sequence and the overall similarity to other high molecular weight PBPs add support to the view that PBP-2 acts, like other high molecular weight PBPs (6, 10, 11), as a bifunctional enzyme of peptidoglycan synthesis.
Peptidoglycan Synthesis by PBP-2 and RodA Protein

PBP-2 and the RodA protein are not the only gene products that were proved to have a role in the determination of the shape of E. coli. The gene envB (39) which is located at 71 min on the E. coli chromosome, and the closely linked and possibly identical mreB gene (5), as well as the mreC (5) gene have also been implicated in the determination of cell shape and the sensitivity of E. coli cells to mecillinam. At present we know little of the function of these genes in the bacterial cell cycle.

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