JOURNAL OF BACTERIOLOGY, Sept. 1992, p. 5772–5779
0021-9193/92/185772-08$02.00/0

Vol. 174, No. 18

Identification of the *Escherichia coli murI* Gene, Which Is Required for the Biosynthesis of D-Glutamic Acid, a Specific Component of Bacterial Peptidoglycan

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Received 16 April 1992/Accepted 29 June 1992

The *murI* gene of *Escherichia coli*, whose inactivation results in the inability to form colonies in the absence of D-glutamic acid, was identified in the 90-min region of the chromosome. The complementation of an auxotrophic *E. coli* B/r strain by various DNA sources allowed us to clone a 2.5-kbp *Eco*RI chromosomal fragment carrying the *murI* gene into multicopy plasmids. The *murI* gene corresponds to a previously sequenced open reading frame, ORF1 (J. Brosius, T. J. Dull, D. D. Sleeter, and H. F. Noller. J. Bacteriol. 148:107–127, 1987), located between the *btuB* gene, encoding the vitamin B_{12} outer membrane receptor protein, and the *rrnB* operon, which contains the genes for 16S, 23S, and 5S rRNAs. The *murI* gene product is predicted to be a protein of 289 amino acids with a molecular weight of 31,500. Attempts to identify its enzymatic activity were unsuccessful. Cells altered in the *murI* gene accumulate UDP-*N*-acetylmuramyl-L-alanine to a high level when depleted of D-glutamic acid. Pools of precursors located downstream in the pathway are consequently depleted, and cell lysis finally occurs when the peptidoglycan content is 25% lower than that of normally growing cells.

The biosynthesis of bacterial cell wall peptidoglycan (murein) is a complex process involving many different cytoplasmic and membrane steps (21, 44). Conditional-lethal mutants of *Escherichia coli* altered at different levels of this metabolic pathway have been described previously, and most of the mutations were mapped in several discrete regions of the chromosome (16, 28, 39, 44, 45, 50, 52). One of them, at 2 min on the *E. coli* map, was studied in great detail since it contained a large cluster of genes from *pbpB* to *envA* that code for proteins involved in different aspects of peptidoglycan synthesis and cell division. Through earlier work from this and other laboratories, the complete physical map and DNA sequence of the whole 17-kbp region were determined (26, 34). Interestingly, seven genes that participate in the pathway for peptidoglycan synthesis from UDP-*N*-acetylglucosamine to the formation of the lipid intermediate *N*-acetylglucosaminyl-*N*-acetylmuramyl(pentapeptide)-pyrophosphate-undecaprenol (*murC*, *murD*, *murE*, *ddl*, *murF*, *mraY*, and *murG*) were identified in this region (23, 36; see also reference 34 and references therein).

However, not all of the genes coding for cytoplasmic peptidoglycan synthetases are located in the 2-min region. In particular, genes *murA* and *murB*, coding respectively for the transferase and reductase activities which catalyze the two-step formation of UDP-*N*-acetylmuramic acid from UDP-*N*-acetylglucosamine, as well as the gene required for the synthesis of D-glutamic acid, have not yet been precisely localized. Earlier data from Miyakawa et al. (39) and Venkateswaran and Wu (50) showed that *murA* and *murB* probably mapped in a completely separate region of the chromosome around 89 min.

D-Glutamic acid is a specific component of the peptidoglycan structure. It is incorporated into peptidoglycan precursors by its initial addition to UDP-*N*-acetylmuramyl-L-alanine (UDP-MurNAc-L-alanine), which is catalyzed by the D-glutamic acid-adding enzyme (the *murD* gene product) (34, 42). A strain of *E. coli* B auxotrophic for this D amino acid has been isolated by Hoffman et al. (20) and studied in some detail by Lugtenberg et al. (28). Attempts to identify the enzymatic defect in this mutant were unsuccessful, but the approximate genetic location of the mutation was determined to be between the *rpsL* marker at 73 min and the origin of transfer of the HfrH strain at 98 min (28). It was therefore tempting to speculate that this mutation might be located in the same chromosomal region as *murA* and *murB*. Such a cluster, named *mrb* (for murein region b), was earlier suggested by Miyakawa et al. (39) to be located in the region close to *argH*, but its physical map and organization were not further investigated.

It appeared essential to study this region of the chromosome thoroughly, and our aim was to more precisely localize the genes related to peptidoglycan metabolism in this area. We report here the identification of the gene required for D-glutamic synthesis in the 90-min region of the *E. coli* chromosome. We named this gene *murI* (for murein gene I) according to the nomenclature previously adopted for the other genes involved in cytoplasmic or membrane steps of peptidoglycan synthesis (44).

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The *E. coli* strains used in this study are listed in Table 1. Plasmid pAC1 (5) carrying the *lamB* gene expressed under the control of the *tac* promoter was a gift from A. Charbit (Institut Pasteur, Paris), and pUC18 and pUC19 vectors were purchased from Pharmacia (Uppsala, Sweden). Low-copy-number plasmid pLG339 (48) was obtained from B. Holland (Institut de Microbiologie, Orsay, France). Phage clones from the *E. coli* miniset library of Kohara et al. (25) and a bank of phasmids constructed by Caillet and Droogmans (7) in vector λSE6

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TABLE 1. *E. coli* strains used

Strain	Genotype	Reference or source[a]
E. coli K-12		
JM83	*ara* Δ(*lac-proAB*) *rpsL thi* φ80 d*lacZ*Δ*M15*	53
FB8	F⁻ prototroph	F. Blasi
AB1133	F⁻ *thr-1 leuB6* Δ(*gpt-proA*)*62 hisG4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL-31 supE44*	1
ET12558	*supE44 endA1 zig102*::Tn*10 glnA1857 thi-1 hsdR17*	B. Bachmann; CGSC 7076
ECL339	Δ(*argF-lac*)*205 supE44 endA1 zig924*::Tn*10* Δ(*rha-pfkA*)*200 thi-1 hsdR17*	B. Bachmann; CGSC 6740
RK4349	*pro-3* Δ(*lac*)*6 entA403 supE44 his-218 metB1 rpsL109 xyl-5 ilvC7 metE163*::Tn*10*	B. Bachmann; CGSC 6403
NK5139	*thi-39*::Tn*10* IN(*rrnD-rrnE*)*1*	B. Bachmann; CGSC 6165
BW6160	Hfr λ⁻ *zdh-57*::Tn*10 relA1 spoT1 metB1* λ[r]	B. Wanner; CGSC 6756
BW7620	Hfr λ⁻ *lac-42 zed-977*::Tn*10 relA1 spoT1 thi-1*	B. Wanner; CGSC 6813
BW5660	Hfr λ⁻ Δ(*gpt-lac*)*5 supE44 srlC300*::Tn*10 relA1? spoT1?*	B. Wanner; CGSC 6753
BW6159	Hfr λ⁻ *relA1 spoT1 ilv-691*::Tn*10 thi-1*	B. Wanner; CGSC 6755
BW6175	Hfr λ⁻ *thr-1 leuB6 azi-15 tonA21 lacY1 supE44 argE86*::Tn*10 thi-1*	B. Wanner; CGSC 6763
BW6164	Hfr λ⁻ *thr-43*::Tn*10 supE42 malB28* (λ[r]) *sfa-4*	B. Wanner; CGSC 6759
NK6051	Hfr λ⁻ Δ(*gpt-lac*)*5 purE79*::Tn*10 relA1 spoT1 thi-1*	B. Wanner; CGSC 6186
BW6165	Hfr *ara-41 lacY1* or *lacY40* λ[ind⁻] *xyl-7 mtl-2 argE86*::Tn*10*	B. Wanner; CGSC 6760
BW6156	Hfr λ⁻ *relA1 spoT1 metB1 zje-2005*::Tn*10*	B. Wanner; CGSC 6754
BW7261	Hfr *leu-63*::Tn*10 tonA22* Δ(*argF-lac*)*169 ompF627 relA1 spoT1* T2[r]	B. Wanner; CGSC 6787
BW7623	Hfr λ⁻ *purE79*::Tn*10 relA1? spoT1?*	B. Wanner; CGSC 6815
BW7622	Hfr λ⁻ *trpB114*::Tn*10 relA1 spoT1 thi-1*	B. Wanner; CGSC 6814
BW5659	Hfr λ⁻ *lacY1 zdh-57*::Tn*10 mglP1 xyl-7*	B. Wanner; CGSC 6752
BW6163	Hfr λ⁻ *zed-977*::Tn*10 relA1 spoT1 thi-1*	B. Wanner; CGSC 6758
BW6169	Hfr λ⁻ *leuB6 tonA2? lacY1* or *lacZ4 tsx-1? supE44 gal-6 argA81*::Tn*10 thi-1*	B. Wanner; CGSC 6762
BW6166	Hfr λ⁻ *supE44 zhf-721*::Tn*10 thi-1 malB16* (λ[r])	B. Wanner; CGSC 6761
β180	MG1655 *metA*Δ::Cm[r]	C. Richaud (18)
E. coli B/r		
WM301	*leu pro trp his arg thyA deoB met lac gal xyl ara mal lam phx rpsL hsdS*-K-12	W. Messer; PC 2341
WM335	WM301 *glt*	W. Messer; PC 2342

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.; PC, Phabagen Collection, Department of Molecular Cell Biology, State University of Utrecht, Utrecht, The Netherlands.

(13) were used as DNA sources for complementation of the D-glutamate-requiring mutant strain.

Growth conditions. Unless otherwise noted, 2xYT (38) as a rich medium and M63 (38) supplemented with 0.4% glucose as a defined minimal medium were used for growing cells. Growth was monitored at 600 nm with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). D-Glutamic acid (50 μg ml^{-1}), thymine (100 μg ml^{-1}), thiamine (1 μg ml^{-1}), and L-amino acids (50 μg ml^{-1}) were also added to meet specific strain requirements. For strains carrying drug resistance genes, the antibiotics ampicillin (100 μg ml^{-1}), streptomycin (50 μg ml^{-1}), kanamycin (25 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), and tetracycline (15 μg ml^{-1}) were used. Broth for plates was solidified with 1.5% agar; to screen plasmid inserts for the absence of α complementation, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added at 40 μg ml^{-1}.

Pool levels of peptidoglycan precursors. WM335 cells (1-liter cultures) were grown exponentially at 37°C in 2xYT medium supplemented with D-glutamic acid. At an optical density of 0.8, cells were rapidly harvested at room temperature, washed with 2xYT medium, and finally suspended at an optical density of 0.2 in prewarmed 2xYT medium containing or lacking D-glutamic acid. In all cases, incubation was continued at 37°C until the optical density of the depleted culture reached a plateau value of 0.5 about 1 h later (Fig. 1). At this time, preceding the onset of cell lysis by only a few minutes, cells were rapidly chilled to 0°C and harvested in the cold. The method for extraction of peptidoglycan precursors and the analytical procedure used for their quantitation were as previously described (31, 32).

Isolation of sacculi and quantitation of peptidoglycan. Ex-

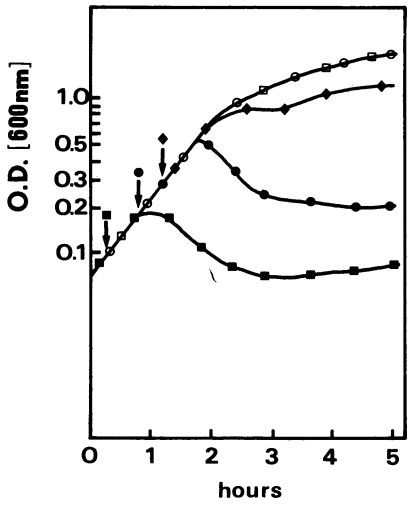

FIG. 1. Effect of a D-glutamic acid deficiency on the growth of strain WM335. Cells growing exponentially at 37°C in 2xYT medium supplemented with D-glutamic acid (50 μg ml^{-1}) were rapidly harvested (at an optical density [O.D.] of 1.0), washed with 2xYT medium, and then resuspended at an appropriate dilution into prewarmed 2xYT medium with or without D-glutamic acid. Growth at 37°C was monitored by measuring the optical density at 600 nm. Symbols: ■, ●, and ◆, growth of mutant strain WM335 in the absence of D-glutamic acid following resuspension of cells at optical densities of 0.1, 0.2, and 0.3, respectively; ○, growth of the mutant in the presence of D-glutamic acid; □, growth of the parental strain WM301 under both conditions.

ponentially growing WM335 cells either were or were not depleted of D-glutamic acid as described above. Harvested cells were washed with a cold 0.85% NaCl solution and centrifuged again. Bacteria were then rapidly suspended under vigorous stirring in 40 ml of a hot (95 to 100°C) aqueous 4% sodium dodecyl sulfate solution for 30 min. After standing overnight at room temperature, the suspensions were centrifuged for 30 min at 200,000 × *g*, and the pellets were washed several times with water. Final suspensions made in 5 ml of water were homogenized by brief sonication. The peptidoglycan content of the sacculi was expressed in terms of its diaminopimelic acid (DAP) content (35, 37). It should be noted that *E. coli* B/r was unable to internalize exogenous DAP, probably because of the lack of a transport system (data not shown). The technique of monitoring the incorporation of radioactive *meso*-DAP into the macromolecule of a *lysA* derivative strain (35) was therefore unusable, and peptidoglycan synthesis was estimated in that case only by measuring the unlabeled DAP content of isolated sacculi.

General DNA techniques and *E. coli* cell transformation. The isolation of phages and phasmids and the extraction and purification of their DNAs were performed by standard techniques (9, 38, 46). Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (46), and plasmids were further purified by using cesium chloride-ethidium bromide gradients. Standard procedures for endonuclease digestions, ligation, filling in of 5′ protruding ends by using the Klenow fragment of DNA polymerase I, and agarose electrophoresis were used (46). Usually, *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (8), but the technique of electroporation was preferentially used for transformation of *E. coli* B/r strain WM335.

Mapping by conjugation and transduction. Crude mapping of D-glutamate auxotrophy by conjugational recombination was performed by using as donors the 16 different Hfr strains that composed the Hfr::Tn*10* kit constructed by Wanner (51) and strain WM335 as the recipient. These Hfr strains have integration sites distributed all around the *E. coli* K-12 map and Tn*10* transposons inserted within the range of 10 to 30 min from the point of origin (51). Conjugation was performed according to standard procedures (38); briefly, exponential-phase donor and recipient cells were mixed together in a 1/20 ratio and incubated at 37°C for 1 h under gentle agitation, followed by 2 h of expression at 37°C under vigorous aeration. Taking advantage of the *rpsL* marker in strain WM335, conjugants that received transposon Tn*10* were selected on 2xYT plates supplemented with D-glutamic acid, streptomycin, and tetracycline. Final replicates on plates with or without D-glutamic acid allowed us to estimate in each case the linkage between D-glutamate prototrophy, origin of transfer of the Hfr strain, and localization of transposon Tn*10*. P1-mediated transduction experiments were performed essentially as previously described (38).

Complementation tests for D-glutamate auxotrophy. (i) With phages. Overnight stationary-phase cells of mutant WM335 carrying plasmid pPDM3 (see below) were washed with 10 mM $MgSO_4$ and plated onto 2xYT-ampicillin plates containing 10 mM $MgSO_4$ and no added D-glutamic acid. Small spots were made with lysates of phages from the genomic library of Kohara et al. (25) (more than 10^{11} ml^{-1}) onto the lawn of bacteria, and the plates were incubated at 30°C. Complementation was judged positive 24 or 48 h later when many colonies were observed at the position of the phage spot (complementation occurred in that case by a recombination event).

(ii) With phasmids. A 200-μl sample of a suspension of WM335(pPDM3) cells in 10 mM $MgSO_4$ (see above) was incubated for 1 h at 30°C without shaking with a lysate corresponding to a whole library of phasmids constructed in vector λSE6 (7, 13). After addition of 400 μl of 2xYT medium supplemented with D-glutamic acid, the mixture was incubated at 30°C for an additional 2 h with shaking to ensure expression of the kanamycin resistance gene (*npt* gene from vector λSE6). Cells were recovered by centrifugation, washed several times with 2xYT medium to remove residual D-glutamic acid, and then plated on 2xYT plates containing ampicillin and kanamycin. Ap^r Km^r transductants were observed after 24 h of incubation at 30°C. Phasmids from these clones were thermally induced (cells growing exponentially at 30°C were shifted to 42°C for 3 h to induce the release of the corresponding phage particles following inactivation of the *c*I857 thermosensitive lambda repressor present on plasmid pPDM3) and used to transduce again strain WM335(pPDM3) to kanamycin resistance. All Km^r colonies tested in that way were D-Glu^+, indicating 100% linkage between Km^r and D-glutamate prototrophy.

(iii) With plasmids. WM335 cells were made competent as described above 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 2xYT medium supplemented with D-glutamic acid was added, and cells were incubated at 37°C for more than 1 h for expression of plasmid genes. Cells were recovered by centrifugation and washed several times with 2xYT medium. A final suspension made in 200 μl of 2xYT medium was separated into two parts, which were plated onto two 2xYT-antibiotic plates with or without D-glutamic acid and incubated at 37°C. Growth was observed after 24 h of incubation.

Construction of plasmids. Plasmid pAC1, carrying the *lamB* gene of *E. coli* K-12 expressed under the control of the *tac* promoter, was previously described (5). Plasmid pPDM3 was generated by inserting the 2.4-kb *Xho*II-*Xho*II lambda fragment that carries the structural gene *c*I857 (encoding a thermosensitive form of the lambda *c*I repressor) into the unique *Stu*I site of pAC1. JM83 clones harboring pPDM3 were easily selected on the basis of their acquired resistance to lambda at 30°C. DNA from phage λ534 of the collection of Kohara et al. (25) was used as the starting material for construction of the plasmids shown in Fig. 3. First, the 9-kb *Eco*RI-*Eco*RI insert corresponding to the right end of the chromosomal fragment present on this phage clone was isolated and introduced into the *Eco*RI site of the pUC19 vector. All of the JM83 transformants chosen as white-appearing colonies on 2xYT plates with X-Gal were shown to contain the plasmid (pPDM14) with the insert in the orientation opposite that of the *lac* promoter (*lacZp*) control. Plasmid pPDM23 was constructed by subcloning the 9.3-kb *Bam*HI-*Bam*HI fragment from λ534 into the *Bam*HI site of low-copy-number plasmid pLG339 (in fact, the cloned chromosomal DNA was a 9.3-kb *Eco*RI-*Bam*HI fragment, since the leftmost *Bam*HI site originated from the polylinker of the λ2001 vector [24]). Plasmids pPDM24 and pPDM25 were obtained by subcloning the 2.8-kb *Sal*I-*Sal*I and the 2.5-kb *Eco*RI-*Eco*RI fragments, respectively, into the same sites of the pUC19 vector. Insertion of these different fragments carrying the *murI* gene was directly selected in mutant strain WM335 by isolating ampicillin-resistant clones that grew in

TABLE 2. Peptidoglycan content and pool levels of its nucleotide precursors in WM335 cells depleted or not depleted of D-glutamic acid[a]

Peptidoglycan or precursor	Amt (nmol/g [dry wt] of bacteria)	
	+D-Glu	−D-Glu
UDP-GlcNAc	700	850
UDP-MurNAc	30	70
UDP-MurNAc-L-Ala	3	1,700
UDP-MurNAc-dipeptide	15	5
UDP-MurNAc-tripeptide	30	10
UDP-MurNAc-pentapeptide	400	30
Peptidoglycan[b]	13,000	9,700

[a] Cells growing exponentially at 37°C in 2xYT medium supplemented with D-glutamic acid were washed and resuspended at an optical density of 0.2 in 2xYT medium supplemented (+D-Glu) or not supplemented (−D-Glu) with D-glutamic acid. Growth was continued at 37°C, and cultures were stopped approximately 1 h later (the time at which the growth rate of the mutant deprived of D-glutamic acid begins to decrease; see Fig. 1). Cells were harvested, and their peptidoglycan and nucleotide precursors were isolated and quantified as detailed in Materials and Methods.

[b] The peptidoglycan content of sacculi was expressed in terms of its DAP content (35, 37).

the absence of D-glutamic acid. Plasmid pPDM26 was obtained by an internal *Pst*I deletion in pPDM25.

RESULTS

Effects of a D-glutamic acid deficiency on peptidoglycan synthesis. The D-glutamic acid-requiring mutant WM335 of *E. coli* B was isolated by Hoffman et al. (20), and some of its properties were described previously (28). When WM335 cells growing exponentially in 2xYT medium supplemented with D-glutamic acid were rapidly washed and resuspended in 2xYT medium without D-glutamic acid, cell lysis was observed after a growth period corresponding to approximately one or two generation times (Fig. 1). D-Glutamic acid-dependent lysis exhibited by strain WM335 thus resembled the phenotype of previously described mutants altered in cytoplasmic or membrane steps of peptidoglycan synthesis (36, 39, 44, 45, 52). The residual growth preceding the onset of cell lysis was interpreted in this case as the time needed by the peptidoglycan-synthesizing machinery to consume the pool of D-glutamic acid previously accumulated by the cells.

The effects of a D-glutamic acid deficiency on the rate of peptidoglycan synthesis and on the pool levels of some of its nucleotide precursors were investigated. Exponentially growing WM335 cells either were or were not depleted of D-glutamic acid as described in Materials and Methods and were harvested when the optical density of the depleted culture reached the characteristic plateau value preceding cell lysis (Fig. 1). The pool levels of the different nucleotide precursors of peptidoglycan and the peptidoglycan content itself were then determined in both types of cells (Table 2). Depleting WM335 cells of D-glutamic acid led to a 500-fold increase in the pool of UDP-MurNAc-L-alanine. This result was qualitatively consistent with earlier data from Lugtenberg et al. (28), but the accumulation of UDP-MurNAc-L-alanine observed by these authors was comparatively very low, probably because considerable cell lysis occurred in the cell resuspension medium that they used (27, 28). UDP-GlcNAc and UDP-MurNAc, which are upstream precursors in the pathway, also accumulated but to much lower levels. Conversely, pools of precursors derived from UDP-MurNAc-L-alanine were drastically depleted, and as a result, the peptidoglycan content of the cells also decreased by 25%.

Attempts to identify the enzymatic defect. Accumulation of UDP-MurNAc-L-alanine could be explained either by a defective enzyme in the biosynthesis of D-glutamic acid or by an altered D-glutamic acid-adding enzyme (the *murD* gene product) with a decreased affinity for its D-amino acid substrate. This latter possibility was unlikely since no variation of the in vitro-determined D-glutamic acid-adding activity had been observed (28). We now definitively eliminated this possibility, since we observed that plasmid pDML13 (34) (which carries the *murD* gene of *E. coli* K-12 under the control of the *lac* promoter) was unable to complement the specific defect of strain WM335. Most probably the biosynthesis of D-glutamic acid was altered in this mutant. In bacteria, D-glutamic acid is produced either by an L-glutamic acid racemase (40, 41) or by a D-glutamate:D-alanine transaminase (29, 49). However, neither of these enzymes has previously been characterized in *E. coli* (28). We tried to assay for these two enzymatic activities in extracts from a prototrophic *E. coli* K-12 strain, FB8. For this purpose, a recently developed high-pressure liquid chromatography procedure allowing rapid and quantitative detection of D-glutamic acid after its derivatization by the Marfey reagent (33) was developed (data not shown). Whatever the technique used for the enzyme extractions (disruption of cells by sonication or lysis of spheroplasts) or for the in vitro assays, no conversion of D-alanine or L-glutamic acid to D-glutamic acid was observed, using either soluble or membrane fraction from these extracts (data not shown). We therefore concluded, as did Lugtenberg et al. (28), that either *E. coli* does not contain the tested enzymatic activities or the conditions used for isolation or assay of these enzymes were still inadequate. One of these enzymes could be present in *E. coli* cells at a level of activity sufficient to sustain the rate of peptidoglycan synthesis and bacterial growth but too low to be efficiently detected in our assay conditions. Cloning of the corresponding gene for construction of strains overproducing this enzymatic activity was therefore considered an eventual solution to this problem.

Crude mapping of the mutation. Since D-glutamic acid is incorporated in bacteria specifically into peptidoglycan (44), we proposed *murI* as the name for the gene required for its biosynthesis, in reference to previously identified genes from *murE* to *murG* that code for proteins involved in intermediate steps of the peptidoglycan pathway.

A preliminary genetic location of the mutation conferring D-glutamic acid auxotrophy to WM335 cells was previously determined to be between 73 and 98 min on the *E. coli* chromosome (28). We localized the *murI* gene more precisely, first by conjugation experiments using the complete set of Hfr::Tn*10* strains constructed by Wanner (51) as donors and WM335 as the recipient strain. These Hfr strains have origins of transfer distributed all around the *E. coli* K-12 map and Tn*10* transposons inserted within the range of 10 to 30 min from the point of origin. As shown in Fig. 2,, linkage of D-glutamic acid prototrophy to the Tn*10* insertion was observed during crosses of WM335 with six Hfr strains which transfer precociously chromosomal DNA originating from the 85- to 95-min region of the chromosome. We obtained a more precise gene localization by P1 transduction experiments in which linkage of *murI* with various selectable markers from the 85- to 95-min region was estimated. The results (Table 3) indicated a close linkage of the mutation with *metA*, *argE*, and *thi* markers, and we concluded that the *murI* gene mapped at approximately 90 min on the *E. coli*

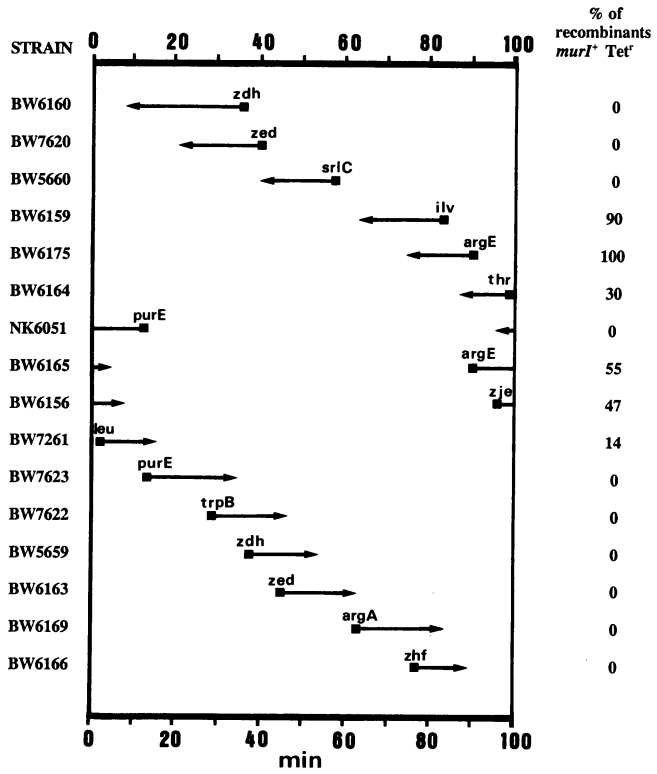

FIG. 2. Crude mapping of the *murI* mutation by conjugation. The map representing the points of origin and direction of transfer (indicated by arrowheads) of the Hfr::Tn*10* mapping strains is essentially redrawn from Wanner (51). Filled boxes show positions of the transposon. See Table 1 for complete strain descriptions. For analysis of recombinants, WM335 was the recipient strain in all cases. Recombinants were first selected for acquisition of transposon Tn*10* and then tested for D-glutamic acid prototrophy.

chromosome. It was also noteworthy that the highest linkage value (71%) was observed with a *thi-39*::Tn*10* marker originating from a strain carrying a ca. 1,000-kb-long inversion of the chromosome between *rrnD* and *rrnE* (2). This result further indicated that the *murI* gene mapped counterclockwise with respect to the *rrnE* operon at 90.4 min.

Complementation of the mutation by phages and phasmids. Two different sources of *E. coli* chromosomal DNA were used for cloning of the *murI* gene: the ordered collection of phages of Kohara et al. (25) and a *Sau*3A library constructed in phasmid vector λSE6 (7, 13). The ability to complement

TABLE 3. P1 transductional mapping of the *murI* mutation[a]

Donor genotype[b]	Chromosomal location of drug resistance markers (min)	Total no. of transductants	% of recombinants *murI*+ Tetr (or Cmr)
zig-102::Tn*10*	87	65	0
zig-924::Tn*10*	88.5	50	4
argE86::Tn*10*	89.5	150	36
thi-39::Tn*10*	90.5	82	71
*metA*Δ::Tn*10*	91	96	25

[a] In all cases, WM335 was the recipient strain and transductants were first selected for acquisition of the drug resistance marker (Tetr or Cmr) and then tested for D-glutamic acid prototrophy.

[b] *murI*+ donors were strains ET12558, ECL339, BW6165, NK5139, and β180, respectively.

D-glutamic acid auxotrophy was taken in all cases as preliminary proof that phage or phasmid clones carried the *murI* gene.

One initially encountered difficulty was that the only available D-glutamic acid-requiring mutant (*E. coli* B/r strain WM335) was also lambda resistant. This fact was correlated with the absence of a functional lambda receptor, and sensitivity was effectively restored when WM335 cells were transformed with plasmid pAC1 (5), which carries the *lamB* gene of *E. coli* K-12. We thus constructed plasmid pPDM3, carrying both *lamB* (receptor to lambda) and *c*I857 (lambda repressor) genes that made WM335 cells sensitive but also immune to lambda phages, a prerequisite to complementation experiments.

To more precisely localize the *murI* gene in the 90-min region of the *E. coli* chromosome, we first infected strain WM335(pPDM3) with different lambda phage clones from the library of Kohara et al. (25) which carry *E. coli* DNA from the 87- to 92-min region of the physical map. Only recombinant phage λE11C11 (clone 534) was apparently able to complement (by recombination) the D-glutamic acid auxotrophy of strain WM335 (Fig. 3). This phage consists of a 15-kb *Eco*RI-*Eco*RI fragment (obtained by partial *Eco*RI digestion of the *E. coli* chromosome) (25) cloned into vector λ2001 (24).

Another approach was to complement the mutant by using the whole *E. coli* genomic library prepared in phasmid vector λSE6 (13). This vector confers kanamycin resistance and is capable of growing lysogenically as a phasmid in an immune host. Samples from three independent batches of the initial library (7) were used to infect WM335(pPDM3) at a multiplicity of 0.1. We selected clones directly for both acquisition of the phasmid and complementation of D-glutamic acid auxotrophy. Only five Apr Kmr clones that could grow at 30°C in the absence of D-glutamic acid were isolated by this procedure. Appropriate controls showed that these prototrophs were obtained at a frequency of 1 in 500 phasmid-containing clones. Phasmids from the five clones (λGLU1 to λGLU5) were thermally induced and used to transduce again WM335(pPDM3) to Kmr. All Apr Kmr colonies tested were now effectively D-Glu+, indicating 100% linkage between Kmr and D-glutamic acid prototrophy.

Phasmid DNA was prepared and analyzed in each case. On the basis of the positions and matching restriction patterns of the λ534 and *murI*+-containing λGLU phasmids, the *murI* gene was placed unambiguously at 90 min on the *E. coli* genetic map, within a 15-kb fragment located between the *argECBH* cluster and the *rpoBC* genes (Fig. 3). All of the phasmids which complemented the auxotrophy for D-glutamic acid carried *Eco*RI-*Eco*RI fragments of 1.5, 2.1, and 2.5 kb (data not shown; Fig. 3 shows only the map of λGLU3, which carries the leftmost chromosomal insert, characterized by the minimum of overlap with the insert from λ534). The fact that all of these phasmid inserts have the same location on the *E. coli* map further indicated that this auxotrophy was probably the result of only one gene alteration, as already suggested by conjugation experiments.

Cloning of the *murI* gene which is required for D-glutamic acid synthesis. Most of the DNA from this chromosomal region has been previously sequenced (17; see also reference 26 and references therein). The corresponding restriction map redrawn from Kohara et al. (25) and revised according to sequences available from the EMBL data base (10, 26, 30) as well as the positions of the genes that were previously identified in this region (2, 6, 12, 15, 17, 19, 22) are shown in the Fig. 3.

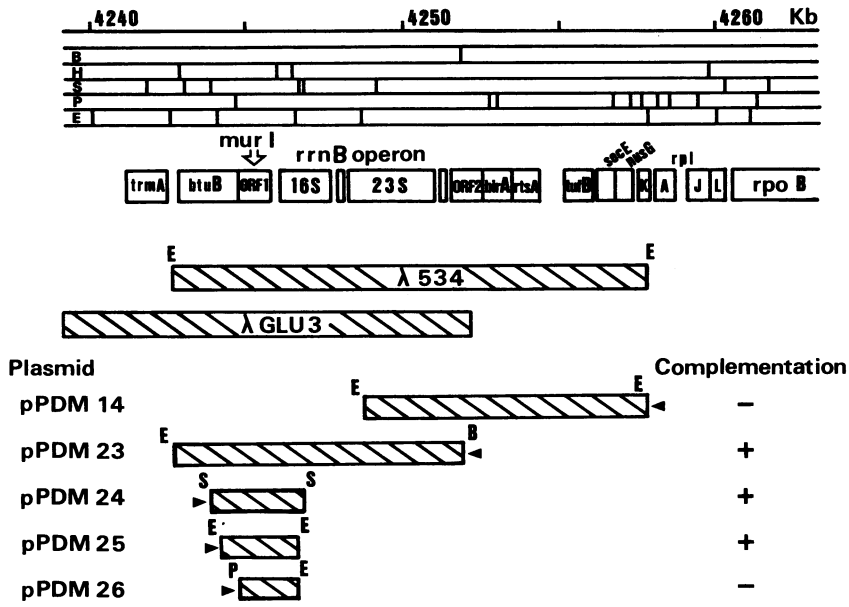

FIG. 3. Identification of the *murI* gene in the 90-min region of the *E. coli* chromosome. Locations of genes from the 90-min region in relation to the physical map of the *E. coli* chromosome (26) are shown at the top. Positions of cleavage sites are shown for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Pst*I (P), and *Sal*I (S). Bacterial DNA present in λ534 and in one (λGLU3) of the phasmids conferring D-glutamic acid prototrophy to WM335 cells is represented (hatched regions). Plasmid inserts derived from λ534 are shown below. The position of the *lac* promoter relative to the insert in each pUC19-derived plasmid is indicated by an arrowhead. In the case of plasmid pPDM23, the arrowhead indicates the position of the promoter of a disrupted tetracycline resistance gene from the pLG339 vector relative to the insert. The capability of plasmids to complement the D-glutamic acid auxotrophy of strain WM335 is indicated on the right (+, complementation; −, no complementation).

The *murI* gene was thus located within a 9-kb fragment corresponding to the overlap between λ534 and λGLU3 inserts. This location was confirmed by the construction of plasmid pPDM23 (Fig. 3), which harbors only this 9.3-kb *Eco*RI-*Bam*HI chromosomal fragment and effectively complemented the specific defect of strain WM335. Analysis of the DNA sequence of this fragment revealed only one open reading frame of unknown function, ORF1 (6), which was flanked on the left by the gene *btuB*, coding for the vitamin B_{12} receptor protein (19), and on the right by the *rrnB* operon, which contains the genes for 16S, 23S, and 5S rRNAs (6). ORF1 was thus considered the only candidate for the *murI* gene required for D-glutamic acid synthesis in *E. coli*. To reduce the size of the ORF1-containing insert, *Sal*I and *Eco*RI digests of λ534 DNA were cloned into the corresponding sites of plasmid vector pUC19. Surprisingly, when plasmids from more than 50 ampicillin-resistant JM83 clones appearing as white colonies on 2xYT–X-Gal plates were analyzed, it appeared that all possible fragments except the 2.8-kb *Sal*I-*Sal*I and 2.5-kb *Eco*RI-*Eco*RI inserts, which both contained ORF1, had been inserted. Boros et al. (4) have also reported that it was difficult to clone and maintain this chromosomal region proximal to the *rrnB* operon into multicopy plasmids, probably because it contains the two strong tandem promoters of rRNA genes. This difficulty was resolved by transforming directly mutant strain WM335 with these ligation mixtures (by electroporation) and selecting ampicillin-resistant transformants that grew in the absence of D-glutamic acid. Plasmids pPDM24, carrying the 2.8-kb *Sal*I-*Sal*I fragment, and pPDM25, carrying the 2.5-kb *Eco*RI-*Eco*RI fragment, both complementing the mutation and expressing ORF1 under the control of the *lac* vector promoter (Fig. 3), were effectively selected by this procedure. Finally, an internal *Pst*I deletion in pPDM25 provided plasmid pPDM26, which was unable to restore the D-Glu$^+$ phenotype (Fig. 3). All of these results were consistent with the identification of the *murI* gene as ORF1, which begins within the end of the preceding *btuB* gene (19) at a putative ATG initiation codon located 25 bp upstream from the *Pst*I restriction site (6).

DISCUSSION

E. coli B/r strain WM335, which is auxotrophic for D-glutamic acid, was used in this work as a tool for cloning the *murI* gene, which is required for D-glutamic acid synthesis in *E. coli* K-12. It resembles other previously described mutants with defects in peptidoglycan synthesis in terms of the lytic phenotype that it exhibits under restrictive growth conditions. In this work, the effects of a D-glutamic acid deficiency on the rate of peptidoglycan synthesis and on the pool levels of nucleotide precursors were investigated. The mutant was shown to accumulate UDP-MurNAc-L-alanine and to a lesser extent UDP-MurNAc, while pools of precursors located downstream in the pathway were significantly depleted. As a result, the rate of peptidoglycan synthesis decreased, and we observed that lysis of cells depleted of D-glutamic acid occurred when their peptidoglycan content was reduced to a value representing 75% of the normal cell level. It might be assumed that the reduced peptidoglycan content determined in the mutant cells just before cell lysis occurs probably represents in this strain the lowest physiological value compatible with cell integrity.

We confirmed in this study that the accumulation of UDP-MurNAc-L-alanine is the consequence of a defective enzyme in the biosynthesis of D-glutamic acid rather than of

an altered D-glutamic acid-adding enzyme. However, previous attempts to demonstrate the presence of D-glutamate:D-alanine transaminase or glutamate racemase activity in *E. coli* cells were unsuccessful. *E. coli* cannot grow in a minimal medium containing D-glutamic acid as the sole nitrogen source but grows well in a medium containing L-glutamic acid (40, 49). This property was used previously for the cloning of genes encoding D-amino acid aminotransferase and glutamate racemase activity from other bacterial species, on the assumption that *E. coli* clones producing these activities would assimilate D-glutamic acid (40, 49). Although these authors reported that the *E. coli* C600 host strain was deficient in both of these enzymes, it could be imagined that the enzyme responsible of the synthesis of D-glutamic acid in *E. coli* is produced in cells at a very low level of activity sufficient to sustain the specific requirements of peptidoglycan synthesis but inadequate for the utilization of this D-amino acid as a nitrogen source.

This paper reports the identification of the gene which is required for the synthesis of D-glutamic acid in *E. coli*. Complementation of the auxotrophic strain WM335 by different DNA sources unequivocally mapped the gene that we named *murI* in the 90-min region of the chromosome, within a 15-kb fragment that was previously sequenced. It is clearly demonstrated that this gene corresponds to the 867-bp ORF1 (6), located between the gene *btuB* (19), which encodes the minor outer membrane protein responsible for the initial steps of entry of vitamin B_{12}, phage BF23, and E colicins, and the *rrnB* operon (6), which contains the genes for 16S, 23S, and 5S rRNAs. It is noteworthy, when one considers the ATG initiation codon proposed for ORF1 (6), that the coding sequence overlaps by 66 bp the end of the preceding *btuB* gene (19). Final proof for the assignment of the initiation codon of *murI* will be provided by the determination of the N-terminal amino acid sequence of the corresponding protein, but it seems to be correct since a deletion of the first 25 bases that follow this putative ATG codon (*Pst*I deletion) effectively abolished gene expression. The predicted *murI* gene product consists of a protein of 289 amino acids that has a calculated molecular weight of 31,504, is slightly acidic (pI 5.6), and contains a large number of hydrophobic residues (6). Synthesis of a protein of the expected size was observed in minicells programmed by recombinant plasmids carrying this region (14). Baliko et al. (3) showed that the protein could be overproduced to a high level in the cytoplasm of *E. coli* cells without a significant effect on bacterial growth. These authors also reported that the functional integrity of the gene seems to be essential because efforts to replace it by a plasmid-encoded copy mutagenized by a transposon failed (3). Unfortunately, no indication as to the function of the *murI* gene product could be obtained from the sequence of the theoretical polypeptide chain, which exhibits no significant homology with other proteins involved in the racemization of amino acids (at least those whose sequences are available in data banks).

We also recently identified (11) the *murB* gene, coding for the UDP-GlcNAc-enolpyruvate reductase, as an open reading frame of unknown function (ORF2) (6, 22) which is located in this region 6 kb clockwise with respect to the *murI* gene (Fig. 3). This finding was in total agreement with recent data of Pucci et al. (43), who reported the identification and DNA sequence of the *murB* gene of *E. coli* B. Apparently, the *murI* and *murB* genes do not belong to a cluster of tightly packed genes of related functions as is the case for the other *mur* genes in the 2-min region of the *E. coli* chromosome (34). The cellular functions of all of the other genes from this particular region (*trmA*, *btuB*, the *rrnB* operon, *birA*, *rts*, *tufB*, *secE*, and *nusG*) have been established (6, 12, 17, 19, 22, 47). The *murA* gene, coding for the phosphoenolpyruvate:UDP-GlcNAc enolpyruvyl transferase, which was tentatively mapped in this region close to *argH* (50), remains to be identified.

ACKNOWLEDGMENTS

We thank B. Bachmann for supplying most of the *E. coli* strains used in this work and Y. Kohara and J. P. Bouché for providing phage clones. We are grateful to J. Caillet for the generous gift of the phasmid library and to A. Charbit for supplying plasmid pAC1.

This work was supported by grants from the Centre National de la Recherche Scientifique (URA 1131) and the Institut National de la Santé et de la Recherche Médicale (900314). P. Doublet was supported by a scholarship from the Ministère de la Recherche et de la Technologie.

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