

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

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A series of defective lambda transducing phage carrying genes from the *lip-leuS* region of the *Escherichia coli* chromosome (min 14 on the current linkage map) has been isolated. The phage defined the gene order as *lac---lip-dacA-rodA-pbpA-leuS---gal*. These included the structural genes for penicillin-binding protein 2 (*pbpA*) and penicillin-binding protein 5 (*dacA*) as well as a previously unidentified cell shape gene that we have called *rodA*. *rodA* mutants were spherical and very similar to *pbpA* mutants but were distinguishable from them in that they had no defects in the activity of penicillin-binding protein 2. The separation into two groups of spherical mutants with mutations that mapped close to *lip* was confirmed by complementation analysis. The genes *dacA*, *rodA*, and *pbpA* lie within a 12-kilobase region, and represent a cluster of genes involved in cell shape determination and peptidoglycan synthesis. A restriction map of the *lip-leuS* region was established, and restriction fragments were cloned from defective transducing phage into appropriate lambda vectors to generate plaque-forming phage that carried genes from this region. Analysis of the proteins synthesized from lambda transducing phage in ultraviolet light-irradiated cells of *E. coli* resulted in the identification of the *leuS*, *pbpA*, *dacA*, and *lip* gene products, but the product of the *rodA* gene was not identified. The nine proteins that were synthesized from the *lip-leuS* region accounted for 57% of its coding capacity. Phage derivatives were constructed that allowed about 50-fold amplification of the levels of penicillin-binding proteins 2 and 5 in the cytoplasmic membrane.

The characteristic shape of bacterial cells is maintained by the structural rigidity of the peptidoglycan layer of the cell wall (30). Little is known about the processes that determine cell shape, but presumably the properties of the enzymes that incorporate peptidoglycan precursors into the preexisting cell wall have a major role in determining the shape of the peptidoglycan, and therefore the shape of the bacterial cell. The enzymes that catalyze this final cross-linking reaction in peptidoglycan synthesis have been identified as the peptidoglycan transpeptidases, and these enzymes are the targets that penicillin and other β -lactam antibiotics inhibit to produce their lethal effects on bacteria (2, 7).

Bacteria contain multiple forms of these penicillin-sensitive enzymes in their cytoplasmic membrane, and they can be detected on sodium dodecyl sulfate (SDS)-polyacrylamide gels as

penicillin-binding proteins (PBPs) (32, 34). One of the seven PBPs of *Escherichia coli* has been shown to have a crucial role in cell shape determination since inactivation of the protein (PBP2), either by the atypical β -lactam antibiotic mecillinam or by mutation, results in the growth of the bacteria as large spherical cells (32, 33, 36, 37). PBP2 has a molecular weight of 66,000 and is the least abundant of the *E. coli* PBPs.

Several mutants that have defects in PBP2 have been described (32, 36, 37). Some of these lack detectable activity of PBP2 and grow under all conditions as spherical cells. Other mutants have highly thermolabile forms of PBP2 and grow as rod-shaped cells at 30°C and as spherical cells at 42°C. Genetic studies with these cell shape mutants have established clearly that the spherical shape of the cells is a direct consequence of the defect in PBP2 activity (36, 37).

All mutations that produce defects in PBP2 map close to *lip* at 14 min on the current linkage

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map (1, 36, 37, 41), and this cell shape gene has been called *pbpA* (35). Other mutations that cause spherical growth of *E. coli* map close to *aroE* at 71 min; these have no effect on PBP2 activity, and have been designated *envB* (43) or *rodY* (12).

Recently we isolated a temperature-sensitive cell shape mutant, SP52, which had a cell shape mutation that mapped very close to *lip*, but was distinguishable from *pbpA* mutants since it had normal PBP2 activity. We show here that this mutant defines a previously unidentified cell shape gene, which we call *rodA*, that maps very close to *pbpA*.

A further gene, *dacA*, which is involved in peptidoglycan metabolism, maps close to the *pbpA* and *rodA* genes; *dacA* has been reported to be 87% cotransducible with *leuS* (41), which is 42% cotransducible with *lip* (16). Inactivation of *dacA* results in loss of the activity of the penicillin-sensitive enzyme D-alanine carboxypeptidase 1a (19). This enzyme corresponds to PBP5 and PBP6 (39), and recently it has been shown that *dacA* is the structural gene for PBP5 (molecular weight of 42,000), since an altered form of this enzyme was found in a *dacA* mutant (20).

We show here that *dacA*, *pbpA*, and *rodA* are a cluster of genes involved in peptidoglycan metabolism and cell shape determination which maps between *lip* and *leuS* and describe the isolation of a series of lambda DNA transducing phage which carry these genes. The transducing phage have been used to derive a restriction map, to analyze the products of the genes in the *lip-leuS* region, and to construct strains of *E. coli* that produce greatly elevated levels of PBP2 and PBP5.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. The bacterial strains used in this work are listed in Table 1. P1 *vir* was used for generalized transduction. λ^+ , λ *imm*⁴³⁴, λ cI857 Sam7, λ cI857 Qam73, λ cI857 Qam73 Sam7, λ *imm*²¹ Qam73, and λ *h*⁸² cI⁻ were kindly provided by Bill Brammar. The lambda phage used as cloning vectors had the following genotypes: λ L41, *chiA*131 Δ (*sr* Δ 1-2) *imm*⁴³⁴ cI⁻ *sr* Δ 4° *nin*5 *shn* Δ 6° *sr* Δ 5°; and λ NM627, Δ (*sr* Δ 1-2) cI857 *sr* Δ 4° *nin*5 *sr* Δ 5° Sam7. The phage were kindly provided by Wilhelmine Loenen and Noreen Murray (via Neil Fairweather), respectively. The nomenclature for restriction endonuclease targets is that used in reference 26, where *sr* Δ 1° and *shn* Δ 1° refer, respectively, to the mutational loss of the *Eco*RI and *Hind*III sites 1 in the lambda genome.

Growth media and buffers. Luria broth (15) was used for routine growth of cells; it was supplemented with 0.2% maltose when optimal lambda phage absorption was required. Phage titers were made on

Trypticase agar (BBL Microbiology Systems, Cockeysville, Md.) (28). Phage lysates were prepared on Luria broth solidified with 1% agar (Difco Laboratories, Detroit, Mich.) for plates or 0.65% for top agars.

Penassay broth (Difco antibiotic medium no. 3) and M9 minimal medium (23), supplemented as required with L-amino acids (20 μ g/ml), adenine (10 μ g/ml), thiamine (10 μ g/ml), biotin (10 μ g/ml), and lipoic acid (1 μ g/ml), were used in some experiments.

Selection of cells containing the Tn10 transposon used nutrient agar (Oxoid Ltd., London) containing tetracycline (20 μ g/ml).

TE buffer contained 10 mM Tris-hydrochloride and 1 mM EDTA, pH 7.4; lambda buffer was 6 mM Tris-hydrochloride, 10 mM MgSO₄, and 50 μ g of gelatin per ml, pH 7.4.

Preparation of cell envelopes and assay of PBPs. Cell envelopes were prepared and PBPs were assayed as described previously (34) except that the separating gel used in the fractionation of the PBPs was modified to 10% acrylamide-0.13% methylenebisacrylamide.

Genetic techniques. Transduction with phage P1 *vir* and conjugation were performed by standard methods (23). Cells of AB1325 *lip*-9 that were to be transduced to *lip*⁺ were prestarved by growing at 37°C for 4 h in minimal medium lacking lipoic acid (10).

Genetic manipulation of the cell shape mutants (*rod* mutants) was simplified by constructing a strain that had the tetracycline resistance transposon, Tn10, inserted into the chromosome close to the *rod* gene(s). This was achieved by mating SP299 with SP13711 and selecting for tetracycline resistance (Tet^r), *lac*⁺, and *rod*⁺. Colonies arising from this mating result from the transposition of Tn10 from its position in the chromosome of SP299 onto F'254 (which carries the genes *lac*⁺ *rod*⁺; see Results) and its transfer into SP13711 (Tet^r *lac* *rod*). Some of the exconjugants should carry an F'254 which has Tn10 inserted sufficiently close to the *rod* gene(s) on the F-prime to be cotransducible with it by P1 phage. About 1,000 exconjugants were therefore pooled; P1 *vir* phage were grown on these cells, and the lysate was used to transduce SP45 to Tet^r and *rod*⁺ (i.e., growth at 42°C on Penassay agar containing tetracycline). Fourteen transductants of this type were obtained, and the linkage of Tn10 to *rod* was determined by P1 transduction. One strain in which Tn10 was about 50% cotransducible with the *rod* gene(s) was used for further work. P1 *vir* grown on this strain was used to transduce Tn10 into the same position in the chromosome of each of the *rod* mutants, TMRL12 and AB1325 *lip*-9, and P1 lysates grown on the resulting strains were used to transduce the *rod*, *leuS*, or *lip* mutations into other strains by cotransduction with Tn10 (see reference 13).

Transposon genetics (13) was also used to put the *rod* mutations onto F'254, to allow complementation analysis. For example, strain SP62 (*rod*-6/F'254 *rod*⁺) was transduced to Tet^r with P1 *vir* grown on Tn10 *rod* strains. Integration of the Tn10-*rod* fragment into F'254 results in spherical cells because of diploidy for *rod*.

In practice, when SP62 was transduced to Tet^r with P1 *vir* grown on SP60, 12% of the transductants were

TABLE 1. *E. coli* strains used

Strain	Genotype ^a	Source or reference
KN126	<i>trp</i> (Am) <i>tyr</i> (Am) <i>ilv supD126</i> (Ts)	34
SP6	KN126 <i>pbpA6 ponA6</i>	32, 39
SP45	KN126 <i>pbpA45</i>	36
SP137	KN126 <i>pbpA137 purE</i>	37
SP52	KN126 <i>rodA52</i>	This paper
AB1325 <i>lip-9</i>	<i>thi his purB proA lacY lip-9 rpsL</i>	J. R. Guest (10)
SP600	AB1325 <i>lip</i> ⁺ <i>pbpA6</i>	This paper
SP4500	AB1325 <i>lip</i> ⁺ <i>pbpA45</i>	36
SP13711	AB1325 <i>lip</i> ⁺ <i>pbpA137</i>	This paper
SP5211	AB1325 <i>lip</i> ⁺ <i>rodA52</i>	This paper
CGSC 4282	<i>metE trpE purE proC leu lacZ rpsL recA/F'254</i> (<i>lac</i> ⁺ <i>purE</i> ⁺ <i>lip</i> ⁺)	B. J. Bachmann
SP62	SP6/F'254	38
SP454	SP45/F'254	This paper
SP1376	SP137/F'254	This paper
SP521	SP52/F'254	This paper
SP60	SP6 <i>zbe::Tn10</i> ^b	This paper
NK5304	Hfr; <i>ilv thi thr srl::Tn10 recA56</i>	N. Kleckner (13)
SP522	SP52 <i>srl::Tn10 recA56</i>	SP52 transduced to <i>srl::Tn10 recA56</i> with P1 phage grown on NK5304 (13)
W3110 <i>lip-9</i>	<i>sup</i> ⁰ <i>zbe::Tn10 lip-9</i>	This paper
TMRL12	<i>arg pro purB his thi leuS lacY sup</i> ⁰	M. Matsuhashi (20)
DU3099	<i>his trp lys lac recA56 zie::Tn10</i>	T. Foster (6)
SP299	DU3099/F'254	CGSC 4282 × DU3099
AB2463	<i>leu thr his proA argE thi lacY recA13</i>	B. M. Wilkins
833	Δ(<i>gal-attλ-bio</i>) <i>his trp tonA</i>	W. J. Brammar
159	<i>gal uvrA</i>	G. Plastow
C600	<i>thr leu thi supE tonA</i>	W. J. Brammar
ED3184	<i>his lac tsx supF</i>	W. J. Brammar

^a Genetic symbols are those used in reference 1. For definition of *pbpA* and *rodA*, see text; *ponA*, penicillin-binding protein 1A gene (41).

^b Transposon nomenclature is that used in reference 13; e.g., *zbe::Tn10* denotes the insertion of *Tn10* into an unknown gene in the 14-min region of the chromosome.

spherical. One of these strains was used to transfer F'254::Tn10 *rod-6* into the *recA* strain AB2463 selecting for *lac*⁺ Tet^r, and the F-prime was transferred from there into SP6, SP45, SP52, and SP137 by selection for Tet^r at 30°C. The morphology of the resulting partial diploids was examined at 30 and 42°C, and complementation was indicated by the recovery of the normal rod shape at 42°C.

Isolation of specialized λ transducing phage. A mixed low-frequency transducing lysate was prepared from strain 833 Δ(*gal-uvrB*) lysogenized with λ cI857 Sam7 as described by Schrenk and Weisberg (31) and was used to transduce SP5211(λ⁺), SP13711(λ⁺), and TMRL12(λ⁺) to temperature resistance by using the selective conditions described below.

Transduction of *leuS*⁺ was measured by selecting for the growth of TMRL12(λ⁺) on minimal agar at 42°C (16). Selection for *lip*⁺ was by the growth of lipoleic acid-starved cells of AB1325 *lip-9* on minimal agar at 30°C (10). Testing for the transduction of the cell shape genes *pbpA*⁺ and *rodA*⁺ (see Results) used strains SP13711(λ⁺) and SP5211(λ⁺), respectively. Cells were plated on Penassay agar containing Sarkosyl NL97 (0.1%), incubated at 30°C for 3 h, and then transferred to 42°C. Addition of Sarkosyl eliminated

the background growth of the temperature-sensitive *pbpA* and *rodA* mutants at 42°C since the spherical cells formed under these conditions were supersensitive to the detergent.

Preparation of phage DNA and digestion with restriction endonucleases. High-frequency transducing stocks of defective transducing phage were prepared by induction from double lysogens of the type AB1325 *lip-9* (or SP600) (λ cI857 Sam7) (λ *dIip5* cI857 Sam7), and were separated from helper phage by CsCl density gradient centrifugation (23). The purified phage were dialyzed against TE buffer and were extracted twice with phenol-chloroform-isoamyl alcohol-8-hydroxyquinoline (100:100:4:0.1). The phage DNA was dialyzed against TE buffer, ethanol precipitated, and redissolved in TE buffer at a concentration of 100 to 200 μg of DNA per ml.

Plaque-forming phage were prepared by induction from lysogens or by confluent lysis on plates, using *E. coli* C600 or ED3184 as host (23). The phage were concentrated by centrifugation into a CsCl block gradient, and DNA was prepared as described above.

Digestion of DNA with restriction endonucleases and analysis of DNA fragments on agarose gels were performed as described (22). The buffers used for

restriction enzymes were those recommended by the suppliers (Bethesda Research Laboratories, Rockville, Md.).

Construction of plaque-forming phage in vitro. Plaque-forming phage carrying genes from the *lip-leuS* region were obtained by cloning fragments of the defective transducing phage λ *dli*p5 into λ vectors.

*Hind*III fragments of λ *dli*p5 were cloned into the replacement vector λ L41 (see Fig. 2A) by using T4 DNA ligase (3) generously provided by Ron Wilson and Barry Ely. Portions of the ligation mixture were used to transfect CaCl_2 -treated cells of C600(P2) (18), and the resulting clear-plaque, *spi*⁻, recombinant phage were tested for their ability to transduce λ *imm*⁴³⁴ lysogens of TMRL12 (*leuS*), SP13711 (*pbpA*), SP5211 (*rodA*), and AB1325 *lip-9*.

*Sal*I fragments of λ *dli*p5 were cloned into the vector λ NM627 (see Fig. 2C), and phage carrying genes from the *leuS-lip* region were detected by transduction of λ ⁺ lysogens of TMRL12, SP13711, SP5211, and AB1325 *lip-9*. The resulting dilyso gens were induced, and the lysates were plated on ED3184. Single plaques of recombinant transducing phage were detected by their ability to transduce chromosomal genes and were purified.

Proteins synthesized from specialized transducing phage. The proteins synthesized from the chromosomal genes on λ transducing phage were analyzed by a method modified from Ptashne (29).

Cells of *E. coli* 159 (λ *ind*⁻) were irradiated with UV light (12,000 ergs/mm²) and infected with transducing phage (multiplicity of infection of 5 to 10), and the proteins synthesized after infection were labeled with [³⁵S]methionine, fractionated on 10 to 16% SDS-polyacrylamide slab gels (34), and detected by fluorography (14). Separation of cells into cell envelopes and cytoplasmic and outer membrane protein fractions was done as described previously (34).

Molecular weights of bands on fluorographs of SDS-polyacrylamide slab gels were estimated by comparison with those produced by a ¹⁴C-methylated protein mixture (The Radiochemical Centre, Amersham, England) which provided molecular weight standards of 14,300, 30,000, 46,000, 69,000, 92,500, 100,000, and 200,000.

Construction of a λ *dli*p5 *ci*857 *Qam*73 *Sam*7 lysogen. λ *ci*857 *Qam*73 *Sam*7 was absorbed (multiplicity of infection of 1) to the defective single lysogen AB1325 *lip-9* (λ *dli*p5 *ci*857 *Sam*7), and phage development was induced by a heat shock. The resulting lysate was used to transduce W3110 *lip-9* to *lip*⁺ at 30°C, and the level of PBPs was measured in the transductants 2 h after phage development had been induced by a heat shock. Lysogens that showed the highest levels of overproduction of PBP2 and PBP5 were tested for the presence of a functional λ *Q* gene by spotting 10⁵ λ *imm*²¹ *Qam*73 onto a lawn of the lysogen. Failure to produce plaques at 30°C showed that the lysogens contained λ *dli*p5 *ci*857 *Qam*73 *Sam*7.

Chemicals. [¹⁴C]benzylpenicillin (54–59 mCi/mmol) and [³⁵S]methionine (>600 Ci/mmol) were purchased from The Radiochemical Centre. Benzylpenicillin was kindly provided by Nigel Curtis of Glaxo Laboratories, Greenford, England. Sarkosyl NL97 was

obtained from Geigy Industries, New York. Restriction endonucleases were purchased from Bethesda Research Laboratories; ampholytes were purchased from Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Properties of cell shape mutants. The mutants SP6, SP45, and SP137 all have defects in the activity of PBP2 and have been described previously (32, 36, 37). SP6 grows under all conditions as spherical cells and lacks detectable levels of PBP2 (32). The mutants SP45 and SP137 are temperature-sensitive cell shape mutants which grow at 30°C or below as rod-shaped cells, but at 42°C as spherical cells (36, 37). The temperature sensitivity of cell shape in these latter two mutants has been shown to be due to the production of highly thermolabile forms of PBP2 (36, 37).

The temperature-sensitive cell shape mutant SP52 was isolated from strain KN126 after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (35). SP52 grew as rod-shaped cells at 30°C or below and as spherical cells at 42°C, and was morphologically indistinguishable from the mutants SP45 and SP137. However, it differed from the latter mutants in that SP52 produced a thermostable PBP2 (data not shown). SP52 contains the temperature-sensitive amber suppressor *supD*126, and the temperature sensitivity of cell shape could have been due to an amber mutation in the PBP2 gene, such that PBP2 was synthesized at 30 but not at 42°C. This was not the situation, since the level of PBP2 activity in cells of SP52 that had been grown for three mass doublings at 42°C was the same as that in cells of the parent strain, KN126, grown under the same conditions (data not shown). Furthermore, the cell shape mutation of SP52 could be readily transferred into strain TMRL12 by cotransduction with *leuS*, and the resulting strains showed the same temperature sensitivity of cell shape as SP52, although the TMRL12 strain was free of amber suppressors as judged by its inability to support the growth of λ *ci*857 *Qam*73 or λ *ci*857 *Sam*7 (data not shown).

Genetic mapping and complementation studies on cell shape mutants. In this section, the cell shape mutations of SP6, SP45, SP52, and SP137 will be described as *rod-6*, *rod-45*, *rod-52*, and *rod-137*, respectively.

The *rod-6* mutation, which results in the loss of PBP2 activity, maps close to *lip* (37), and the cell shape of SP6 was corrected by introduction of F⁺254 which carries chromosomal genes between approximately 7 and 15 min on the genetic map (38). F⁺254 was transferred by conjugation from strain CGSC 4282 into the cell shape mu-

tants SP45, SP52, and SP137 to obtain the partial diploids SP454, SP521, and SP1376, respectively. Both SP454 and SP1376 regained thermostable PBP2 and normal rod shape at 42°C, establishing that the *rod* mutations in these strains were recessive, and mapped within the region covered by F'254. The *rod-52* mutation also mapped within this region and was recessive, since the partial diploid SP521 was rod shaped at 42°C.

P1 vir phage was grown on strain SP6, SP45, SP52, and SP137, and the lysates were used to transduce AB1325 *lip-9* to *lip*⁺ at 30°C. The frequency of cotransduction of the cell shape mutations *rod-6*, *rod-45*, *rod-52*, and *rod-137* were 86, 84, 92, and 89%, respectively (50 to 100 transductants tested in each cross).

Since the mutations *rod-6*, *rod-45*, and *rod-137* all mapped very close to *lip*, but differed in their effects on the activity of PBP2, complementation analysis was used to establish whether they were all mutations in the same *rod* gene. The *rod-6*, *rod-45*, and *rod-137* mutations were incorporated on F'254 and were transferred into strains SP6, SP45, SP52, SP137, and KN126 as described in Material and Methods. Complementation was indicated by the growth of the partial diploids as rod-shaped cells at 42°C. Partial diploids containing any combination of the *rod-6*, *rod-45*, and *rod-137* mutations failed to show complementation of cell shape, establishing that these three mutations, which all result in defects of PBP2, were allelic. However, introduction of F'254 carrying the *rod-6*, *rod-45*, or *rod-137* mutations into SP52, or into a *recA56* derivative of SP52 (SP522), resulted in complementation of cell shape, indicating that the *rod-52* mutation, which has no effect on PBP2 activity, was in a separate gene to the other *rod* mutations.

We have proposed (35) that the designation *pbpA* be used for mutations in the structural gene for PBP2, and the mutations of SP6, SP45, and SP137 will henceforth be called *pbpA6*, *pbpA45*, and *pbpA137*, respectively. The designation *rodA* has been used to describe spherical mutants that map close to *lip* (21), and we propose that this designation be now used for the second cell shape gene that maps in this region. The mutation of SP52 is therefore renamed *rodA52*.

Isolation of specialized λ transducing phage carrying the *lip-leuS* region. The cell shape mutants in their original genetic background (strain KN126) were found to be resistant to phage λ , and the cell shape mutations were transferred into the λ -sensitive strain AB1325 *lip-9* by cotransduction with *lip* to yield strains SP600 (*pbpA6*), SP4500 (*pbpA45*),

SP13711 (*pbpA137*), and SP5211 (*rodA52*). Specialized transducing phage that could transduce SP13711(λ ⁺) to *pbpA*⁺ were isolated, and high-frequency lysates were prepared as described in Materials and Methods. Nine transducing phage were obtained by using this selection, a further six were obtained by selecting for transduction of SP5211(λ ⁺) to *rodA*⁺, and an additional five were obtained by selection for transduction of TMRL12(λ ⁺) to *leuS*⁺.

The presence of the genes *leuS*, *pbpA*, *rodA*, and *lip* on these transducing phage was determined by spot tests on the appropriate mutants. No simple transductional test was available for the presence of the *dacA* gene on the phage. The presence of *dacA* was detected by the ability of the phage to program the synthesis of PBP5 on infection of UV-irradiated *E. coli* (see below). Table 2 shows the five classes of phage that could be distinguished, and these define unambiguously the gene order as *lac*---*lip*-*dacA*-*rodA*-*pbpA*-*leuS*---*gal*. (The orientation of *lip* and *leuS* is that established in the forthcoming genetic map; B. J. Bachmann, personal communication.) The *dacA* gene was mapped between *lip* and *pbpA* since phage that carried the complete *lip-rodA-pbpA-leuS* region synthesized PBP5 in infection experiments, whereas phage that only carried the *pbpA-leuS* region did not. Phage that carried *rodA-pbpA-leuS* fell into two classes, either producing (e.g., λ *ddacA51*) or not producing (e.g., λ *drodA1*) PBP5. *dacA* must therefore map between *lip* and *rodA*.

High-frequency lysates of a representative of each of the five classes of transducing phage were diluted and plated on strain ED3184 to give single plaques. None of the phage from single plaques (20 plaques tested for each phage) was found to transduce TMRL12(λ ⁺) to *leuS*⁺, indicating that each of the transducing phage had lost genes essential for plaque formation.

All of the phage carried the *leuS* gene, but they differed in the genes counterclockwise to *leuS* that were present. This suggested that all

TABLE 2. *Classes of λ transducing phage isolated*

Class	Chromosomal genes on phage ^a					No. of class isolated	Example of phage class
	<i>lip</i>	<i>dacA</i>	<i>rodA</i>	<i>pbpA</i>	<i>leuS</i>		
1	+	+	+	+	+	9	λ <i>dlip5</i>
2	—	+	+	+	+	5 ^b	λ <i>ddacA51</i>
3	—	—	+	+	+		λ <i>drodA1</i>
4	—	—	—	+	+	4	λ <i>dpbpA108</i>
5	—	—	—	—	+	2	λ <i>dleuS27</i>

^a Presence of chromosomal genes was determined by transduction of *lip*, *rodA*, *pbpA*, and *leuS* and by programming of the synthesis of the *dacA* gene product.

^b Presence of the *dacA* gene was not determined for all phage.

of the phage were formed by the aberrant excision of λ cI857 Sam7 from the same secondary attachment site clockwise from *leuS*. The phage have been named according to the most distant gene from *leuS* that they are known to carry; e.g., λ *drodA* has the chromosomal genes *leuS*, *pbpA*, and *rodA* but not *dacA* or *lip*.

The presence of the *pbpA* gene on the transducing phage was initially determined by the transduction of SP13711(λ^+) to *pbpA*⁺, and 18 out of 20 of the phage carried this gene. All 18 of these phage also transduced the other *pbpA* mutants (SP600 and SP4500) to *pbpA*⁺, whereas the two phage that failed to transduce SP13711(λ^+) also failed to transduce the other *pbpA* mutants to *pbpA*⁺. This result confirms the assignment of the *pbpA6*, *pbpA45*, and *pbpA137* mutations into a single gene. Similarly, the isolation of the λ *dpbpA* phage that transduced all three of the *pbpA* mutants to *pbpA*⁺, but did not transduce the *rodA* mutant (SP5211) to *rodA*⁺, confirmed that *pbpA* and *rodA* are separate genes.

Restriction mapping of the *lip-leuS* region. DNA prepared from λ cI857 Sam7, λ *dlip5*, λ *ddacA51*, λ *drodA1*, and λ *dpbpA108* was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Sal*I, *Sst*I, *Xba*I, and *Xho*I, and the resulting fragments were fractionated on agarose gels. In each transducing phage, the λ DNA fragments that derive from the right of the attachment site (*att*) were present, but some, or all, of those to the left of *att* were absent, indicating that the chromosomal DNA in the transducing phage had replaced DNA from *att* into the phage left arm (see Fig. 1).

Since all of the transducing phage appeared to have been formed by the aberrant excision of λ cI857 Sam7 from the same secondary attachment site, but differed in the extent of the chromosomal replacement in the phage left arm, it was possible to order the cleavage sites for the restriction endonucleases in the chromosomal DNA of the phage by comparing the fragments produced by the digestion of each phage DNA. The order of cleavage sites was confirmed by digestion with combinations of the restriction enzymes, and the sites established for the enzymes *Bam*HI, *Eco*RI, *Hind*III, *Kpn*I, *Sal*I are shown in Fig. 1. No cleavage sites for the enzymes *Sst*I, *Xba*I, or *Xho*I were found in the *lip-leuS* region.

The distribution of targets for restriction endonucleases in λ *dlip5* indicated that plaque-forming phage carrying genes from the *lip-leuS* region could be obtained by cloning *Sal*I fragments or the 15-kilobase (kb) *Hind*III fragment

of the chromosomal DNA of the phage into appropriate vectors.

Construction of plaque-forming phage carrying genes from the *lip-leuS* region. The 15-kb *Hind*III fragment from the chromosomal DNA of λ *dlip5* was cloned into the replacement vector λ L41 (Fig. 2A) as described in Materials and Methods. The resulting *att*⁻ *int*⁻ *spi*⁻, *cI*⁻ *imm*⁴³⁴ phage, λ pBS10 (Fig. 2B), transduced the *rodA*, *pbpA*, and *leuS* genes but did not transduce the *lip* gene. λ pBS10 also carried the *dacA* gene, since PBP5 (the *dacA* gene product) was synthesized after infection of UV-irradiated *E. coli* with this phage (see below). Digestion of λ pBS10 DNA with *Hind*III established that the middle *Hind*III fragment of λ L41 (Fig. 2A) had been replaced with the 15-kb *Hind*III fragment from λ *dlip5* (Fig. 2B and 3). The 15-kb chromosomal DNA fragment in λ pBS10 contained an asymmetric *Bam*HI site, and digestion of λ pBS10 DNA with this enzyme was used to show that the orientation of the *dacA-leuS* genes in λ pBS10 was the reverse of that in λ *dlip5*.

*Sal*I fragments of λ *dlip5* DNA were cloned into the vector λ NM627 (Fig. 2C). Plaque-forming phage that transduced both the *rodA* and *pbpA* genes were isolated, but we were not able to isolate phage that transduced *lip* or *leuS* with this method. One of the plaque-forming phages that transduced *rodA* and *pbpA* (λ pBS99) was studied further. λ pBS99 did not carry the *dacA* gene since PBP5 was not synthesized after infection of UV-irradiated *E. coli* with the phage (see below). Digestion of λ pBS99 DNA with *Sal*I showed that the region between the *Sal*I targets in λ NM627 had been replaced with the 7.1-kb *Sal*I fragment from the chromosomal DNA of λ *dlip5* (Fig. 2D and 3). In addition, the right arm of λ pBS99 was larger than that in the vector, λ NM267, as the *nin5* deletion in the vector had been lost by in vivo recombination with λ^+ during the isolation of this phage (see Materials and Methods).

The 7.1-kb *Sal*I fragment of chromosomal DNA in λ pBS99 contained an asymmetric *Kpn*I site, and digestion of the phage DNA with a combination of *Kpn*I and *Eco*RI established that the orientation of the *rodA* and *pbpA* genes in λ pBS99 was the same as that in λ pBS10 and the reverse of that in λ *dlip5* (Fig. 2D).

λ pBS99 was *red*⁻ *gam*⁻ owing to the loss of the 0.49-kb region between the *Sal*I targets of the vector phage (42), and since the phage lacked a *chi* site, it grew extremely poorly (40). A phage cross between λ pBS10 and λ pBS99 (Fig. 2B,D) allowed the construction of derivatives of λ pBS99 that had gained the *chiA131* mutation

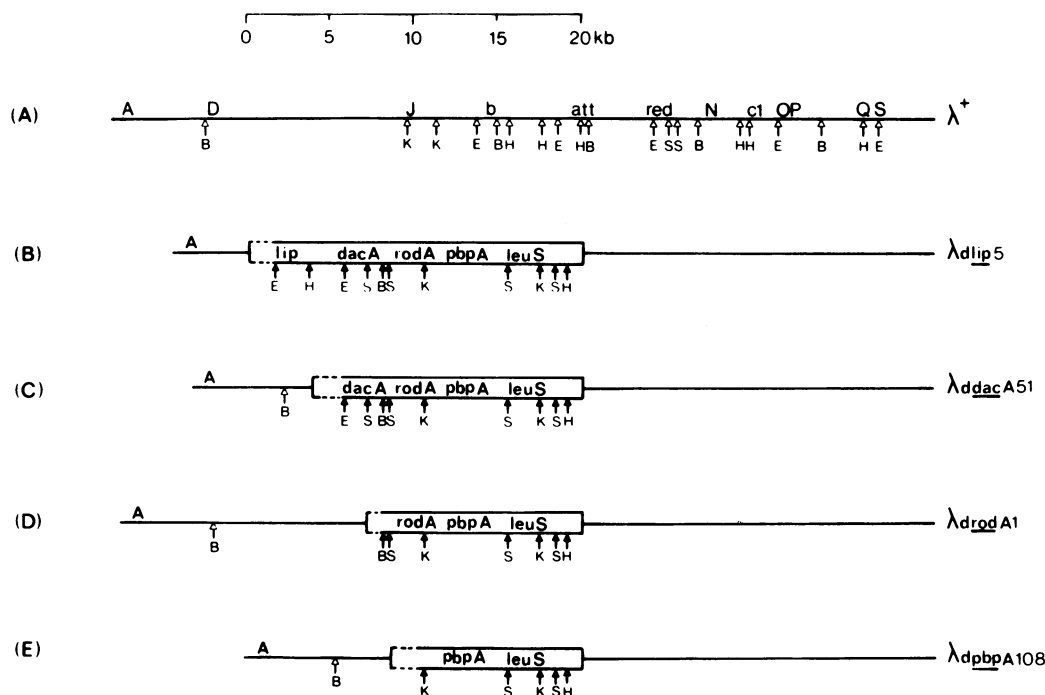


FIG. 1. Structure of defective transducing phage carrying genes from the *lip-leuS* region. (A) λ^+ , (B) λ *dlip5*, (C) λ *ddacA51*, (D) λ *drodA1*, (E) λ *dpbpA108*. (∇) Targets for restriction endonucleases in λ DNA. ($\blacktriangle$) Targets in the chromosomal DNA: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I. Targets in the right arm of the transducing phage are not shown since they are identical to those in λ^+ . The phage right arm and the remaining parts of the left arm are shown by single lines. The chromosomal DNA extending from *att* into the phage left arm is shown within a box. The precise junction points between the left end of the chromosomal DNA and the remainder of the lambda left arm are unknown and are indicated approximately by the broken lines. The positioning of the known genes in relation to the restriction enzyme targets is approximate.

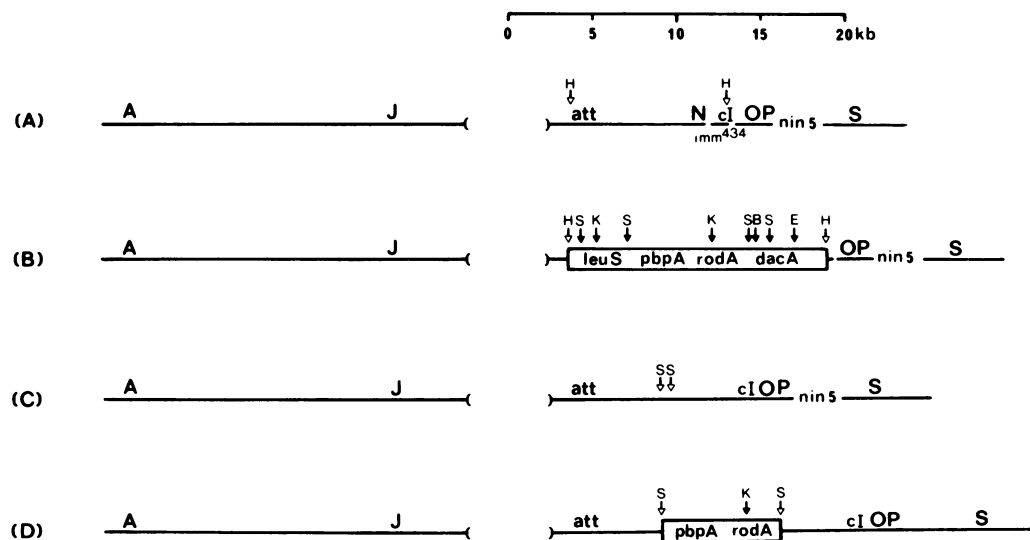


FIG. 2. Structure of plaque-forming phage carrying genes from the *lip-leuS* region. (A) λ L41, (B) λ pBS10, (C) λ NM627, (D) λ pBS99. The targets for restriction endonucleases are represented as in Fig. 1. The targets in the lambda DNA of the transducing phage are not shown. The space between brackets in the left arm of the phage represents the deletion of DNA between *Eco*RI sites 1 and 2 of lambda. Full genotypes of λ L41 and λ NM627 are given in Materials and Methods.

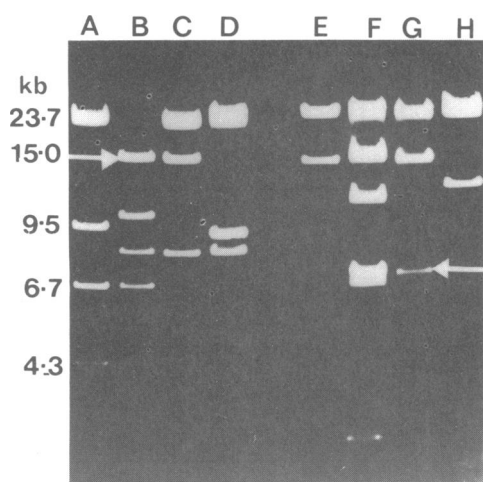


FIG. 3. Electrophoretic analysis of restriction enzyme digests of DNA from plaque-forming λ transducing phage. DNA from λ cI857 Sam7 (A), λ dIip5 (B), λ pBS10 (C), and λ L41 (D) was digested with *Hind*III; DNA from λ cI857 Sam7 (E), λ dIip5 (F), λ pBS99 (G), and λ NM627 (H) was digested with *Sal*I, and the fragments were separated on a 0.5% agarose gel. The arrows indicate the 15-kb *Hind*III and 7.1-kb *Sal*I chromosomal DNA fragments inserted in the plaque-forming phage λ pBS10 and λ pBS99, respectively. DNA fragments below 4 kb are not visible in the photograph. The largest fragment in track (F) is from contaminating λ cI857 Sam7 helper phage present in this preparation of λ dIip5 DNA.

from λ pBS10 (selection for *spi*⁻ *imm* λ cI857 recombinants). These derivatives (e.g., λ pBS104 = *chiA*131 Δ (*sr* Δ 1-2) *pbpA*-*rodA* Δ (*red-gam*) cI857 Sam7) were fully *spi*⁻ and grew well. Since the chromosomal genes in λ pBS10 and λ pBS99 were in the same orientation, recombination between the chromosomal genes resulted in the formation of a new class of plaque-forming phage that transduced *leuS* in addition of *pbpA* and *rodA* (e.g., λ pBS102 = *chiA*131 Δ (*sr* Δ 1-2) *leuS* *pbpA* *rodA* Δ (*att-int-red-gam*) cI857 Sam7).

Proteins synthesized from the *lip-leuS* region. Infection of UV-irradiated *E. coli* 159 (λ *ind*⁻) with λ dIip5 resulted in the synthesis of 11 proteins (Fig. 4). Two of these proteins (molecular weights of 27,000 and 29,000) were also produced after infection with λ cI857 Sam7 and were the products of the phage *cI* and *rex* genes, which are the only phage genes expressed in the presence of lambda repressor (42). The other nine proteins were not synthesized in uninfected control cells (Fig. 4A) or in cells infected with λ cI857 Sam7, and were the products of the bacterial genes in λ dIip5.

Table 3 lists the proteins synthesized from λ dIip5 and from other phage carrying genes from

the *lip-leuS* region of the chromosome. Infection experiments with each of the transducing phage were also carried out with a UV-irradiated nonlysogen of *E. coli* 159 since, in the absence of lambda repressor, chromosomal genes that have very weak promoters, or have lost their promoters on insertion into transducing phage, can be expressed from phage promoters. With all of the transducing phage, the only proteins synthesized in the nonlysogen that were not also synthesized in the lysogen were identified as lambda proteins by comparison with those produced in a parallel infection with λ cI857 Sam7 (data not shown).

Comparison of the genes known to be on each of the transducing phage with the proteins synthesized in infection experiments, together with a knowledge of the molecular weights and properties of some of the gene products, has allowed the identification of some of the proteins synthesized by the transducing phage.

The protein of 105,000 molecular weight (105K protein) was identified as the *leuS* product (leucyl tRNA synthetase) since it was cytoplasmic, had the previously reported molecular weight (8), and was synthesized only in infection

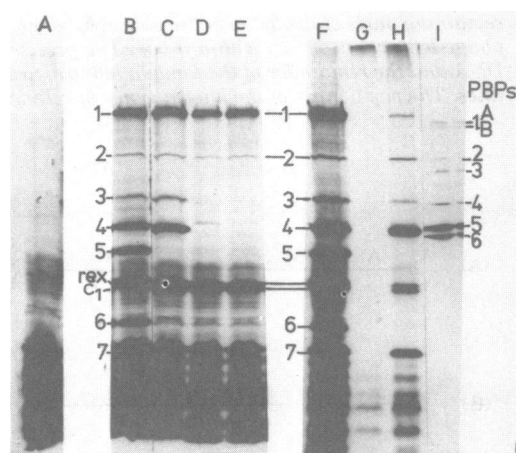


FIG. 4. SDS-polyacrylamide gel electrophoresis of proteins synthesized from λ transducing phage. Cells of *E. coli* 159 (λ *ind*⁻) were UV irradiated, and the proteins synthesized after infection with λ transducing phage were labeled with [³⁵S]methionine. The total cell proteins (A-F) or cell envelope fractions (G, H) were fractionated on a 12% SDS-polyacrylamide gel and fluorographed. (A, G) controls (uninfected); (B, F, H) λ dIip5; (C) λ ddacA51; (D) λ drodA1; (E) λ dIip5A108. (I) PBPs of *E. coli* KN126 labeled with [¹⁴C]benzylpenicillin. The molecular weights of the seven proteins marked in this photograph and of two additional low-molecular-weight proteins, which are only detected on 16% SDS-polyacrylamide gels, are shown in Table 3.

TABLE 3. Proteins synthesized from λ transducing phage carrying genes from the *lip-leuS* region

Protein	Mol wt	Synthesis of protein on infection of UV-irradiated <i>E. coli</i> with:						
		λ^+	λ <i>dlip5</i>	λ <i>ddacA51</i>	λ <i>drodA1</i>	λ <i>dppbA108</i>	λ pBS10	λ pBS99
1	105,000	—	+	+	+	+	+	—
2	66,000	—	+	+	+	+	+	+
3	54,000	—	+	+	—	—	+	—
4	41,000	—	+	+	—	—	+	—
5	35,000	—	+	—	—	—	—	—
<i>rex</i> product	29,000	+	+	+	+	+	— ^a	+
<i>cl</i> product	27,000	+	+	+	+	+	— ^a	+
6	20,000	—	+	—	—	—	+	—
7	15,000	—	+	+	+	+	+	—
8	12,000	—	+	+	+	+	+	+
9	11,000	—	+	—	—	—	+	—

^a *cl* and *rex* genes were deleted in λ pBS10.

experiments with phage that transduced the *leuS* gene. The *pbpA* gene product (PBP2) was identified as the 66K protein, since it was synthesized by all of the phage that transduced *pbpA*, was, as expected, located in the cell envelope (Fig. 4H), and had precisely the same mobility on SDS-polyacrylamide gels as PBP2 detected by the PBP assay using [¹⁴C]benzylpenicillin (Fig. 4I).

The *dacA* gene, which is reported to be 87% cotransducible with *leuS* (41), was found to be in the *lip-leuS* region carried by λ *dlip5*, since induction of this phage from a lysogen resulted in massive overproduction of PBP5, the *dacA* gene product (see below), and because strains that overproduce PBP5 have been obtained by cloning fragments of λ *dlip5* DNA into the multicopy plasmid pBR325 (B. G. Spratt, unpublished experiments).

The 41K protein synthesized from λ *dlip5* had almost the same mobility on SDS-gels as PBP5 detected by the PBP assay and, as expected, was located in the cell envelope (Fig. 4H). The mobility of the 41K protein was, however, consistently slightly faster than that of PBP5. There is, however, little doubt that the 41K protein is identical with PBP5, and the slight difference in mobility on SDS-polyacrylamide gels may indicate that posttranslational modification of PBP5 occurs in vivo, but not in the UV-irradiated cell system.

No information about the *lip* gene product appears to have been published. The *lip* gene was transduced by λ *dlip5* but not by any of the other phage, and the *lip* gene product should therefore be a protein synthesized after infection of UV-irradiated *E. coli* with the former but not with the latter phage. The 35K protein (Table 3) was the only protein synthesized exclusively by λ *dlip5*, and this cytoplasmic protein was tentatively assigned as the *lip* gene product.

Further experiments will be necessary to confirm this assignment.

The *rodA* gene product should be a protein synthesized by λ *drodA1* but not by λ *dppbA108*. However, no differences were found in the proteins synthesized by these phage.

The genes corresponding to the 54K, 20K, 15K, 12K, and 11K proteins have yet to be identified.

Figure 5 shows the approximate alignment of the proteins synthesized from the *lip-leuS* region with the known genes and restriction enzyme targets. The sizes of the genes required to code for the known proteins are indicated, based on the assumption that 1 kb of DNA can code for a 35K protein. The total molecular weight of the proteins synthesized from the *lip-leuS* region in λ *dlip5* was 359,000, which would require a coding capacity of 10.2 kb. The size of the chromosomal DNA in λ *dlip5* is about 18 kb, and the known proteins therefore account for 57% of the coding capacity.

Overproduction of PBP2 and PBP5 during λ *dlip5* development. High levels of expression of genes cloned in λ transducing

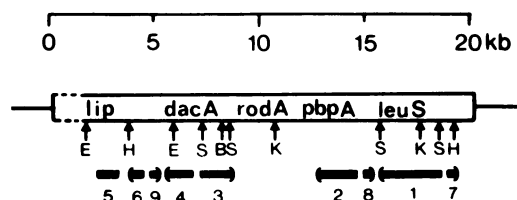


FIG. 5. Alignment of the genes, gene products, and restriction enzyme targets in the *lip-leuS* region. The proteins synthesized from the *lip-leuS* region of the chromosome are numbered according to Table 3 and Fig. 4. The boxes above the numbers represent the estimated size of the genes required to code for each protein. The order of pairs of proteins between brackets is undetermined.

phage have been achieved by allowing phage DNA replication to occur but preventing the packaging of the DNA into phage particles by an amber mutation in the λ *Q* gene (25).

Figure 6A shows the level of the PBPs in W3110 *lip-9* (λ *dlip5* *cl857* *Qam73* *Sam7*) grown at 30°C and in the same strain 2 h after induction of the phage from the lysogen (Fig. 6B). The overproduction of PBP2 and PBP5 was difficult to estimate accurately since their levels were so different in induced and uninduced cells, but the level was about 50-fold for both PBPs. (Exposure of the gel to X-ray film for a short period showed that the large amount of radioactivity at

the position of PBP5/6 was due to overproduction of PBP5 rather than PBP6.) PBP4' was prominent in induced cell envelopes; this PBP is a (proteolytic?) product of PBP2 (33). The level of some of the other PBPs decreased after phage induction (e.g., PBP1B and PBP4), and this probably reflects a decreased rate of transcription of their genes during λ development.

DISCUSSION

The mutations *pbpA6*, *pbpA45*, *pbpA137*, and *rodA52* all mapped very close to *lip* and resulted in the growth of the bacteria as large spherical cells under the restrictive conditions. Complementation analysis established that the *pbpA6*, *pbpA45*, and *pbpA137* mutations were in the same cell shape gene. This is consistent with the finding that all of these mutations caused alterations of PBP2. Since the latter two mutations resulted in a decreased thermostability of PBP2 (36, 37), the *pbpA* gene is the structural gene for this protein. The mutation *rodA52* had no detectable effect on the activity or synthesis of PBP2, and complementation analysis established that it defined a second cell shape gene close to *lip* and *pbpA*. The allocation of cell shape mutants that mapped near *lip* into two classes was supported by the isolation of specialized λ transducing phage that transduced all of the *pbpA* mutants, but not the *rodA52* mutant, to normal cell shape. S. Tamaki, H. Matsuzawa, and M. Matsushashi (personal communication) have shown independently, by complementation analysis, the existence of two cell shape genes close to *lip*.

Several cell shape mutations that map close to *lip* have been reported previously (9, 11, 12, 21, 32). The temperature-sensitive mutant (*lss-21*) of Henning et al. (9) appears to be a *pbpA* mutant, since it produces a highly thermolabile PBP2 (37). The mutant of Matsuzawa et al. (21) appears to be a *rodA* mutant since it has a normal PBP2 and complements *pbpA* mutants (Tamaki et al., personal communication). Other cell shape mutations that map close to *lip* could be classified as *pbpA* or *rodA* on the basis of the activity of PBP2 or, preferably, by complementation analysis or transductional tests with λ *drodA* and λ *dpbpA* phages. It is, of course, possible that further cell shape genes map close to *lip*.

The determination of the gene order as *lac---lip-dacA-rodA-pbpA-leuS---gal* raises the possibility that *dacA*, *rodA*, and *pbpA*, which are presumably all involved in the final stages of peptidoglycan synthesis, are contiguous genes. Certainly the three genes are within a 12-kb region, but further detailed physical studies are needed to settle this point. None of the other

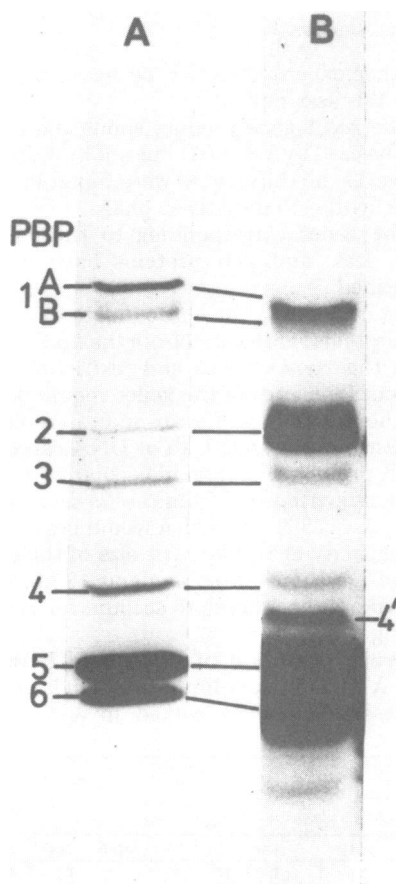


FIG. 6. Overproduction of PBP2 and PBP5 during λ *dlip5* development. *E. coli* W3110 *lip-9* (λ *dlip5* *cl857* *Qam73* *Sam7*) was grown at 30°C in Luria broth containing glucose (0.5%) to an absorbancy at 550 nm of 0.2; the culture was shifted to 42°C for 10 min, to induce phage development, and was grown for a further 2 h at 37°C. Cell envelopes were prepared, and the levels of PBPs were determined in cells grown at 30°C (A) or after phage induction (B). PBP4' is believed to be proteolytic product of PBP2 (33).

PBP genes maps in, or close to, the *lip-leuS* region (41). The gene for PBP6 has not yet been located, but it is not in the *lip-leuS* region, since PBP6 was not synthesized by λ *dli5* phage. Furthermore, a 6.2-kb *EcoRI* fragment containing the PBP6 gene has been cloned into the plasmid pSF2124, and the restriction map around the PBP6 gene showed no similarities with that of the *lip-leuS* region (B. G. Spratt and R. Diaz, unpublished data).

The close linkage of the *dacA*, *rodA*, and *pbpA* genes is intriguing, and represents a cluster of genes involved in peptidoglycan synthesis and cell shape determination. Several of the unascribed proteins that were synthesized from the region between *lip* and *leuS* were located in the cytoplasmic membrane (unpublished experiments), and some of these may be the products of additional cell shape-peptidoglycan synthesis genes in the *dacA-rodA-pbpA* cluster. Although none of the other PBP genes has been shown to be clustered, those for the early steps in peptidoglycan synthesis are clustered in two groups at 2 and 89 min on the genetic map (24, 44). The group at 2 min appears to be a cell division-peptidoglycan synthesis cluster, in many ways analogous to the cell shape-peptidoglycan synthesis cluster described in this paper (5, 44). It includes the gene for PBP3 (a cell division protein; 35), at least two further cell division genes, the genes *sulB* and *envA*, and genes for some of the soluble steps in peptidoglycan synthesis (5, 17, 44).

The products of the *leuS*, *pbpA*, and *dacA* genes, and the probable *lip* gene product, were identified among the nine proteins that were synthesized from the chromosomal genes in λ *dli5*. The *rodA* gene product has not yet been identified. The 7.1-kb *SalI* fragment, which contained the *rodA* and *pbpA* genes, programmed the synthesis of only two proteins in the UV-irradiated cell system. One of these was the *pbpA* gene product (PBP2) and the other, the 12K protein, was unlikely to be the *rodA* gene product, since it was synthesized by λ *dpbpA108*, which did not transduce *rodA*. The proteins synthesized from the 7.1-kb *SalI* fragment accounted for only 32% of its coding capacity and there may be several proteins, including the *rodA* gene product, which for some reason are not expressed in the UV-irradiated cell system. Alternatively, the *rodA* gene product may lack methionine, or the gene may be part of a transcriptional unit that is expressed from a promoter some distance to the left of the *rodA* gene. In this case, the gene product could be the 54K protein or one of the proteins that appeared to be synthesized from a region to the left of *rodA*. These possibilities are being investigated.

The *dacA* gene product has been identified as PBP5, which is known to catalyze a D-alanine carboxypeptidase 1 reaction in vitro; its function in peptidoglycan synthesis in vivo is obscure, since mutants that lack the activity of the enzyme grow without obvious growth defects (19, 20). This appears to suggest that PBP5 has a minor role in peptidoglycan synthesis, but the possibility that these mutants have significant levels of enzyme activity in vivo cannot be eliminated. The availability of *E. coli* strains with Tn10 inserted close to *dacA* should allow (13) the isolation of *E. coli* strains with complete deletions of the *dacA* gene if the gene product is nonessential for peptidoglycan synthesis. Derivatives of this kind are being sought.

The enzymatic, or regulatory, function of PBP2 is unknown. The number of molecules of this important protein per bacterial cell has been estimated to be about 10 to 20 (34), and its purification from the cytoplasmic membrane in substantial amounts is difficult. The considerable level of amplification of PBP2 that has been achieved after induction of λ *dli5* cI857 Qam73 Sam7 from a lysogen will be extremely useful for purification purposes. This phage also greatly overproduce PBP5, but derivatives of λ pBS99, which overproduce PBP2, but lack the PBP5 (*dacA*) gene, have been constructed. The expression of PBP2 from these phage will be described elsewhere.

The purification of PBPs from the cytoplasmic membrane, where presumably they are intimately associated with the other enzymes catalyzing the membrane-bound steps of peptidoglycan synthesis, is likely to have drastic effects on their activity. Overproduction of PBP2 in mutants that lack the activity of the major *E. coli* penicillin-sensitive enzymes should allow the detection of any mecillinam-sensitive activities catalyzed by PBP2 without the need for purification from its normal membrane environment.

Hybrid ColE1 plasmids that complement *pbpA* were not found among the Carbon-Clarke collection (4), although plasmids that carried the *lip* gene were obtained (27). This is almost certainly because of the instability of *E. coli* strains carrying multiple copies of the genes in the *lip-leuS* region, since overproduction of the products of these genes results in severe distortions of bacterial cell shape (unpublished data).

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