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Organization of the *murE-murG* Region of *Escherichia coli*: Identification of the *murD* Gene Encoding the D-Glutamic-Acid-Adding Enzyme

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The 2-min region of the *Escherichia coli* genome contains a large cluster of genes from *pbpB* to *envA* that code for proteins involved in peptidoglycan biosynthesis and cell division. From pLC26-6 of the collection of Clarke and Carbon (L. Clarke and J. Carbon, Cell 9:91–99, 1976) plasmids carrying different fragments from the 8-kilobase-pair region downstream of *pbpB* were constructed and analyzed for their ability to direct protein synthesis in maxicells, to complement various thermosensitive mutations, and to overproduce enzymatic activities. We report the localization of the previously unidentified *murD* gene coding for the D-glutamic acid-adding enzyme within this region. Our data show that the genes are in the order *pbpB-murE-murF-X-murD-Y-murG*, where *X* and *Y* represent chromosomal fragments from 1 to 1.5 kilobase pairs, possibly coding for unknown proteins. Furthermore, the *murE* and *murF* genes, encoding the *meso*-diaminopimelic acid and D-alanyl-D-alanine-adding enzymes, respectively, may be translationally coupled when transcription is initiated upstream of *murE*, within the preceding structural gene *pbpB* coding for penicillin-binding protein 3.

The biosynthesis of bacterial cell wall peptidoglycan is a complex process involving many different cytoplasmic and membrane steps (27). The main cytoplasmic precursors are a series of uridine nucleotides which have been characterized in *Escherichia coli* (27) as uridine diphosphate–*N*-acetylglucosamine (UDP-GlcNAc), UDP-GlcNAc-enolpyruvate, UDP–*N*-acetylmuramic acid (UDP-MurNAc), and its four peptide derivatives from UDP-MurNAc–L-Ala to UDP-MurNAc–L-Ala–γ–D-Glu–*meso*-DAP–D-Ala–D-Ala, where *meso*-DAP is *meso*-diaminopimelic acid. Their sequential formation from UDP-GlcNAc, phosphoenolpyruvate NADPH, L-alanine, D-glutamic acid, *meso*-DAP, and D-alanyl–D-alanine is catalyzed by a set of eight highly specific enzymes which have been studied in detail previously (8, 15–17, 27).

Conditional lethal mutants of *E. coli* with certain of these enzymes altered have been described and most of the mutations have been mapped (6, 27, 28, 34). In particular, at 2 min on the *E. coli* chromosome (1), a large cluster of genes, involved in different aspects of cell envelope growth and division, was shown to contain the genes *murE*, *murF*, *murC*, and *ddl* coding for *meso*-DAP-, D-alanyl–D-alanine-, and L-alanine-adding enzymes and D-alanine–D-alanine ligase, respectively (6, 12, 22, 34) (Table 1). These genes for the synthesis of soluble nucleotide precursors were also shown to be flanked on the left by a cell division gene named *pbpB* (1, 30) or *ftsI* (21) that codes for penicillin-binding protein 3 which is involved in septum formation (9), and on the right, by a group of three contiguous genes *ftsQ*, *ftsA*, *ftsZ* (2), which are involved in the late steps of the cell division process but whose functions have not yet been identified.

Takeda et al. (32) have earlier shown by genetic complementation that pLC26-6 from the collection of Clarke and Carbon (4) contained the genes *pbpB*, *murE*, and *murF*. From the data concerning the precise localization and DNA sequencing of the *pbpB* gene (21) and from the partial restriction map of this plasmid (32), it was calculated that approximately 8 kilobases (kb) of *E. coli* chromosomal insert were located downstream of the *pbpB* gene on pLC26-6. Since a *murC* mutation was not complemented by pLC26-6 (unpublished data) and since the only two identified genes that to date have been located clockwise from *pbpB* were *murE* and *murF* (6, 14, 38), some space remains available in this intermediate region between *murF* and *murC* for other genes possibly also involved in cell envelope growth or division. The gene coding for the D-glutamic acid-adding enzyme was designated as *murD*, and it was speculated that it may belong to the same gene cluster (34). To date however, no evidence has been provided for its localization in this chromosomal region or elsewhere. Analysis of the proteins produced under the direction of a λ bacteriophage carrying genes *envA*, *ftsZ*, *ftsA*, *ddl*, and *murC* revealed the presence between *murF* and *murC* of an unidentified gene coding for a 48,000-molecular-weight protein, and it was proposed that it may be the *murD* gene product (12). In another work (28), a gene named *murG* was located just upstream of *murC* and its undetermined function seemed to be related with peptidoglycan synthesis. Furthermore, it was speculated (31) that the 48,000-molecular-weight protein detected by Lutkenhaus and Wu (12) may be the product of *murG*.

It was therefore essential to determine whether *murD* belongs to this chromosomal region, and if so, to ascertain its relationship with the two genes (12, 28) described as located between *murF* and *murC*. For these reasons, we have decided to investigate in more detail the region between the *pbpB* and *murC* genes preceding the *ddl* and *ftsQAZ* genes, which are otherwise well documented (2, 3). The aim of this study was to conclude investigating this incompletely

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TABLE 1. Correlation between the genetic and enzymatic designations

Gene	Activity of the gene product	Plasmids carrying the gene
murC	L-Alanine-adding enzyme	
murD	D-Glutamic acid-adding enzyme	pDML7 to pDML10, pDML13, pDML14, and pDML21
murE	*meso*-DAP-adding enzyme	pKP1, pKP2, pKE1, pKE2, pDML4, and pDML7 to pDML12
murF	D-Alanyl-D-alanine-adding enzyme	pCP10 and pDML7 to pDML12
murG	Unknown	pDML13 to pDML20, pDML7, and pDML8
ddl	D-Alanine–D-alanine ligase	

elucidated region by determining its complete physical map and by analyzing its coding regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used in this study are shown in Table 2. Plasmid pLC26-6 from the collection of Clarke and Carbon (4) was a gift of B. Bachmann, and plasmid vectors pUC18 and pUC19 were obtained from Pharmacia (Uppsala, Sweden). LB medium (19) was usually used for growing cells, with the addition of ampicillin (100 μg/ml) for strains carrying plasmids encoding the drug resistance gene, and growth was monitored at 600 nm with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Broth for plates was solidified with 1.5% agar. When screening plasmid inserts for the absence of α-complementation, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were added at final concentrations of 40 μg/ml and 40 μM, respectively.

Recombinant DNA techniques and transformation of *E. coli* cells. Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (13) and eventually further purified in cesium chloride-ethidium bromide gradients. Standard procedures were used for endonuclease digestions, ligation, filling in of 5′ protruding ends with the Klenow fragment of DNA polymerase I, and agarose gel electrophoresis (13). *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (5).

TABLE 2. *E. coli* strains used in this study

Strain	Genotype	Source or reference
JM83	*ara* Δ(*lac-proAB*)*rpsL thi strA* φ80 d*lac ZΔM15*	39
JM109	*recA1* Δ(*lac-proAB*)*endA1 gyrA96 thiA hsdR17 supE44 relA1*(F′ *traD36 proAB lacI*^{q} *ZΔM15*)	39
PC2453	*purE murC phx lam*(*lam*) *rpsL*	Phabagen collection[a]
PC2336	*car thi pyrF codA nadC murE lacY tonA phx rpsL recA vtr sup*	Phabagen collection[a]
PC1242	*thr leu thi pyrF codA thyA argG ilvA his murF lacY xyl tonA tsx phx supE ths dra sus uvrB vtr*	Phabagen collection[a]
GS58	F$^-$ *ilv his thyA deo ara*(Am) *lac-125*(Am) *gal U42*(Am) *galE trp*(Am) *tsx*(Am) *tyrT murG*(Am) *supF-A81*(Ts)	28

[a] Phabagen collection, Department of Molecular Cell Biology, State University of Utrecht, The Netherlands.

Preparation of crude enzyme. Plasmid-containing strains were grown with vigorous aeration at 37°C in LB-ampicillin broth (50 ml) to an optical density of 0.7 (250 mg of bacteria [dry weight] per liter of culture). Cells rapidly chilled in ice to 0 to 4°C were harvested in the cold, washed with 0.02 M potassium phosphate buffer (pH 7.0) containing 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, and were centrifuged again. Final suspensions in 2.5 ml of the same buffer were sonicated in the cold until complete disruption of the cells. After centrifugation at 200,000 × *g* for 15 min in a centrifuge (TL-100; Beckman Instruments, Inc., Fullerton, Calif.), the resulting supernatants were directly used for the enzymatic assays. Protein content was determined by the method of Lowry et al. (11), with bovine serum albumin as the standard.

Chemicals. UDP-MurNAc-peptides, D-Ala–D-Ala, and D-Ala–D-[^{14}C]Ala were obtained as previously described (7, 15, 16, 18). DL-[^{14}C]Glu (2 TBq/mol), *meso*-[^{14}C]DAP (13 TBq/mol), and L-[^{35}S]methionine (44 TBq/mmol) were purchased from CEA (Saclay, France), and D-[^{14}C]Ala (0.75 TBq/mol) was from Amersham International (Amersham, England). Restriction enzymes and T4 DNA ligase were obtained from Pharmacia (Uppsala, Sweden) or from New England BioLabs, Inc., (Beverly, Mass.). Ampicillin and IPTG were purchased from Serva (Heidelberg, Federal Republic of Germany), D-cycloserine was from Sigma Chemical Co. (St. Louis, Mo.), and X-Gal was from Promega Biotec (Madison, Wis.).

Enzymatic assays. The D-glutamic acid-adding enzyme was assayed by monitoring the appearance of UDP-MurNAc–L-Ala–D-[^{14}C]Glu in a mixture containing 0.1 M Tris hydrochloride buffer (pH 8.6), 20 mM $MgCl_2$, 5 mM ATP, DL-[^{14}C]glutamic acid (200,000 cpm), 0.05 mM D-glutamic acid, 0.1 mM UDP-MurNAc–L-Ala and enzyme (30 μg of protein) in a final volume of 75 μl.

The *meso*-DAP-adding enzyme was assayed by monitoring the appearance of UDP-MurNAc–L-Ala–γ-D-Glu–*meso*-[^{14}C]DAP in a mixture containing 0.1 M Tris hydrochloride buffer (pH 8.6), 100 mM $MgCl_2$, 5 mM ATP, *meso*-[^{14}C]DAP (0.1 mM; 100,000 cpm), 0.2 mM UDP-MurNAc–L-Ala–D-Glu, and enzyme (35 μg of protein) in a final volume of 75 μl.

The D-Ala–D-Ala-adding enzyme was assayed by monitoring the appearance of UDP-MurNAc–L-Ala–γ-D-Glu–*meso*-DAP–D-Ala–D-[^{14}C]Ala in a mixture containing 0.1 M Tris hydrochloride buffer (pH 8.6), 100 mM $MgCl_2$, 5 mM ATP, D-Ala–D-[^{14}C]Ala (0.1 mM; 100,000 cpm), 0.2 mM UDP-MurNAc–L-Ala–γ-D-Glu–*meso*-DAP, and enzyme (25 μg of protein) in a final volume of 75 μl.

The amounts of protein indicated for the assays correspond to wild-type levels of these enzymatic activities, which were appropriately decreased for overproducing strains. In all cases, mixtures were incubated at 37°C for 30 min and reactions were terminated by the addition of 20 μl of acetic acid. Reaction products were separated by high-voltage electrophoresis on filter paper (3469; Schleicher & Schuell, Inc., Keene, N.J.) in 2% formic acid (pH 1.9) for 50 min at 40 V/cm, and the radioactive spots (substrate and product) were cut out and counted in an Intertechnique SL30

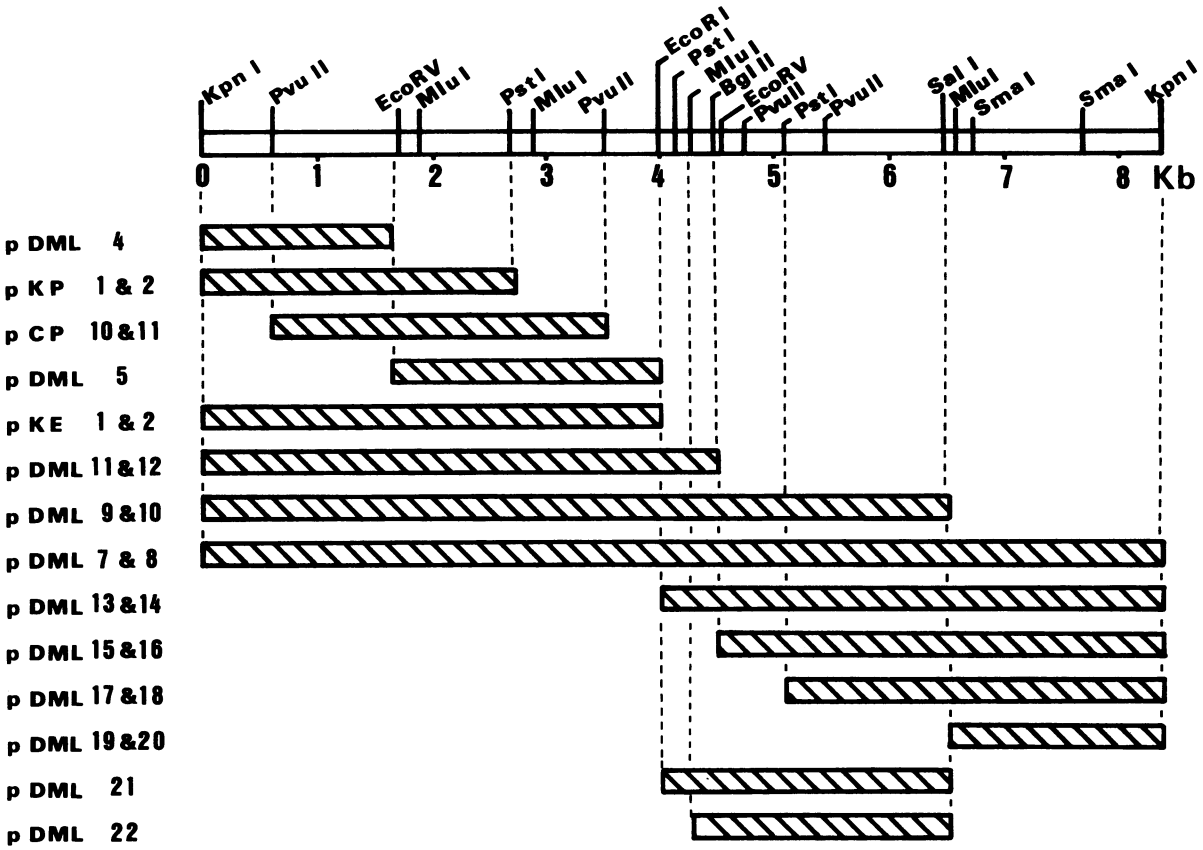

FIG. 1. Restriction map of the *pbpB-murG* chromosomal region of *E. coli* indicating the fragments cloned into plasmids.

liquid scintillation spectrometer with a solvent system consisting of 2 ml of water and 14 ml of Aqualyte mixture (J. T. Baker, Deventer, Holland). One unit of each enzyme activity was defined as the amount which catalyzed the addition of 1 μmol of amino acid or dipeptide per min to the appropriate nucleotide precursor.

Labeling of proteins directed by plasmids. The maxicell method described by Sancar et al. (29) was used in labeling the proteins encoded by plasmids. In all cases, *E. coli* JM109 was used as the host strain for the plasmid to be tested. Routinely, a 10-ml volume of a log-phase culture (A_{600} = 0.5 to 0.6) grown at 37°C in M9 minimal medium (19) supplemented with 0.4% glucose, 1% Casamino Acids (Difco Laboratories, Detroit, Mich.), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and ampicillin (100 μg/ml) was irradiated with UV light and further incubated for 3 h. Since the host strain used does not present the Phr^- (nonphotoreactivable) phenotype (29), all the following steps were conducted in the dark. After the addition of D-cycloserine (100 μg/ml), the culture was incubated overnight at 37°C. Cells from 1.5 ml of each culture were washed with M9 medium, suspended in 0.8 ml of M9 supplemented with 0.4% glucose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 100 μg/ml each of threonine, arginine, proline, and leucine, and incubated for 1 h at 37°C. IPTG (2 mM [final concentration]) and, 10 min later, L-[^{35}S]methionine (44 TBq/mmol; 1.1 MBq/ml) were added to the sulfate-starved culture, and the incubation was continued for 1 h. The labeled cells were harvested, washed with M9 minimal medium, and finally suspended in 50 μl of the sample buffer, followed by heating for 2 min at 100°C. L-[^{35}S]methionine incorporation into proteins was estimated in each case by measuring the radioactivity retained on filters (GF/C; Whatman, Inc., Clifton, N.J.) after precipitation of samples with cold 5% trichloroacetic acid. Finally, the total cell proteins (30,000 to 100,000 cpm) were fractionated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and the labeled proteins were located by fluorography.

Complementation of mutations. The different *E. coli* thermosensitive mutants were made competent as described previously (5) and transformed by the various plasmids to be tested. The cell suspension (200 μl), mixed with DNA, was kept on ice for 2 h before heating for 3 min at 42°C. Then 400 μl of LB medium was added and cells were incubated at 30°C for over 1 h for expression of plasmid-coded genes. Cells were recovered by centrifugation, and a final suspension made in 200 μl of LB medium was separated into two parts which were plated onto two prewarmed LB-ampicillin plates, one incubated at 30°C and the other at 42°C. Growth was observed after 24 or 48 h of incubation.

RESULTS

Construction of plasmids. Plasmid pLC26-6 from the collection of Clarke and Carbon (4) was used as the starting material for the construction of the different plasmids shown in Fig. 1. First, the 8-kb *Kpn*I-*Kpn*I fragment corresponding to the right end of the chromosomal insert present on this synthetic ColE1 hybrid plasmid (32) was isolated and introduced into the *Kpn*I site of the pUC18 vector. Surprisingly, all the JM83 transformants chosen (about 20) as white-appearing colonies on LB plates with X-Gal were shown to contain the plasmid (pDML8) with the insert in the same

orientation, opposite to that of the *lac* promoter (*lacZp*) control. It was thus speculated that overproduction of some activity present on this insert, through transcription from the *lac* promoter, was not tolerated by the cells and that insertion in the opposite direction was favored by differences in the growth capacity of the two possible types of transformants. This was further verified by forcing the insertion of the *Kpn*I-*Kpn*I fragment (isolated from pDML8 under a *Bam*HI-*Sac*I form) into both plasmid vectors pUC18 and pUC19 cut by the corresponding restriction enzymes. By this procedure, transformants harboring plasmid pDML7, with the *Kpn*I-*Kpn*I insert in the opposite orientation, were effectively isolated but appeared on LB plates as small colonies that were remarkably rough and translucent compared with the smooth and opaque pDML8-containing *E. coli* JM83 clones.

The detailed restriction maps of the *Kpn*I-*Kpn*I region and the different segments, which were subsequently cloned into plasmids, are shown in Fig. 1. Plasmids pKP1 and pKP2, pCP10 and pCP11, pKE1 and pKE2, and pDML9 to pDML12 were constructed by subcloning the 2.7-kb *Kpn*I-*Pst*I, 3-kb *Pvu*II-*Pvu*II, 3.9-kb *Kpn*I-*Eco*RI, 4.5-kb *Kpn*I-*Bgl*II, and 6.5-kb *Kpn*I-*Sal*I fragments, respectively, into the same (or compatible) sites of the polylinker of both pUC18 and pUC19 vectors. pDML4 and pDML5 were obtained from pKE1 by internal deletion. pDML13 and pDML14 were obtained by insertion into pUC18, in either orientation, of the 4.5-kb *Eco*RI-*Eco*RI fragment from pDML7. Plasmids pDML15 to pDML20 were obtained from pDML7 or pDML8 by the different internal deletions shown in Fig. 1. Plasmid pDML21 is an internal *Sal*I deletion from pDML13, and pDML22 was obtained from pDML9 by an internal *Mlu*I-*Eco*RI deletion, fully filling in the resulting ends, and ligation. In most cases, insertion of chromosomal fragments within plasmids was performed in both directions and the orientation of inserts compared with that of the *lac* promoter control is indicated in Table 3.

Alteration of colony morphology for plasmid-containing clones. As mentioned above, the in vivo expression under the *lac* promoter of the *Kpn*I-*Kpn*I fragment resulted in an unusual rough and translucent aspect of the colonies on LB-ampicillin plates compared with clones with the same fragment inserted in the opposite orientation. In liquid medium, when cultures of JM83(pDML7) were inoculated from stationary-phase overnight cultures, they were characterized by a 2- to 3-h lag in the onset of growth relative to that for JM83(pDML8). This explained the initially observed preferential isolation of clones with pDML8, instead of pDML7. However, after growth had begun, the generation time of both strains was quite similar, 45 min at 37°C in LB medium. Furthermore, it should be noted that by observation with an optical microscope, log-phase JM83(pDML7) cells appeared as small filaments. When considering the series of constructed plasmids shown in Fig. 1, only pKE1, pDML9, and pDML11 caused the same effects on cell morphology as those which were initially detected with pDML7. This result indicated that the overproduced activity responsible for the phenomena observed was coded on the 3.9-kb *Kpn*I-*Eco*RI chromosomal fragment. Moreover, high-level expression of the corresponding gene, presumably obtained only under the control of *lac* promoter, was required, since such effects were no longer detectable when strain JM83 harbored pKE2, pDML10, or pDML12, whose corresponding inserts are in the opposite direction. Finally, it should be noted that the unusual aspect of colonies on LB plates was observed at 37 or 42°C, but not at 30°C.

Complementation of mutations and overproduction of enzymatic activities coded by plasmid clones. Plasmids shown in Fig. 1 were analyzed for their capability to complement mutations affecting the *murE*, *murF*, *murG*, and *murC* genes, respectively, as well as to direct overproduction of the D-glutamic acid-, *meso*-DAP-, and D-alanyl–D-alanine-adding enzymes. The results concerning both types of experiments are reported in Table 3.

Since the small pDML4 plasmid complemented the *murE* mutation, the *murE* gene was located unambiguously on the 1.7-kb *Kpn*I-*Eco*RV chromosomal fragment, a finding in agreement with the recently published data of Maruyama et al. (14). This result was further supported by the 20-fold overproduction of *meso*-DAP-adding enzyme measured in pDML4-containing strains (Table 3). It should be remembered that the initial *Kpn*I site lies within the *pbpB* gene at approximately 280 base pairs (bp) from its end (21). Furthermore, an open reading frame starting 60 bp after the end of *pbpB* and possibly corresponding to the *murE* gene was deduced from the sequence published by Nakamura et al. (21), and our DNA sequencing of the *murE* region shows that this gene ends 65 bp upstream of the *Eco*RV site (data not shown) (see Fig. 3).

A *murF* mutation could be complemented by pKE1 but not by pDML5 carrying the smaller *Eco*RV-*Eco*RI insert under *lacZp* control (Table 3), indicating that *murF* is initiated upstream of the *Eco*RV site. Our DNA sequencing data (23) and the N-terminal amino acid sequence of the corresponding adding enzyme (K. Duncan, personal communication) show that the *murF* gene starts 60 bp upstream of the *Eco*RV site and is separated from the *murE* termination codon by only 4 bp. Furthermore, the complementation of *murF* mutants by pCP10, but not by pKP1, localized the end of this gene within the 0.8-kb *Pst*I-*Pvu*II intermediate fragment. In all cases, results from complementation tests agree completely with the enzymatic assays, which showed that the D-alanyl-D-alanine-adding enzyme was efficiently accumulated (up to 14-fold) in the cells harboring those plasmids containing the complete *murF* gene under the control of the *lac* promoter (Table 3).

No mutation in the *murD* gene coding for the D-glutamic acid-adding enzyme has been described in the literature until now. For this reason, the localization of this gene within the 8-kb *Kpn*I-*Kpn*I chromosomal insert was studied only by looking for an eventual overproduction of the D-glutamic acid-adding activity in the different plasmid-containing strains. By this method, a 20-fold accumulation of this enzyme was effectively observed in pDML9- or pDML13-harboring strains (Table 3), therefore indicating that the *murD* gene was located within the 2.5-kb *Eco*RI-*Sal*I fragment. This result was further confirmed by construction of pDML21, which contains only this latter insert (Table 3). In addition, the fact that pDML22 did not generate any accumulation of *murD* gene product in the cell restricted the initiation codon of this gene within the small 0.3-kb region separating the *Eco*RI and *Mlu*I sites (Table 3) (see Fig. 3).

The different pLC26-6 derivative plasmids were also tested for their capability to complement the *murG* mutation, mapped a few years ago immediately upstream from the *murC* gene and affecting an unidentified protein supposed to be also involved in peptidoglycan biosynthesis (28). As previously shown by Salmond et al. (28), shifting a *murG* mutant growing in LB medium from 30 to 42°C led to filamentation and other abnormal shapes before cell lysis occurred a few hours later. Since the gene product function was unknown, only complementation experiments could be

TABLE 3. Complementation of mutations and overproduction of enzymatic activities by the various pLC26-6-derived plasmids

Plasmid	Orientation of the *lac* promoter[a]	Complementation of mutants[b]				Overproduction of enzymatic activities[c]		
		murE	*murF*	*murG*	*murC*	D-glutamic acid-adding enzyme (*murD* product)	*meso*-DAP-adding enzyme (*murE* product)	D-Ala-D-Ala-adding enzyme (*murF* product)
pUC19		−	−	−	−	1	1	1
pKP1	L	+	−	−	−	1	16	1
pKP2	R	+	−	−	−	1	5	1
pKE1	L	+	+	−	−	1.1	5	4
pKE2	R	+	+	−	−	1	4	3
pDML4	L	+	−	−	−	1.2	20	1.2
pDML5	L	−	−	−	−	1.1	1.1	1
pCP10	L	−	+	−	−	1	0.9	2.5
pCP11	R	−	−	−	−	1	1.1	1
pDML9	L	+	+	−	−	19	15	14
pDML10	R	+	+	−	−	6.5	3.5	4
pDML11	L	+	+	−	−	1.2	15	14
pDML12	R	+	+	−	−	1	2.5	3
pDML13	L	−	−	+	−	20	1	1.1
pDML14	R	−	−	+	−	3	1	1
pDML15	L	−	−	+	−	1.1	0.9	1
pDML16	R	−	−	+	−	1.2	1	1
pDML17	L	−	−	+	−	1	1	1.1
pDML18	R	−	−	+	−	1.1	1	1
pDML19	L	−	−	+	−	1.1	1	1
pDML20	R	−	−	+	−	1	1	1
pDML21	L	−	−	−	−	13	1	1.1
pDML22	L	−	−	−	−	0.9	1	1

[a] L (left) and R (right) indicate the location of the *lac* promoter according to the inserts defined in Fig. 1.
[b] +, Complementation; −, no complementation.
[c] Enzymatic assays were carried out as described in the text. For D-glutamic acid-adding enzyme, a value of 1.0 corresponds to 2.5 nmol/min per mg of protein, the specific enzyme activity detected in crude extracts of JM83. Similarly, a value of 1.0 corresponds to 2.7 and 3.3 nmol/min per mg of protein for the *meso*-DAP- and D-alanyl-D-alanine-adding enzymes, respectively.

used to search and locate the *murG* gene within this region. Among the series of plasmids shown in Fig. 1, many clones were shown to effectively complement the *murG* mutation. They all contained a 1.8-kb *Sal*I-*Kpn*I chromosomal fragment corresponding to the extreme right end of the investigated region (Table 3).

Finally, plasmids were assayed for their ability to complement a *murC* mutation; the result was negative in all cases, as was the case with the starting pLC26-6 plasmid material (Table 3). This finding was not very surprising, since Lutkenhaus and Wu (12) have already described one *Hin*dIII site and two *Eco*RI sites within the DNA sequence of the *murC* gene, restriction sites which are not encountered in the chromosomal region presently being studied. However, these data do not exclude the presence of a truncated *murC* gene at the end of the 8-kb *Kpn*I-*Kpn*I insert. In fact, this is probably the case, since the partial restriction map of the entire *E. coli* chromosome published by Kohara et al. (10) revealed such *Hin*dIII and *Eco*RI sites located downstream, but very close to the distal *Kpn*I site of our insert.

Localization of promoters and direction of transcription. To determine the orientation of transcription of genes *murE*, *murF*, *murD*, and *murG* and to locate their respective promoters, most of the chromosomal fragments shown in Fig. 1 have been cloned in both pUC18 and pUC19 vectors to obtain the two different orientations, dependent or not dependent on *lac* promoter control. Each resulting pair of plasmids was examined for its ability to complement the various mutations or to overproduce the different enzymatic activities considered.

By this procedure, the *murE* gene product appears to be effectively expressed in both orientations (compare, for instance, results obtained with pKE1 and pKE2 or with pDML9 and pDML10). Nevertheless, overproduction of the *meso*-DAP-adding enzyme was more pronounced when the *lac* promoter was located on the left side of the corresponding inserts (Table 3). Since the leftmost *Kpn*I site of these inserts is located within the end part of the preceding *pbpB* gene, these results show that *murE* is transcribed from left to right, independently expressed with a proper promoter which should be located between the *Kpn*I site and the initiation codon of the *murE* gene. In fact, analysis of the DNA sequence of the *pbpB* gene, published earlier by Nakamura et al. (21), reveals two such potential promoter sequences, located at positions 2218 (TGTTAT) and 2345 (AAAAAT) downstream of the *Kpn*I restriction site (position 2114), within the distal part of the *pbpB* gene ending at position 2395 (data not shown).

When considering the *murF* gene, a comparison was made between the expression of pCP10 and pCP11 plasmids which contain the complete *murF* gene preceded by 1.1 kb of chromosomal insert corresponding to the end of the neighboring *murE* gene. Complementation of a *murF* mutation and overproduction of the D-alanyl-D-alanine-adding enzyme were observed with pCP10, but not with pCP11 (Table 3). Consequently, the expression of the *murF* gene appeared

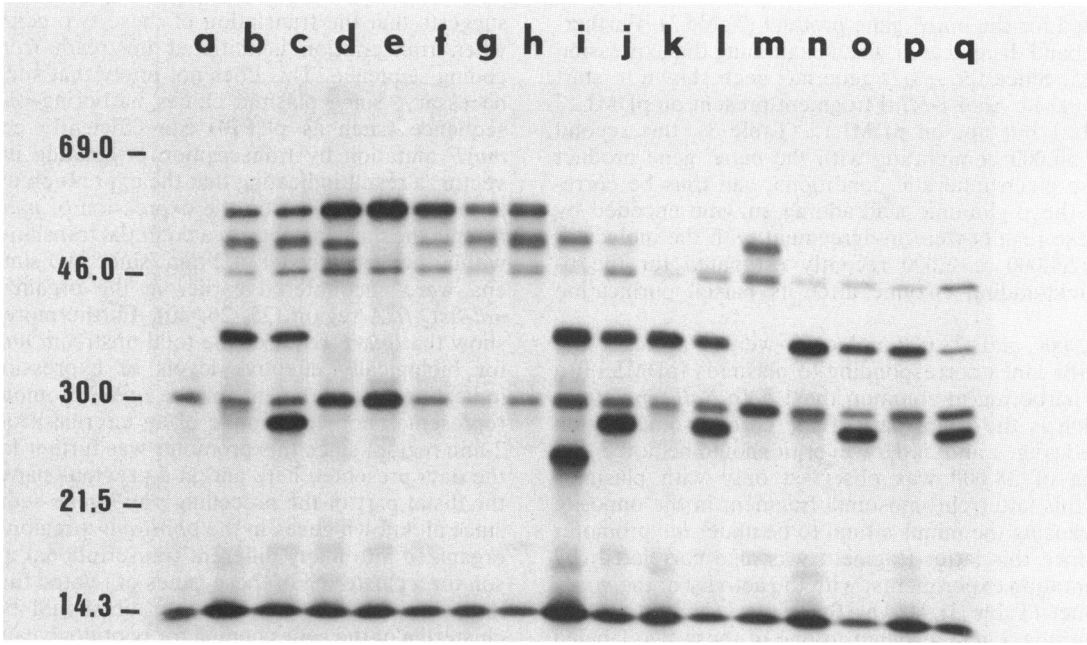

FIG. 2. Radiolabeling of plasmid-encoded proteins in maxicells. Cells of *E. coli* JM109 and its plasmid-harboring derivatives were UV irradiated, and the proteins encoded by the plasmids were labeled with L-[^{35}S]methionine. The total cell proteins were fractionated on a 12% sodium dodecyl sulfate-polyacrylamide gel and fluorographed. Molecular weight standards indicated on the left are as follows: bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000). Lanes: a, JM109(pUC18); b, JM109(pDML7); c, JM109(pDML8); d, JM109(pKE1); e, JM109(pDML4); f, JM109(pDML11); g, JM109(pDML9); h, JM109(pDML10); i, JM109(pDML13); j, JM109(pDML14); k, JM109(pDML15); l, JM109(pDML16); m, JM109(pDML21); n, JM109 (pDML17); o, JM109(pDML18); p, JM109(pDML19); q, JM109(pDML20). Chromosomal inserts and their orientation within these plasmids are shown in Fig. 1 and Table 2, respectively.

to be dependent on a promoter located upstream from the leftmost *Pvu*II site of the pCP10 insert. Since this *Pvu*II site lies within *murE*, only 300 bp from its initiation codon, it seems very likely that the expression of *murF* is under the control of the *murE* promoter. This assumption is further supported by the fact that *murF* expression in the *lacZp*-independent orientation could be effectively recovered in plasmids such as pKE2, pDML10, or pDML12 containing, in addition, the complete *murE* gene with its promoter (Table 3).

A threefold accumulation of the D-glutamic acid-adding enzyme was obtained with pDML14 carrying the 4.5-kb *Eco*RI-*Kpn*I insert in the orientation opposite to *lac* transcription (Table 3). Furthermore, it should be remembered that the initiation codon of the *murD* gene has been unambiguously located in the very initial part of this insert, namely, within the 0.3-kb *Eco*RI-*Mlu*I fragment (see Fig. 3; Table 3). Since there is not enough space on this fragment for a complete gene sequence upstream from *murD*, the efficient expression of the *murD* gene observed in pDML14 strongly suggests the presence in this small area of an independent promoter controlling the transcription of *murD*.

Finally, the observation that enzyme overproduction is in all cases much higher when the *lac* promoter is located on the left side of the cloned fragments (Table 3) clearly indicates that the direction of transcription is clockwise for *murE*, *murF*, and *murD*.

Furthermore, the hypothesis that *murG* is also controlled by its own promoter could be deduced from the complementation tests with plasmids pDML19 and pDML20 (Fig. 1 and Table 3). The 1.8-kb *Sal*I-*Kpn*I insert was effectively shown to complement in either orientation the amber *murG* mutation from strain GS58, but such qualitative results could not be further interpreted in terms of transcription direction for this gene.

Radiolabeling of plasmid-encoded proteins. To determine the molecular weights of the *murE*, *murF*, *murD*, and *murG* gene products and thus to deduce the size of the corresponding genes, radiolabeling of the proteins coded by the different plasmids shown in Fig. 1 was performed by using the maxicell technique of Sancar et al. (29) with *E. coli* JM109 as the host strain (Fig. 2). With plasmid pDML7 carrying the whole 8-kb *Kpn*I-*Kpn*I insert, only three apparent L-[^{35}S]methionine-labeled protein bands corresponding to approximate molecular weights of 55,000, 50,000 and 38,000 were observed in addition to the 30,000 band, which consists of the ampicillin resistance (*bla*) gene product encoded by pUC vectors. A protein band of 28,000 was also observed when examining pDML8 harboring the same insert in the opposite orientation.

The two upper bands were conserved in the lanes corresponding to plasmids pKE1, pDML9, and pDML11, whereas only the highest molecular weight protein of 55,000 was directed by plasmid pDML4 (Fig. 2). These results, correlated with the data concerning the characterization of the enzymatic activities encoded by these different plasmids (Table 3), clearly show that the 55,000 and 50,000 proteins correspond to the *meso*-DAP- and D-alanyl–D-alanine-adding enzymes, respectively, encoded by *murE* and *murF*. This finding is in complete agreement with the recently published data of Maruyama et al. (14). Furthermore, Michaud et al. (18) have partially purified the D-alanyl–D-alanine-adding enzyme from *E. coli* K-12 and have shown that its molecular weight is 51,000 ± 2,000. Interestingly, a 50,000-molecular-weight protein also appeared in lanes corresponding to pDML13 or pDML21 (Fig. 2), while these plasmids no

longer coded for the *murF* gene product (Table 3). Furthermore, this band disappeared when examining the expression of pDML15. Since the *murD* gene has been shown to start within the 0.3-kb *Eco*RI-*Mlu*I fragment present on pDML13 and pDML21 but not on pDML15 (Table 3), this second protein of 50,000 comigrating with the *murF* gene product under these electrophoretic conditions, can thus be correlated with the D-glutamic acid-adding enzyme encoded by *murD*. These results were in agreement with the molecular weight of 54,000 ± 2,000 recently estimated for the D-glutamic acid-adding enzyme after its partial purification (18).

The 38,000- and 28,000-molecular-weight proteins appeared in the lanes corresponding to plasmids (pDML13 to pDML19) harboring in common the 1.8-kb *Sal*I-*Kpn*I fragment, which is the distal part of the chromosomal region investigated (Fig. 1 and 2). However, it should be noted that the protein of 28,000 was observed only with plasmids harboring this latter chromosomal fragment in the opposite direction causing the minus strand to be under *lac* promoter control. Since this latter fragment was also correlated, by complementation experiments, with the activity of the *murG* gene product (Table 3), it was therefore very tempting to identify the *murG* gene product to one of these two labeled protein bands. The small 25,000-molecular-weight protein which only appeared in lane i (Fig. 2) may correspond to a fusion protein resulting from reading through the vector and indicates that a truncated gene might also be present at the end of the pDML13 insert. This band was not detected with plasmids pDML7, pDML15, pDML17, and pDML19, which have an identical *Kpn*I site end as pDML13 but which were constructed in a different way (see Materials and Methods). The band around 46,000 observed in all the lanes is an artifact related to [^{35}S]methionine utilization. According to a personal communication from A. Higgs (Amersham International) a product resulting from the radiolysis of [^{35}S]methionine reacts specifically with a cell protein which has not yet been identified. All these results, which are summarized in Fig. 3, suggest that *murE*, *murF*, and *murD* are approximately 1.5, 1.4, and 1.4 kb in length, respectively, size estimates which are in good agreement with the more-or-less precise localization of these genes within the restriction map.

DISCUSSION

This paper reports a detailed study of the proximal part of a remarkable *E. coli* gene cluster at 2 min that is involved in cell envelope growth and division. From the pLC26-6 plasmid of Clarke and Carbon (4) various DNA fragments that carry the genes located in the 8-kb region downstream of *pbpB* coding for penicillin-binding protein 3 were subcloned and analyzed for their ability to direct protein synthesis in UV-irradiated cells, to complement various thermosensitive mutations, and to overproduce different enzymatic activities.

In particular, utilization of these plasmids made possible the localization of the previously unidentified *murD* gene coding for the D-glutamic acid-adding enzyme and gave more information on the organization of the genes located in the *pbpB-murG* region. It has been possible to localize very precisely the *murE*, *murF*, *murD*, and *murG* genes and to determine the approximate location of their potential respective promoters and the direction of their transcription. The observation that the termination codon of *murE* is separated from the initiation codon of *murF* by only 4 bp strongly suggests that the translation of these two genes is coupled when transcription is initiated upstream from the *murE* coding sequence. This does not imply that such coupling is necessary, since plasmid clones harboring only the *murF* sequence (such as pCP10) can efficiently complement a *murF* mutation by transcription originating in the cloning vector, a result indicating that the expression of *murF* is not absolutely dependent on the expression of *murE*. This represents the third instance of a potential translational coupling within this gene cluster at 2 min, since two similar phenomena were encountered earlier in the organization of the *ddl-ftsQ-ftsA* region (25, 26, 40). Furthermore, our results show that *murF* requires the total upstream *murE* sequence for biologically effective levels of expression, therefore indicating a requirement for the *murE* promoter. This also represents another example of an internal promoter in this 2-min region, since this promoter was further localized from the data presented here and in a previous paper (21) within the distal part of the preceding *pbpB* gene sequence. Thus, since all known genes in the *pbpB-envA* region appear to be organized into many different transcriptional units, the reason for a clustering of these genes of related functions is not immediately apparent. However, it should be noted that clustering of the genes coding for peptidoglycan cytoplasmic synthetases in the 2-min region is not a general feature; *murA* (37) and *mrbA* (20) genes coding for the PEP:UDP-GlcNAc-enolpyruvyl transferase and the UDP-GlcNAc-enolpyruvate reductase, respectively, have been mapped to a completely separated region around 90 min, whereas the *alr* gene coding for the alanine racemase was located earlier at 93 min (35, 36). Furthermore, the genes coding for the enzymatic activities which catalyze the very early cytoplasmic steps of peptidoglycan synthesis leading from glucosamine-6-phosphate to the formation of UDP-GlcNAc (33) have not yet been mapped.

Our present knowledge of the organization of the chromosomal region we have investigated is summarized in Fig. 3. From the data shown in this paper, it is now clear that the still unidentified product of *murG* (28) is not the D-glutamic acid-adding enzyme, since the gene *murD* encoding this activity has been unambiguously located 2.5 kb upstream from *murG*. The order of the genes in this region is *pbpB-murE-murF-X-murD-Y-murG*, where *X* and *Y* represent chromosomal fragments of 1 to 1.5 kb. We were unable to detect any protein corresponding to the *X* and *Y* regions with the maxicell technique, and so have no proof that these chromosomal fragments are actually coding regions. However, it is known that all the genes of the downstream region from *murC* to *envA* are tightly packed, the chromosomal DNA in that region being almost 100% utilized (26, 31). It can thus be speculated that this is also the case in the *pbpB-murG* region studied here. *X* and *Y* would then represent chromosomal fragments of great interest, which most probably code for proteins also involved in cell envelope-related functions. It should be remembered that the expression from the *lac* promoter of inserts containing the *murE-murF-X* region resulted in an unusual rough and translucent aspect of the corresponding clones. In fact, the phenomena observed are not dependent of *murE* expression, since JM83(pDML4) clones containing a 20-fold-increased level of *meso*-DAP-adding enzyme have quite normal-appearing colonies. However, it is not yet known if they are dependent on the expression of *murF* or that of the *X* region, since all the constructed plasmids that provide a significant accumulation of D-alanyl-D-alanine-adding enzyme also carry the whole *X* region. An overproduction of the *murF* gene product could

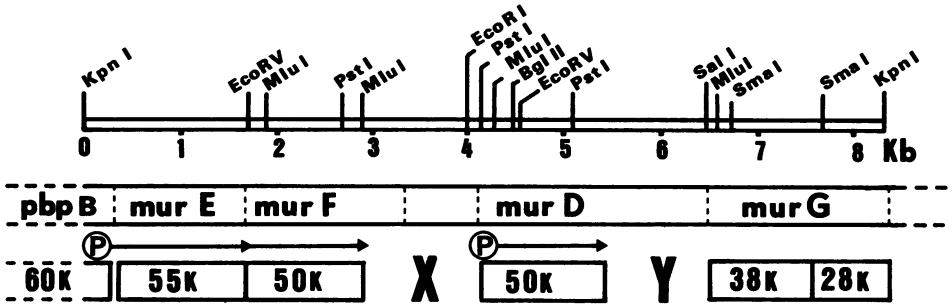

FIG. 3. Organization of the genes located in the 8-kb *Kpn*I-*Kpn*I chromosomal region subcloned from pLC26-6 into pDML7. The positions of the genes on this chromosomal fragment were deduced from the data presented in this work, including complementation of mutations and detection of enzyme overproduction. Orientation is clockwise from left to right. Vertical broken lines between the genes indicate the maximum identified length that could contain the genes. The sizes of the DNA fragments required to code for these various proteins were estimated from maxicell experiments and are indicated by the rectangles. The approximate position of potential promoters is designated by the letter P, and the arrows indicate the direction of transcription. The end of the *pbpB* gene has been previously located by Nakamura et al. (21) at 0.28 kb downstream from the initial *Kpn*I site. The relative positions of the 28,000-molecular-weight (28K) and 38K proteins were determined by the fact that the 38K protein was lost after deletion of the small *Sal*I-*Mlu*I fragment in plasmids pDML19 and pDML20.

decrease the pool level of its UDP-MurNAc-tripeptide substrate. Pisabarro et al. (24) earlier proposed that this latter nucleotide precursor could also participate in the membrane steps of peptidoglycan synthesis involving penicillin-binding protein 3. The unusual morphology of clones accumulating the *murF* gene product from 5- to 14-fold could then reflect some modification in the structure of peptidoglycan resulting from a decreased availability of UDP-MurNAc-tripeptide for this latter physiological process. Considering the *Y* region now, Ishino et al. (F. Ishino, H. K. Jung, M. Doi, A. Ohta, I. Shibuya, K. Begg, and M. Matsuhashi, Abstr. Bacterial Cell Surfaces Bioscience Congr., p. 55–57, 1987) have recently described a thermosensitive *fts17* mutation, located closely upstream from the *murG* gene, that may correspond to the region we have named *Y* in Fig. 3.

Consequently, this preliminary study opens the way to the characterization of at least two other potential activities located in the *X* and *Y* regions. Furthermore, the *murG* gene product whose function is not known also remains to be identified, since two proteins of 38,000 and 28,000 were expressed in maxicells from the 1.8-kb *Sal*I-*Kpn*I fragment which efficiently complements the *murG* mutation.

Finally, the purification to homogeneity of the *meso*-DAP-, D-alanyl-D-alanine-, and D-glutamic acid-adding enzymes and the nucleotide sequencing of this 8-kb region of the chromosome will provide final proof for assigning the *murE*, *murF*, and *murD* genes. Furthermore, since this latter region probably also contains a truncated *murC* gene, utilization of larger clones that also harbor the downstream *murC-ddl-fts* region will be necessary to estimate the distance separating *murG* from *murC* and to thus reach a complete knowledge of how all these genes are organized and their expression is regulated. However, since the various cytoplasmic synthetases involved in UDP-MurNAc-pentapeptide formation were earlier shown to be constitutively produced in the cell at a relatively high level independent of the growth conditions used (15–17), it seems that an eventual regulation of their in vivo activity will more likely be the result of specific effectors acting on the enzyme activities, rather than a modulation of their genetic expression.

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