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Molecular Cloning and Characterization of the Genes (*pbpA* and *rodA*) Responsible for the Rod Shape of *Escherichia coli* K-12: Analysis of Gene Expression with Transposon Tn*5* Mutagenesis and Protein Synthesis Directed by Constructed Plasmids

SADAMITSU ASOH,[1] HIROSHI MATSUZAWA,[1]* MICHIO MATSUHASHI,[2] AND TAKAHISA OHTA[1]

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

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Two cell shape-determining genes of *Escherichia coli* K-12, *pbpA*, the structural gene for penicillin-binding protein 2, and *rodA*, whose protein is unknown, were subcloned into plasmid vectors from the transducing phage λMAd *lip*24, which carries the *lip-leuS* region of the *E. coli* chromosome. Plasmids with restriction enzyme-created deletions or transposon Tn*5* insertions were isolated, and studies of genetic complementation of these plasmids with chromosomal mutations were carried out. Thus, a physical and genetic map of the *rodA-pbpA* region was established. The genes *rodA* and *pbpA* lie side by side within a 4.4-kilobase-pair region. The size of the *rodA* gene has been shown to be between 0.86 and 1.6 kilobase pairs; such DNA would encode a protein with a molecular weight between 32,000 and 59,000. Since Tn*5* mutagenesis of the *rodA* gene did not affect the expression of the *pbpA* gene and vice versa, the genes *rodA* and *pbpA* seem to have independent promoters. Analysis of the proteins synthesized from the constructed plasmids in maxicells revealed that the plasmid carrying the *pbpA* gene encoded penicillin-binding protein 2 and amplification of the protein occurred. The product of the *rodA* gene was not identified.

Several mutations are known to cause the formation of spherical cells from rod-shaped *Escherichia coli*. Mutants with temperature-sensitive penicillin-binding protein (PBP) 2 grow as spherical cells at high temperature and are resistant to mecillinam, an amidinopenicillin (19). The structural gene for PBP2 has been designated as *pbpA* (20) or *mrdA* (23). The *rodA* mutant also grows as spheres (14) and is resistant to mecillinam, but it has normal PBP2 activity (13). The *rodA* gene is probably identical to the *mrdB* gene (23). The locations of the *pbpA* and *rodA* genes are such that they form a cluster, and the gene arrangement is as follows: *lip* (14.2 min)-*rodA* (*mrdB*)-*pbpA* (*mrdA*)-*leuS* (14.6 min) (21, 23) on the *E. coli* genetic map (1).

PBP2 appears in *E. coli* cells in very small amounts, and its enzymatic function is only considered to be involved in the formation of peptidoglycan, a supramolecule covering the entire surface of the cell (12). This protein can be produced in about 50-fold excess in cells carrying a λ transducing phage containing the *lip-leuS* region of the chromosome (21). The *rodA* protein has not yet been identified, and its enzymatic function is still unknown. Thus, an attempt was made to subclone the genes *pbpA* and *rodA* into the plasmid vectors pBR322 and pACYC184, to determine the physical and genetic map of the cloned DNA, and to investigate the products of the two genes and the mechanism regulating their expression.

MATERIALS AND METHODS

Bacterial strains. The properties of the *E. coli* K-12 strains used are summarized in Table 1.

Growth media. Modified L broth (14) supplemented with 20 μg of thymine and 50 ng of lipoic acid per ml (LL broth) was usually used for growing the cells. Cultures were grown at 30°C with shaking. Tryptone medium (1% tryptone [Difco Laboratories]–0.25% NaCl, pH 7.0, with NaOH) supplemented with 0.2% maltose and 10 mM $MgSO_4$ was used for the preparation and titration of λ phage lysates. K10 minimal medium (14) containing 10 mM $MgSO_4$ was used for the transduction experiment with the λ transducing phage. The amino acids and others required were added as described previously (13). The antibiotics used were ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), tetracycline (25 μg/ml), and kanamycin (30 μg/ml). Broth was solidified with 1.5% agar for the plates and 0.5% for the top agar.

Isolation of the specialized λ transducing phage carrying the *lip-pbpA* region of the chromosome. The proce-

TABLE 1. Strains of *E. coli* K-12 used

Strain	Genotype[a]	Source or reference
AB1325 *lip-9*	F⁻ *lip-9 thi-1 his-4 purB15 proA2 mtl-1 xyl-5 galK2 lacY1 rpsL35*	J. R. Guest strain
TMM3	As AB1325 *lip-9* but *pbpA3*(Ts)	23
TMM13	As TMM3 but *recA1 his*⁺	23
S2	As AB1325 *lip-9* but *rodA51*(Ts) *lip*⁺	14
SJC21	As S2 but *recA56 srlC300*::Tn*10*	Recombinant from S2 × JC10240 (8)
RL1	As AB1325 *lip-9* but *leuS31* (Ts) *lip*⁺	D. Söll strain
JC10240	Hfr PO45 *recA56 srlC300*::Tn*10 thr-300 ilv-318 rpsE300*	8
Ymel	F⁺ *mel-1 supF58 supE57*	K. Mizobuchi
KS302	HfrH Δ(*gal-att*λ-*bio*) *supO thi rpsL*	K. Mizobuchi
D6434	F⁻ *argE*(Am) Δ(*lac-pro*)XIII *gyrA rpoB* Su6⁺	10
CSR603	F⁻ *recA1 uvrA6 phr-1 thr-1 leu-6 proA2 argE3 thi-1 rpsL31*	17

[a] The genetic symbols used are those described in reference 1. For definition of *pbpA* and *rodA*, see the text.

dure for isolation of the specialized λ transducing phage was essentially the same as that described by Schrenk and Weisberg (18). Bacteriophage λ *c*I857 *S*am7 (from K. Mizobuchi) was prepared by lytic growth on the *supF* strain, Ymel. The host range phage λ *c* Δ*int h*80 (from K. Yoda) was used for the λ-immunity selections. A mixed low-frequency transducing phage lysate was prepared from strain KS302 Δ(*gal-att*λ-*bio*) lysogenized with λ *c*I857 *S*am7 and was used to transduce strain TMM13 *lip-9 pbpA3* lysogenized with λpapa (from H. Uchida) to Lip⁺ and PbpA⁺. The *pbpA*(Ts) mutant was sensitive to Sarkosyl (a gift from Ciba-Geigy) at 42°C as described previously (21). The Lip⁺ PbpA⁺ transductants were selected for growth on tryptone agar (Difco) containing Sarkosyl (0.05%) with no lipoate at 42°C. The cell shapes of the transductants and the transformants were examined with a phase-contrast microscope. Production of a high-frequency transducing phage was investigated after UV irradiation. We succeeded in isolating one defective transducing phage, λMAd *lip*24.

Preparation of phage DNA. Phage lysate was prepared by the method of Miller (15). DNA of the transducing phage λMAd *lip*24 was prepared from double lysogen AB1325 *lip-9*(λMAd *lip*24 and λ *c*I857 *S*am7). The double lysogen was constructed by using transducing strain AB1325 *lip-9* lysogenized with λ *c*I857 *S*am7 to Lip⁺ with λMAd *lip*24 at 30°C. Phage particles were collected by sedimentation in the presence of 10% polyethylene glycol 6000 and 0.5 M NaCl as described by Yamamoto et al. (26). The phage DNA was extracted with phenol, precipitated with ethanol, and dissolved in TES buffer (10 mM Tris-hydrochloride [pH 7.6]–1 mM EDTA–10 mM NaCl). This DNA sample, a mixture of the transducing and helper phage DNA, was used for subcloning the *pbpA* and *rodA* genes into vectors pBR322 (3) and pACYC184 (5).

Preparation of plasmid DNA. The preparation method for cleared lysates was based on that described by Clewell and Helinski (6). Cultures of cells carrying plasmids were amplified with chloramphenicol (150 μg/ml) or, in the case of chloramphenicol-resistant (Cm^r) strains, spectinomycin (350 μg/ml). Plasmid DNA was purified on a hydroxylapatite (Bio-Rad Laboratories; DNA grade) column as described by Colman et al. (7). After dialysis against TE buffer (10 mM Tris-hydrochloride [pH 7.5]–1 mM EDTA) and ethanol precipitation, plasmid DNA was redissolved in TE buffer. For rapid preparation of plasmid DNA, the method described by Bukhari et al. (4) was used.

Transformation. Preparation of competent cells and transformation with plasmid DNA were carried out by the method of Dagert and Ehrlich (9).

Analysis of DNA with restriction endonucleases. The methods used for restriction endonuclease cleavage were those described by Bukhari et al. (4). Electrophoresis of DNA was carried out on a 0.8% agarose (Sigma Chemical Co. type II) gel. The electrophoresis buffer used was 89 mM Tris-borate buffer (pH 8.3) containing 2.5 mM disodium EDTA.

T4 DNA polymerase reaction. This reaction was carried out by the procedure of Morris et al. (16). The buffer solution used was 67 mM Tris-hydrochloride (pH 8.8)–6.7 mM $MgCl_2$–5 mM dithiothreitol–16.7 mM ammonium sulfate–6.7 μM disodium EDTA–0.02% bovine serum albumin plus 33 μM concentrations of each of the four deoxyribonucleotide triphosphates. T4 DNA polymerase (1 unit) was added to 20 μl of the reaction mixture containing 0.5 to 1 μg of DNA, and the mixture was incubated a 37°C for 1 h.

T4 DNA ligase reaction. The ligase reaction mixture (25) contained 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.1 mM ATP, and digested DNA (1 to 20 nM termini), which was incubated at 30°C for 15 min to separate preannealed fragments. T4 DNA ligase (1 unit) was added to the reaction mixture (50 to 300 μl) which was incubated at 11°C for 12 to 18 h.

Transposition of Tn*5* into plasmid pHS202. Bacteriophage λ *c*I857 *rex*::Tn*5* *O*am29 *P*am80 *b*221 (from J. Beckwith) was prepared by lytic growth on Su6⁺

indicator strain D6434. Strain SJC21 or TMM13 harboring pHS202 grown in tryptone broth (Difco) was infected with this phage at a multiplicity of infection of 10 as described previously. After 30 min for adsorption of phage, the cells were washed and then were grown in LL broth at 30°C for 2 h. They were concentrated and plated at 37°C onto K10 minimal plates of LL plates containing kanamycin and chloramphenicol. Transposition of Tn*5* occurred at a frequency of 10^{-4} to 10^{-3} per infected cell, and about 10^4 to 10^5 independent kanamycin-resistant (Km^r) and Cm^r colonies were pooled. They were adequately diluted, plated on LL plates containing kanamycin, chloramphenicol, and mecillinam (10 to 30 μg/ml), and incubated at 42°C. The plasmid DNAs were prepared from the pooled Km^r, Cm^r, and mecillinam-resistant colonies (10^3 to 10^4 colonies) to transform SJC21 and TMM13 into Km^r Cm^r.

Labeling of proteins directed by plasmids. The maxicell method (17) was generally followed in labeling the proteins directed by plasmids. Strain CSR603 was used as a host for the plasmid to be tested. A 2-ml volume of a log-phase culture (ca. 2×10^8/ml) grown in M9 medium supplemented with 1% Casamino Acids (Difco) at 37°C was irradiated with UV light and further incubated for 1 h. After the addition of D-cycloserine (100 μg/ml), the culture was incubated for 12 h at 37°C. L-[^{35}S]methionine (Amersham Corp.; 1,130 Ci/mmol) was added at a final concentration of 33 μCi/ml to the sulfate-starved culture, and incubation was continued for 1 h. The labeled cells were collected and suspended in 50 μl of the sample buffer, followed by heating for 2 min at 100°C and sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Labeled proteins were located by fluorography.

Chemicals. Ampicillin was kindly provided by Meiji-Seika Co., Tokyo; spectinomycin was provided by Japan Upjohn, and mecillinam was provided by F. Lund, Leo Pharmaceutical Products. Chloramphenicol, tetracycline, and restriction endonuclease *Bam*HI were purchased from Boehringer Mannheim GmbH; the enzymes *Sal*I, *Eco*RI, *Mlu*I, *Pvu*II, *Bgl*II, *Kpn*I, *Sma*I, and T4 DNA ligase were purchased from Takara Shuzo Co., Kyoto; T4 DNA polymerase was purchased from P-L Biochemicals, Inc.; kanamycin sulfate was purchased from Sigma Chemical Co.; and D-cycloserine was purchased from Aldrich Chemical Co. Other chemicals were commerically obtained standard products.

RESULTS

Cloning of the *pbpA* and *rodA* genes into the plasmid vectors pBR322 and pACYC184 and restriction endonuclease mapping of the cloned DNA. The transducing phage λMAd *lip*24 carrying the *lip-pbpA* region of the *E. coli* chromosome was isolated as described above. This phage was also capable of transforming the *rodA* and *leuS* mutants. This indicates that the specialized λ transducing phage, carrying the *lip-rodA-pbpA-leuS* region of the chromosome and similar to that isolated by Spratt et al. (21), was obtained. After this, we subcloned the *pbpA* and *rodA* genes from the chromosomal DNA of λMAd *lip*24 into the plasmid vectors pBR322 and pACYC184.

The phage DNA was prepared from strain AB1325 *lip-9* lysogenized with both λMAd *lip*24 and λ *c*I857 *S*am7, digested with the restriction endonuclease *Sal*I, and then mixed with *Sal*I-digested pBR322. The mixture was then ligated with T4 DNA ligase. This ligated mixture was used to transform strain TMM13 *pbpA3 recA1* into a strain growing at 42°C on plates containing both ampicillin and Sarkosyl. Plasmid DNA was prepared from one such transformant and used for transforming strain SJC21 *rodA51 recA56*. This plasmid, pMA101, also complemented the *rodA51* mutation (Table 2), indicating that both the *pbpA* and *rodA* genes of the chromosomal DNA were subcloned from λMAd *lip*24 into pBR322. The plasmid pMA101, which contains 16 kilobase pairs (kb) of DNA, was found to consist of two copies of pBR322 (4.3 kb of DNA), a 7.2-kb *Sal*I fragment of the chromosomal DNA, and 0.4 kb of *Sal*I foreign DNA, from analysis of the DNA fragment after diges-

TABLE 2. Genetic complementation of various plasmids with strains carrying the *rodA* or *pbpA* mutation[a]

Plasmid[b]	SJC21 (*rodA51*)	TMM13 (*pbpA3*)
pMA101	+	+
pMA102	+	+
pMA105	+	−
pMA106	+	−
pMA107	−	−
pMA108	−	−
pMA109	+	−
pMA110	+	+
pMA111	+	−
pMA113	−	−
pHS201	+	+
pHS202	+	+
pHS202::Tn*5* Ω501	−	+
pHS202::Tn*5* Ω502	−	+
pHS202::Tn*5* Ω503	−	+
pHS202::Tn*5* Ω504-1	+	−
pHS202::Tn*5* Ω504-2	+	−
pHS202::Tn*5* Ω505	+	−
pHS202::Tn*5* Ω506	+	+

[a] Mutant strains were transformed with plasmid DNA, and transformants were selected at 42°C on LL medium containing antibiotics as follows: pMA101 to pMA113, ampicillin (100 μg/ml); pHS201 and pHS202, chloramphenicol (30 μg/ml); and pHS202::Tn*5* Ω501 to pHS202::Tn*5* Ω506, chloramphenicol and kanamycin (30 μg/ml each). Complementation of cell shape was determined after 1 to 2 days of growth with a phase-contrast microscope.

[b] The plasmid pMA109 was prepared from pMA105 with elimination of the *Hin*dIII-*Bam*HI fragment containing the promoter region for the Tc^r gene of vector pBR322. pHS201 contains the 7.2-kb *Sal*I fragment opposite in direction to that in pHS202 (Fig. 3). Other plasmids are shown in Fig. 1 and 3. For pMA101, see the text and the legend to Fig. 1.

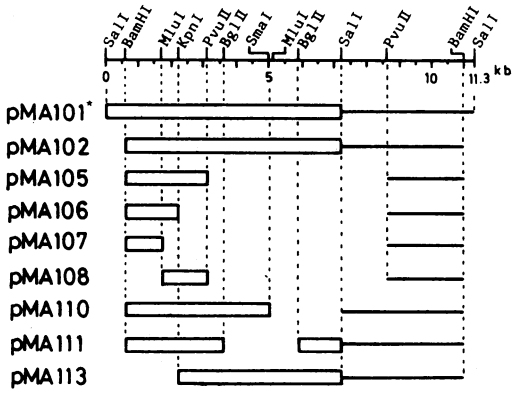

FIG. 1. A physical map and derivations of constructed plasmids. Blocks and solid lines represent DNAs from the *E. coli* chromosome and vector pBR322, respectively. Deletions are shown as gaps. pMA101* is part of pMA101, which consists of pMA101* plus a 0.4-kb *Sal*I fragment and another pBR322 aligned in this order. The size (kb) of each plasmid is as follows: pMA101*, 11.3; pMA102, 10.4; pMA105, 4.9; pMA106, 4.0; pMA107, 3.5, pMA108, 3.8; pMA110, 8.2; pMA111, 8.1; and pMA113, 8.8. For detailed derivations of each plasmid, see the text.

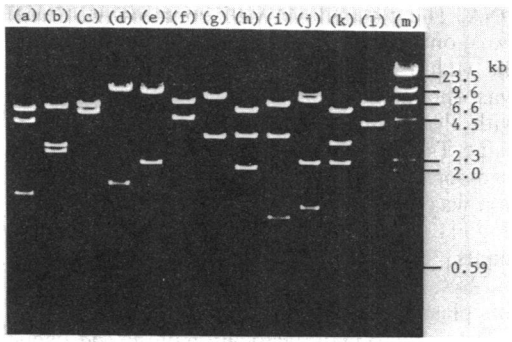

FIG. 2. Cleavage patterns of plasmid pMA102 DNA with various restriction enzymes. Lanes: (a) *Pvu*II and *Sal*I, (b) *Pvu*II and *Bam*HI, (c) *Kpn*I and *Sal*I, (d) *Kpn*I and *Bam*HI, (e) *Sma*I and *Sal*I, (f) *Sma*I and *Bam*HI, (g) *Sma*I and *Mlu*I, (h) *Mlu*I and *Sal*I, (i) *Mlu*I and *Bam*HI, (j) *Bgl*II and *Sal*I, (k) *Bgl*II and *Bam*HI, and (l) *Bam*HI and *Sal*I were used. (m) *Hin*dIII-digested λ DNA was included for calibration. The samples were electrophoresed on a 0.8% agarose gel at room temperature.

tion of *Bam*HI, *Eco*RI, *Hin*dIII, and *Sal*I (data not shown). The 7.2-kb *Sal*I fragment was considered to contain both the *pbpA* and *rodA* genes as reported previously (21). A part of the plasmid pMA101 (pMA101*), consisting of the 7.2-kb *Sal*I fragment and pBR322, is shown in Fig. 1. To obtain a single pBR322 carrying the *pbpA* and *rodA* genes, the pMA101 DNA was digested with *Bam*HI and religated with T4 DNA ligase. Ampicillin-resistant (Ap^r) and Sarkosyl-resistant transformants of TMM13 were selected as described above. Plasmid pMA102 was prepared from one of the transformants and was capable of transforming strain SJC21 into Ap^r and rod-shaped cells (Table 2). The restriction map of the plasmid pMA102 (Fig. 1) was derived from analysis of DNA fragments with agarose gel electrophoresis after digestion by various combinations of the restriction enzymes *Bam*HI, *Mlu*I, *Kpn*I, *Pvu*II, *Bgl*II, *Sma*I, and *Sal*I (Fig. 2). The plasmid pMA102 contained the 6.6-kb *Bam*HI-*Sal*I fragment with the unique *Kpn*I, *Pvu*II, and *Sma*I sites, and the two sites each of *Mlu*I and *Bgl*II. It is this fragment that carries the genes *pbpA* and *rodA*.

The 7.2-kb *Sal*I fragment was similarly subcloned from λMAd *lip*24 into the *Sal*I site of the plasmid vector pACYC184, using the selection of Cm^r and Sarkosyl-resistant transformants. Two types of plasmids, pHS201 and pHS202 (Fig. 3), differing in the direction of the cloned fragment, were obtained; both were capable of complementing *pbpA3* and *rodA51* (Table 2). The direction of the *Sal*I fragment in pHS202 was identical to that in pMA101 and its derivatives (Fig. 1). These results suggest that a promoter(s) for both the *pbpA* and *rodA* genes is included in the cloned fragment.

Construction of plasmids derived from pMA102 with deletions in the *pbpA* or the *rodA* gene region and localization of genes in cloned

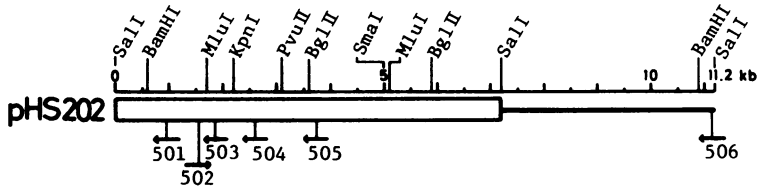

FIG. 3. The location of Tn*5* insertions in plasmid pHS202. The block and the solid line represent DNA from the *E. coli* chromosome and vector pACYC184, respectively. Relevant restriction sites are also shown. The arrows represent the location and orientation of the transposone Tn*5* inserted at the indicated sites. The direction of the arrow defines the order of the unique *Bam*HI, *Sal*I, and *Sma*I sites in Tn*5* as shown *Bam*HI *Sal*I *Sma*I. For clarity, the Tn*5* elements are not drawn to scale.

DNA. The plasmid pMA102 contains two *Pvu*II sites, one in the cloned fragment and the other in the vector (Fig. 1). The larger *Pvu*II fragment was eliminated from the plasmid by digestion with *Pvu*II and religating the restricted DNA, using T4 DNA ligase. Strain SJC21 cells were transformed with the religated DNA, and selection was made for Ap^r strains. Plasmid pMA105 (Fig. 1) was obtained from one of the transformants. This plasmid was able to complement *rodA51*, but *pbpA3* was not complemented by this plasmid (Table 2).

Determination of the location of the *rodA* gene was carried out by deletion of the restricted DNA from the *Bam*HI-*Pvu*II fragment of pMA105. The pMA105 DNA was digested by such enzyme combinations as *Kpn*I and *Pvu*II, *Mlu*I and *Pvu*II, and *Bam*HI and *Mlu*I, and digested DNAs were treated with T4 DNA polymerase to convert their termini into fully base-paired ends. The treated DNAs were religated with T4 DNA ligase. Plasmids pMA106, pMA107, and pMA108 (Fig. 1) were obtained separately from the Ap^r transformant of SJC21 strain, with the corresponding deletion of *Kpn*I-*Pvu*II, *Mlu*I-*Pvu*II, and *Bam*HI-*Mlu*I fragments, as confirmed by agarose gel electrophoresis (data not shown). A study of the complementation with these plasmids indicated that the *rodA* gene was expressed in pMA106, but not in pMA107 and pMA108 (Table 2). This shows that the *rodA* gene is located in the 1.6-kb *Bam*HI-*Kpn*I fragment of the cloned DNA (Fig. 4).

To determine the location of the *pbpA* gene, pMA102 DNA was digested with both *Sma*I and *Sal*I simultaneously to eliminate the *Sma*I-*Sal*I fragment. Plasmid pMA110 (Fig. 1), thus obtained, was able to complement *pbpA3* as well as *rodA51* (Table 2). The plasmid pMA102 has two *Bgl*II sites in the cloned DNA, and plasmid pMA111 (Fig. 1), which has the deletion of 2.3-kb *Bgl*II fragment from pMA102, was obtained after *Bgl*II digestion. This plasmid could not complement *pbpA3* but could complement *rodA51* (Table 2). Plasmid pMA113 (Fig. 1), which has the deletion of 1.6-kb *Bam*HI-*Kpn*I fragment from pMA102, was similarly prepared and was unable to complement both *rodA51* and *pbpA3* (Table 2). These results indicate that the *pbpA* gene is located in the 3.3-kb *Mlu*I-*Sma*I fragment of the cloned DNA (Fig. 4). The DNA length encoding the *pbpA* protein PBP2, with a molecular weight of 66,000 (21), is calculated to be about 1.8 kb. Therefore, the 1.4-kb *Kpn*I-*Bgl*II fragment (Fig. 4) contains about 80% of the coding regin for the *pbpA* protein.

Plasmid pMA109 was also prepared from pMA105 eliminating the *Hin*dIII-*Bam*HI fragment containing the promoter region for the tetracycline resistance (Tc^r) gene of the vector pBR322 (22). The *rodA* gene was expressed in pMA109 (Table 2). This suggests that a promoter for the *rodA* gene is included in the cloned fragment.

Transposon Tn*5* mutagenesis of the *pbpA* and *rodA* genes cloned into pACYC184. Insertion of the Km^r transposon Tn*5* into a gene destroys gene function due to insertional inactivation and exerts a polar effect on genes within an operon distal to its insertion (2). To determine the locations and sizes of the *pbpA* and *rodA* genes and to know whether both genes are part of a single transcriptional unit, the plasmid pHS202 (Fig. 3) was mutagenized with Tn*5*.

Strain SJC21 or TMM13 harboring the plasmid pHS202 was infected with λ *c*I857 *rex*::Tn*5* *O*am29 *P*am80 *b*221. Km^r and Cm^r (a resistance characteristic of the pHS202) colonies were obtained, and plasmid DNAs were prepared from the mixed colonies. By transducing strain SJC21 or TMM13 with the isolated plasmid DNAs and selecting for Km^r and Cm^r, 25 plasmids carrying an insertion of Tn*5* were obtained. The insertion sites and directions of Tn*5* in the plasmid pHS202 (Fig. 3) were determined from analysis of DNA fragments after digestion by *Bam*HI, *Sal*I, or *Sma*I and by a combination of *Xho*I and *Bam*HI or *Xho*I and *Mlu*I (data not shown). There is no *Xho*I site on pHS202, and the restriction sites of other enzymes used are shown in Fig. 3. Tn*5* has unique *Bam*HI, *Sal*I, and *Sma*I sites, three *Xho*I sites (11), and no *Mlu*I site. Tn*5* insertion sites were distributed in six positions from insertion site (Ω) 501 to 506 (Fig. 3). The number of the isolated plasmids carrying Tn*5* at each insertion site is as follows: Ω501, 1; Ω502, 12; Ω503, 1; Ω504, 8; Ω505, 1; and Ω506, 2. The inserted direction of Tn*5* was common among Ω502, Ω504, and Ω506. Genetic complementation of some of these plasmids with the chromosomal mutation *rodA51* or *pbpA3* was examined (Table 2). Plasmids pHS202::Tn*5* Ω501, Ω502, and Ω503 were unable to complement *rodA51* but did complement *pbpA3*. In contrast, plasmids pHS202::Tn*5* Ω504-1, Ω504-2, and Ω505 were able to complement *rodA51*

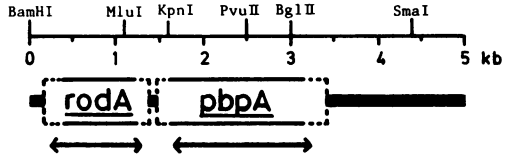

FIG. 4. Physical and genetic map of the *rodA-pbpA* region of the *E. coli* chromosome. The *Mlu*I site was present in the *rodA* gene. The *pbpA* gene carried the sites of *Kpn*I, *Pvu*II, and *Bgl*II. The ends of both genes have not yet been definitely determined. The *rodA* and *pbpA* genes are separate transcriptional units.

but not *pbpA3*. Berg et al. (2) have reported that 26 of 41 Tn*5* transposons in the *lacZ* gene exert a strong polar effect on the *lacY* gene. If this observation can be extrapolated to the relatively small size in the present situation, the finding that all three of the transposons in the *rodA* gene did not affect the expression of the *pbpA* gene and vice versa suggests that both genes have independent promoters. On the location of the genes, these results are consistent with the finding that the *rodA* and *pbpA* genes are located in the 1.6-kb *Bam*HI-*Kpn*I and the 3.3-kb *Mlu*I-*Sma*I fragments, respectively, as described above.

Plasmid pHS202::Tn*5* Ω506, in which the Tn*5* insertion site is downstream from the promoter for the Tc^r gene of the vector pACYC184 (22), was able to complement both *rodA51* and *pbpA3* (Table 2). This suggests that the promoter for the Tc^r gene is not involved in the expression of the *rodA* and *pbpA* genes.

Analysis of proteins synthesized from constructed plasmids. The synthesis of proteins directed by constructed plasmids was examined in UV-irradiated whole cells of strain CSR603 harboring the plasmid (Fig. 5). The plasmid pMA102 encoded PBP2 (66,000 molecular weight) (Fig. 5, lane a) and allowed amplification of the level of the protein (data not shown), whereas the protein could not be detected with plasmid pMA113 (Fig. 5, lane b) or pMA111 (Fig. 5, lane c). These results are consistent with the results of genetic complementation analysis (Table 2). On the other hand, when plasmid pMA113 was used, new bands of proteins with molecular weights of 57,000 and 41,000 could be observed (Fig. 5, lane b). These bands may have appeared because the coding region of carboxyl terminal of PBP2 was deleted in pMA113. If so, on the 1.4-kb *Kpn*I-*Bgl*II fragment in the *pbpA* region (Fig. 4), the *Bgl*II site is proximal and the *Kpn*I site is distal to the promoter for the *pbpA* gene. We are now sequencing the *pbpA* gene, and we will be able to determine the location of the promoter for the gene soon.

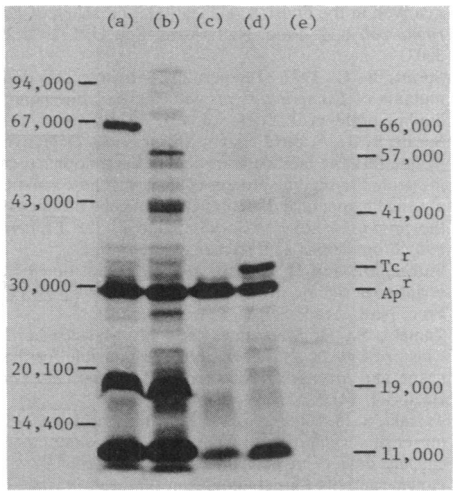

FIG. 5. Gel electrophoretic patterns of proteins synthesized from constructed plasmids. Cells of *E. coli* CSR603 and its plasmid-harboring derivatives were UV irradiated, and the proteins directed by the plasmid were labeled with L-[^{35}S]methionine. The total cell proteins were fractionated on a 13% sodium dodecyl sulfate-polyacrylamide gel and fluorographed. Phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) were used as molecular weight standards. Tc^r and Ap^r are the proteins of the tetracycline and ampicillin resistance genes, respectively, on vector pBR322. Lanes: (a) CSR603(pMA102), (b) CSR603(pMA113), (c) CSR603(pMA111), (d) CSR603(pBR322), and (e) CSR603.

When plasmids pMA102 and pMA113 were used, two other bands of proteins with molecular weights of 19,000 and 11,000 were found (Fig. 5, lanes a and b). These bands could not be observed when pMA111 was used (Fig. 5, lane c). Therefore, these proteins seem to be encoded by the region in the 2.3-kb *Bgl*II DNA of plasmids pMA102 and pMA113 (Fig. 1). The genes corresponding to these proteins have yet to be identified. No *rodA* protein could be identified in cells carrying pMA102 or pMA111 (Fig. 5), or any other plasmid prepared in this study (data not shown).

The production of PBP2 in normal growth conditions was found to be about 20 times more in cells carrying either pHS201 or pHS202 than in cells carrying no plasmid (data not shown).

DISCUSSION

The 4.4-kb *Bam*HI-*Sma*I fragment contained both the *rodA* and *pbpA* genes (Fig. 4). The *rodA* gene was found to be located in the 1.6-kb *Bam*HI-*Kpn*I fragment and to be more than 860 base pairs of nucleotides (the distance between Ω*501* and Ω*503* in Fig. 3). On the basis of the DNA length, the molecular weight of the *rodA* protein was calculated to be more than 32,000 and less than 59,000. Spratt et al. (21) reported that a *Kpn*I site is in the *rodA* gene. However, our result shows that the *Kpn*I site was included in the *pbpA* gene, indicating that the 0.5-kb *Mlu*I-*Kpn*I fragment carries the ends of both genes. The DNA length encoding the *pbpA* protein should be about 1.8 kb. Thus, the 3.4-kb (1.6 kb of the *Bam*HI-*Kpn*I fragment plus 1.8 kb) cloned DNA, containing the *Bam*HI site at one end and the restriction sites of *Mlu*I, *Kpn*I, *Pvu*II, and *Bgl*II, would be almost enough to cover both genes.

A mutation of either *pbpA* or *rodA* causes a similar phenotype at 42°C; the cells grow spherically and are mecillinam resistant (13, 23). Moreover, both genes were found to be present side by side (Fig. 4). These facts make it feasible for the genes *pbpA* and *rodA* to form a single transcriptional unit. However, judging from Tn*5* insertional mutagenesis of the genes, *pbpA* and *rodA* seem to belong to separate transcriptional units.

Another cluster of genes, *mreA*, *-B*, and *-C* (12), which are considered to be involved in the formation of the rod-shaped peptidoglycan sacculus, is localized at about 71 min on the *E. coli* genetic map and has been cloned into a λ phage. *mreA* is a regulatory gene involved in the synthesis of PBP1A and PBP2 (24), and the gene product is thought to function as a repressor. Mutation of *mreB* and *mreC* results in the formation of spherical cells and mecillinam resistance, respectively.

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LITERATURE CITED

1. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44**:1–56.
2. **Berg, D. E., A. Weiss, and L. Crossland.** 1980. Polarity of Tn*5* insertion mutations in *Escherichia coli*. J. Bacteriol. **142**:439–446.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene (Amst) **2**:95–113.
4. **Bukhari, A. I., J. Sambrook, R. M. Harshey, R. Greene, and A. Skalka.** 1981. Laboratory manual for a practical course on recombinant DNA techniques. Banares Hindu University, Varanasi, India.
5. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
6. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. **62**:1159–1166.
7. **Colman, A., M. J. Byers, S. B. Primrose, and A. Lyons.** 1978. Rapid purification of plasmid DNAs by hydroxyapatite chromatography. Eur. J. Biochem. **91**:303–310.
8. **Csonka, L. N., and A. J. Clark.** 1980. Construction of an Hfr strain useful for transferring *recA* mutations between *Escherichia coli* strains. J. Bacteriol. **143**:529–530.
9. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene (Amst) **6**:23–28.
10. **Felton, J., S. Michaelis, and A. Wright.** 1980. Mutations in two unlinked genes are required to produce asparagine auxotrophy in *Escherichia coli*. J. Bacteriol. **142**:221–228.
11. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
12. **Matsuhashi, M., J. Nakagawa, F. Ishino, S. Nakajima-Iijima, S. Tomioka, M. Doi, and S. Tamaki.** 1981. Penicillin-binding proteins: their nature and functions in the cellular duplication and mechanism of action of β-lactam antibiotics in *Escherichia coli*, p. 203–223. *In* S. Mitsuhashi (ed.), Beta-lactam antibiotics. Japan Scientific Societies Press, Tokyo.
13. **Matsuzawa, H., S. Asoh, T. Ohta, S. Tamaki, and M. Matsuhashi.** 1980. Further studies on *rodA* mutant: a round morphological mutant of *Escherichia coli* K-12 with wild-type penicillin-binding protein 2. Agric. Biol. Chem. **44**:2937–2941.
14. **Matsuzawa, H., K. Hayakawa, T. Sato, and K. Imahori.** 1973. Characterization and genetic analysis of a mutant of *Escherichia coli* K-12 with rounded morphology. J. Bacteriol. **115**:436–442.
15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 320. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Morris, C. F., H. Hama-Inaba, D. Mace, N. K. Sinha, and B. Alberts.** 1979. Purification of the gene 43, 44, 45, and 62 proteins of the bacteriophage T4 DNA replication apparatus. J. Biol. Chem. **254**:6787–6796.
17. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.
18. **Schrenk, W. J., and R. A. Weisberg.** 1975. A simple method for making new transducing lines of coliphage λ. Mol. Gen. Genet. **137**:101–107.
19. **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.
20. **Spratt, B. G.** 1977. Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin-binding proteins. J. Bacteriol. **131**:293–305.
21. **Spratt, B. G., A. Boyd, and N. Stoker.** 1980. Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the *lip-dacA-rodA-pbpA-leuS* region of the *Escherichia coli* chromosome. J. Bacteriol. **143**:569–581.
22. **Stüber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. U.S.A. **78**:167–171.
23. **Tamaki, S., H. Matsuzawa, and M. Matsuhashi.** 1980. Cluster of *mrdA* and *mrdB* genes responsible for the rod shape and mecillinam sensitivity of *Escherichia coli*. J. Bacteriol. **141**:52–57.
24. **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.
25. **Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson.** 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. J. Biol. Chem. **243**:4543–4555.
26. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–744.