JOURNAL OF BACTERIOLOGY, May 1993, p. 2970–2979
0021-9193/93/102970-10$02.00/0

Vol. 175, No. 10

The *murI* Gene of *Escherichia coli* Is an Essential Gene That Encodes a Glutamate Racemase Activity

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Received 16 December 1992/Accepted 14 March 1993

The *murI* gene of *Escherichia coli* was recently identified on the basis of its ability to complement the only mutant requiring D-glutamic acid for growth that had been described to date: strain WM335 of *E. coli* B/r (P. Doublet, J. van Heijenoort, and D. Mengin-Lecreulx, J. Bacteriol. 174:5772–5779, 1992). We report experiments of insertional mutagenesis of the *murI* gene which demonstrate that this gene is essential for the biosynthesis of D-glutamic acid, one of the specific components of cell wall peptidoglycan. A special strategy was used for the construction of strains with a disrupted copy of *murI*, because of a limited capability of *E. coli* strains grown in rich medium to internalize D-glutamic acid. The *murI* gene product was overproduced and identified as a glutamate racemase activity. UDP-*N*-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala), which is the nucleotide substrate of the D-glutamic-acid-adding enzyme (the *murD* gene product) catalyzing the subsequent step in the pathway for peptidoglycan synthesis, appears to be an effector of the racemase activity.

The biosynthesis of bacterial cell wall peptidoglycan (murein) is a complex process involving many different cytoplasmic and membrane steps (18, 37). In *Escherichia coli*, D-glutamic acid is one of the specific components of the peptidoglycan structure. It is incorporated specifically into this metabolic pathway by its addition to UDP-*N*-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala), a reaction catalyzed by the D-glutamic-acid-adding enzyme (the *murD* gene product) (28, 35). The enzymatic mechanism by which D-glutamic acid is produced in bacteria has been investigated for a limited number of species. Two different routes for D-glutamic acid biosynthesis have been identified: (i) a transamination process catalyzed by a D-amino acid transaminase with D-alanine and α-ketoglutarate, as demonstrated in *Bacillus* species (24, 40), or (ii) a direct conversion of L-glutamic acid to D-glutamic acid catalyzed by a glutamate racemase, as observed with *Lactobacillus* and *Pediococcus* species (33, 34). However, attempts to identify one of these two enzymatic activities in *E. coli* were until the present unsuccessful (11, 22, 33, 40).

We recently investigated in more detail this problem and in particular the genetics of D-glutamic acid biosynthesis in *E. coli* (11). For this purpose, we used the only known mutant requiring D-glutamic acid that had been described to date: strain WM335 of *E. coli* B/r (17, 22), which resembled other previously described mutants with defects in peptidoglycan synthesis by the lytic phenotype it exhibited under restrictive growth conditions. In particular, we identified the gene we named *murI* whose alteration apparently provoked the auxotrophy for D-glutamic acid in the mutant strain (11). The gene was unambiguously mapped in the 90-min region of the *E. coli* chromosome and corresponded to a previously sequenced open reading frame ORF1 (6), flanked on the left by the *btuB* gene encoding the vitamin B_{12} outer membrane receptor protein (1, 16) and on the right by the *rrnB* operon of genes for 16S, 23S, and 5S rRNAs (5, 6, 14). From the DNA sequence of this chromosomal region (6), the *murI* gene product was predicted to be a protein of 289 amino acids with a molecular weight of 31,500.

It was previously shown that *murI* was the only gene capable of complementing the specific defect of strain WM335 (11). However, this last observation was not sufficient to assess that only one gene product could sustain D-glutamic acid synthesis in *E. coli*. Two genes for alanine racemases as well as two genes for D-alanine:D-alanine ligases, which also catalyzed side reactions in this metabolic pathway, were previously identified in *E. coli* and *Salmonella typhimurium* (43, 44, 47). We report experiments of insertional mutagenesis of the *murI* gene demonstrating that it is essential for the biosynthesis of D-glutamic acid in *E. coli*. A special strategy for the construction of strains with a disrupted copy of *murI* in the chromosome was used, because of an apparently limited capability of *E. coli* strains to take up D-glutamic acid in rich medium. Furthermore, the *murI* gene product was identified as a glutamate racemase activity.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. The *E. coli* strains used in this study are listed in Table 1. Plasmid pPDM3 carrying the *lamB* gene of *E. coli* coding for the lambda receptor and the *cI857* gene that codes for a thermosensitive form of the lambda repressor was previously described (11). pUC18, pUC19, and pTTQ19 vectors and the Kan^r genblock originating from the pUC4K plasmid were purchased from Pharmacia, and plasmid pBTac1 was obtained from Boehringer. Plasmid pSKS114 (39) was used as a source of the *cat* cartridge. Plasmid pMAK705 (15) bearing a thermosensitive replicon was obtained from S. R. Kushner via Y. Mechulam (Laboratoire de Biochimie, Ecole Polytechnique, Palaiseau, France). Phage λ*darg13* carrying a 20-kb chromosomal fragment from *E. coli* extending from *ppc* to *murI* was previously described (25). Phage clone λE11C11 (clone 534) from the *E. coli* miniset library of Kohara et al. (20) initially

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype or marker	Source or reference[a]
E. coli K-12 strains		
JM83	*ara*Δ(*lac-proAB*) *rpsL thi* φ80 d*lacZ*Δ*M15*	46
JM109	*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thiA hsdR17 supE44 relA1* (F′ *traD36 proAB lacI*^{q}ZΔ*M15*)	46
AB1133	F$^-$ *thr-1 leuB6* Δ(*gpt-proA*)*62 hisG4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL-31 supE44*	2
JC7623	AB1133 *tsx-33 recB21 recC22 sbcB15*	45
NFB366	JC7623 (λ199 and λ*darg13*)	This work
FB8	F$^-$ prototroph	F. Blasi
JE1011	F$^-$ *thr leu trp his thy thi lac gal xyl mtl rpsL azi*	32
ST5	JE1011 *mrbA* (*murB*)	B. Bachmann CGSC 6442
NK5139	thi-39::Tn*10* IN(*rrnD-rrnE*)*1*	B. Bachmann CGSC 6165
BW6175	Hfr λ$^-$ *thr-1 leuB6 azi-15 tonA21 lacY1 supE44 argE86*::Tn*10 thi-1*	B. Bachmann CGSC 6763
BW6165	Hfr *ara-41*, *lacY1*, or *lacY40* λ^{ind-} *xyl-7 mtl-2 argE86*::Tn*10*	B. Bachmann CGSC 6760
NFB279	*thr-1 leuB6* Δ(*gpt-proA*) *62 hisG4 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 btuB202*::Tn*10 rpsL31 supE44*	This work
β180	MG1655 *metA* Δ::Cm^r	C. Richaud
GRA83	JM83 *murI*::*kan* (pMAKI)	This work
E. coli B/r strains		
WM301	*leu pro trp his arg thyA deoB met lac gal xyl ara mal lam phx rpsL hsdS-K12*	W. Messer PC 2341
WM335	WM301 *glt* (*murI-335*)	W. Messer PC 2342
GRA101	WM335 *murI-335 argE86*::Tn*10*	This work
GRA102	WM335 *murI-335 btuB202*::Tn*10*	This work
GRA103	WM335 *murI-335 thi-39*::Tn*10*	This work
GRA104	WM335 *murI*$^+$ *metA*Δ::Cm^r	This work

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

selected for complementation of the D-glutamic-acid-requiring mutant strain was used as the DNA source for the cloning of the *murI* gene, as previously described (11).

Growth conditions. Unless otherwise noted, 2YT (31) as a rich medium and M63 (31) 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 (100 μg · ml^{-1}), thymine (100 μg · ml^{-1}), thiamine (1 μg · ml^{-1}), and L-amino acids (50 μg · ml^{-1}) were also added in the case of specific strain requirements. For strains carrying drug resistance genes, antibiotics were used at the following concentrations (in micrograms per milliliter): ampicillin (100), streptomycin (50), kanamycin (30), chloramphenicol (25), and tetracycline (15). Broth for plates was solidified with 1.5% agar, and when plasmid inserts were screened 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. Cells of GRA83 (1-liter cultures) were grown exponentially at 30°C in 2YT medium. At the appropriate cell concentration (3 × 10^6 · ml^{-1}), the temperature of the culture was either maintained at 30°C or increased to 43°C. Incubation was continued until the optical density of the culture at 43°C reached a plateau value of 0.5 about 3 h later (see Fig. 2). 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. As a control, similar cultures were made with strain JM83 (pMAKI) harboring an intact chromosomal copy of *murI*. The extraction of peptidoglycan precursors and the analytical procedure used for their quantitation were as previously described (26, 27).

Separation of isomers of glutamic acid. The relative amounts of both isomers of glutamic acid were estimated in cell extracts and in reaction mixtures (see assays for glutamate racemase activity below) by a recently developed high-performance liquid chromatography (HPLC) technique, after derivatization of aliquots by Marfey's chiral reagent. Diastereoisomers of L- and D-glutamic acid obtained after reaction with 1–fluoro–2,4–dinitrophenyl–5–L-alanine–amide as described by Marfey (23) were separated by HPLC as described in the legend to Fig. 5.

Isolation of sacculi and quantitation of peptidoglycan. Exponential-phase cells of GRA83 were grown at 30°C or first at 30°C and then at 43°C 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 (SDS) 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. Aliquots were hydrolyzed and analyzed as previously described (26, 30), and the peptidoglycan content of the sacculi was expressed in terms of its diaminopimelic acid content. The specific incorporation of radioactive *meso*-diaminopimelic acid into peptidoglycan of an *lysA* derivative strain (29) was also used with similar results.

General DNA techniques and *E. coli* cell transformation. The isolation of phages and the extraction and purification of their DNAs were carried out by standard techniques (9, 38). Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (38), and plasmids were eventually further purified with cesium chloride-ethidium bromide gradients. Standard procedures for endonuclease digestions, ligation, filling in of 5′-protruding ends by using the Klenow

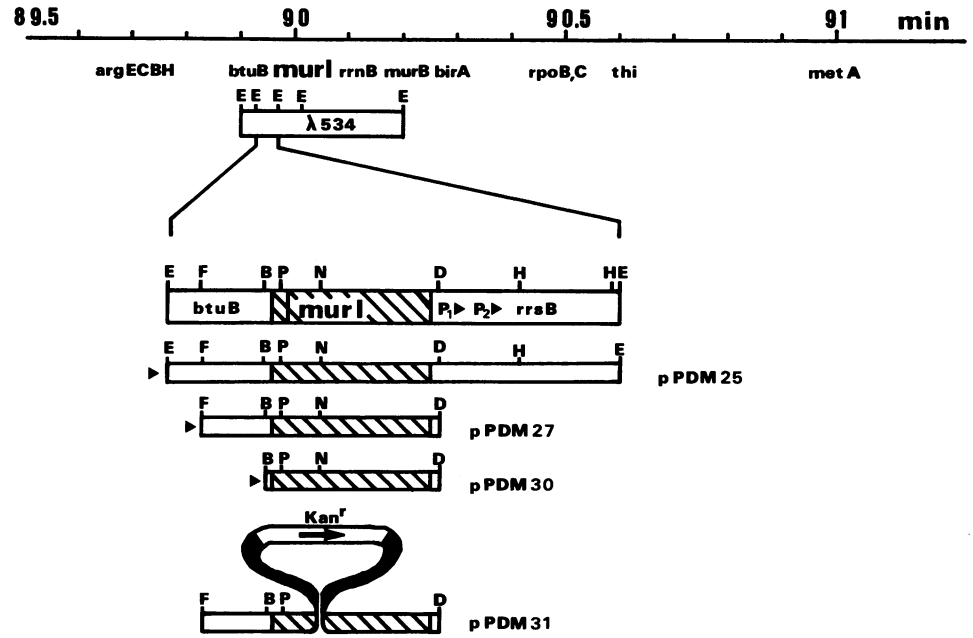

FIG. 1. Localization of the *murI* gene in the 90-min region of the *E. coli* chromosome. Locations of some other genes (3) used in our transduction experiments are indicated at the top. Bacterial DNAs present in phage clone λ534 from the library of Kohara et al. (20) and in the derived plasmid inserts are indicated below. The *murI* gene is represented as a hatched region, and the position of the *lac* promoter relative to the insert in each pUC18- or pUC19-derived plasmid is indicated by an arrow. Positions of cleavage sites are shown for *Bst*EII (B), *Dra*I (D), *Eco*RI (E), *Eco*RV (F), *Hin*dIII (H), *Nae*I (N), and *Pst*I (P).

fragment of DNA polymerase I, and agarose electrophoresis were used (38). Usually, *E. coli* cells were made competent for transformation with plasmid DNA by the method described by Dagert and Ehrlich (8), but the technique of electroporation was preferentially used when strain WM335 of *E. coli* B/r was transformed.

Complementation tests of the D-glutamate auxotrophy. (i) λ*darg13* phage particles. A 200-μl volume of a suspension of WM335(pPDM3) cells in 10 mM $MgSO_4$ was incubated for 1 h at 30°C without shaking with a lysate containing a mixture of λ199 (carrying the *cI857* gene) and λ*darg13* (a λ199-transducing derivative) phage particles ($10^{10} \cdot ml^{-1}$, at a multiplicity of infection of 10). After addition of 400 μl of 2YT medium supplemented with D-glutamic acid, the mixture was incubated at 30°C with shaking for an additional 2-h period for expression of phage genes. Cells were recovered by centrifugation, washed several times with 2YT medium to remove residual D-glutamic acid, and then plated on 2YT plates not supplemented or supplemented with D-glutamic acid. Growth was observed after 24 h of incubation at 30°C on plates lacking D-glutamic acid, and clones were also tested for lysogenization by lambda phages (thermosensitive growth).

(ii) 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 being heated for 3 min at 42°C. Then, 400 μl of 2YT medium supplemented with D-glutamic acid was added, and cells were incubated at 37°C for over 1 h for expression of plasmid genes. Cells were recovered by centrifugation and washed several times with 2YT medium. A final suspension made in 200 μl of 2YT medium was separated into 2 parts which were plated onto two 2YT-antibiotic plates not supplemented or supplemented with D-glutamic acid and incubated at 37°C. Growth was observed after 24 h of incubation.

Construction of plasmids. DNA from phage λE11C11 (clone 534) from the collection of Kohara et al. (20) was used as the starting material for the construction of the different plasmids used in this study (see Fig. 1). Plasmid pPDM25 carrying the 2.5-kb *Eco*RI chromosomal fragment that contains the *murI* gene of *E. coli* has been previously described (11). The size of the *murI*-containing insert was reduced to 1.3-kb *Eco*RV-*Dra*I and 0.96-kb *Bst*EII-*Dra*I fragments that were cloned into the *Hin*cII site of the pUC18 vector to generate plasmids pPDM27 and pPDM30, respectively (Fig. 1). For an expression of *murI* under the control of the lambda p_R promoter, plasmid pPDM33 was constructed by inserting the 2.4-kb *Xho*II lambda fragment (carrying the structural gene *cI857* encoding a thermosensitive form of the lambda *cI* repressor, the strong p_R promoter, and the Shine-Dalgarno sequence of *cro*) into the unique *Bam*HI site of plasmid pPDM30. Plasmid pPDM34 was constructed by inserting the initial 2.5-kb *Eco*RI fragment into the corresponding site of the pTTQ19 vector, in the orientation dependent on the *tac* promoter control. A *cat* cartridge originating from plasmid pSKS114 was inserted in both possible orientations into the unique *Nae*I site of plasmid pPDM34 (lying in the middle of the *murI* gene sequence) to generate plasmids pPDM35 and pPDM36. Similarly, a *kan* cartridge originating from pUC4K was inserted in both possible orientations into the *Nae*I site of plasmid pPDM27, generating pPDM31 and pPDM32 (Fig. 1). The pMAKI*kan* plasmid was obtained by insertion of the 2.5-kb *Eco*RI-*Sph*I fragment from pPDM31 into the *Hin*dIII site of pMAK705 (15). The pMAKI plasmid was isolated as described in the text during the excision process that followed integration of the pMAKI*kan* plasmid at the chromosomal *murI* locus. The

construction of the pTACI2 plasmid carrying the *murI* gene under the control of the *tac* promoter is diagrammed in Fig. 3.

Disruption of the chromosomal *murI* gene. The wild-type chromosomal copy of the *murI* gene of *E. coli* strains was replaced by a disrupted one by following two alternative methods. (i) The procedure described by Hamilton et al. (15), which uses pMAK705, a plasmid bearing a thermosensitive replicon, was used. pMAKI*kan*, a pMAK705 derivative carrying the disrupted *murI* gene, was transformed into JM83. Integration of the plasmid into the chromosome was selected by plating the cells at 44°C on 2YT plates containing 20 μg of chloramphenicol ml^{-1}. Several clones were picked up, and the integration of pMAKI*kan* at the *murI* locus was verified by Southern analysis (data not shown). The plasmid was then excised from the chromosome as follows. Cointegrants were grown at 30°C in 2YT-chloramphenicol for at least 30 doubling times and were subsequently plated at 30°C. Individual clones were then screened for sensitivity to chloramphenicol at 44°C, which is indicative of plasmid excision. The structures of the excised plasmids from several clones were determined by DNA restriction analysis. One clone containing a plasmid bearing the wild-type *murI* gene (called pMAKI) was chosen. Finally, the replacement in that strain (named GRA83) of the chromosomal *murI* copy by the inactivated one was verified by Southern analysis (data not shown). (ii) The procedure described by Winans et al. (45) was used. Plasmid pPDM35 or pPDM36 harboring a disrupted copy of the *murI* gene (by a *cat* cartridge) was linearized with *Bam*HI and used to transform strain JC7623, a *recB recC sbcB* strain that can be transformed by linear DNA (45). Exchange of the mutated copy of *murI* with the chromosomal wild-type gene was selected for by plating of transformed bacteria on 2YT plates in the presence of chloramphenicol and D-glutamic acid.

Preparation of crude enzyme. Cells from JM109(pBTac1) and JM109(pTACI2) (constructs diagrammed in Fig. 3) were grown exponentially (1-liter cultures, in duplicate) at 37°C in LB medium (31) supplemented with ampicillin. When the optical density of the cultures reached 0.1, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to one of the cultures at a final concentration of 1 mM, and growth was continued for 3 h. Cells were harvested in the cold and washed with 40 ml of cold 0.02 M potassium phosphate buffer (pH 7) containing 0.1% β-mercaptoethanol (standard buffer). The wet cell pellet (ca. 1.5 g) was suspended in 12 ml of the same buffer and disrupted by sonication (Sonicator 150; T. S. Ultrasons, Annemasse, France) for 10 min with cooling. The resulting suspension was centrifuged at 4°C for 30 min at 200,000 × *g* with a Beckman TL100 centrifuge. The supernatant was eventually dialyzed overnight at 4°C against 100 volumes of the standard buffer, and the resulting solution (300 mg of protein per 11 ml), designated the crude enzyme, was stored at −20°C. SDS-polyacrylamide gel electrophoresis (PAGE) for the analysis of the proteins from the crude extracts was performed as previously described (21) with 13% polyacrylamide gels.

Enzymatic assays. Glutamate racemase activity in crude enzyme preparations was determined by two different procedures. (i) In the coupled assay, the D-glutamic acid produced from the L-isomer by the glutamate racemase is converted to UDP–MurNAc–L-Ala–D-Glu by the D-glutamic-acid-adding enzyme also present at a high level of activity in crude extracts (30, 35). The standard assay mixture contained 0.1 M Tris-hydrochloride buffer (pH 8.6), 20 mM $MgCl_2$, 10 mM ATP, 0.02 mM [L-^{14}C]glutamic acid (100,000 Bq; Amersham), 0.04 mM UDP–MurNAc–L-Ala, and crude enzyme (30 to 300 μg of protein) in a final volume of 80 μl. The mixtures were incubated at 37°C for 1 h, and reactions were terminated by the addition of 20 μl of acetic acid. Reaction products were separated by high-voltage electrophoresis on Whatman 3MM filter paper in 2% formic acid (pH 1.9) for 45 min at 40 V/cm with an LT36 apparatus (Savant Instruments, Hicksville, N.Y.), and the radioactive spots were located by overnight autoradiography with type R2 films (3M, St. Paul, Minn.) or with a radioactivity scanner (model Multi-Tracermaster LB285; Berthold France, Elancourt, France). The two radioactive spots (glutamic acid and UDP-MurNAc-dipeptide) were cut out and counted in an Intertechnique SL 30 liquid scintillation spectrophotometer with a solvent system consisting of 2 ml of water and 13 ml of Aqualyte mixture (J. T. Baker Chemicals, Deventer, The Netherlands). It was verified that [D-^{14}C]glutamic acid had in all cases been quantitatively converted to the nucleotide form by the D-glutamic-acid-adding activity. (ii) By direct assay, UDP–MurNAc–L-Ala and ATP-Mg^{2+} were omitted from the reaction mixture, and the ratio of L- to D-isomer of glutamic acid was determined by the HPLC procedure described above.

RESULTS

Attempts to transduce the *murI* mutation in bacterial strains other than the D-glutamic-acid-requiring mutant strain WM335. The *murI* gene of *E. coli*, which is required for the biosynthesis of D-glutamic acid, was recently identified in the 90-min region of the chromosome (11). This function was apparently altered in the only auxotrophic mutant described to date, strain WM335 of *E. coli* B/r (17, 22). We tried to transduce the mutation (named *murI-335*) into *E. coli* K-12. This was done by using *argE*, *btuB*, or *thi* markers (with the Tn*10* transposon inserted), chosen for their high frequencies of cotransduction with the wild-type *murI*$^+$ gene (35, 95, and 75%, respectively). In each case, clones of WM335 that acquired tetracycline resistance but remained auxotrophic for D-glutamic acid were isolated (strains GRA101 to GRA103). P1 phage lysates produced on these strains were used to transduce again the Tn*10* transposon into a prototrophic strain, FB8, of *E. coli* K-12. Transductants were selected on 2YT plates supplemented with tetracycline and D-glutamic acid. Surprisingly, all of the isolated *argE*::Tn*10*, *btuB*::Tn*10*, and *thi*::Tn*10* clones (more than 300 in each case) also appeared prototrophic for D-glutamic acid. This unexpected finding was not due to a difference between B and K-12 species in the organization of the genes in this region of the chromosome, since the same was observed if the parental strain WM301 of *E. coli* B/r was used as the recipient strain. It was verified that our phage stocks carried the *murI-335* allele. Strain WM335 was first converted to prototrophy by acquisition of the *murI*$^+$ gene, which was cotransduced in that case at 25% with a *metA*::Cm marker. Then, we observed that the latter clone (GRA104) returned to the initial auxotrophic status when transduced to Tet^r by the phage stocks Tet^r *murI-335* described above.

These results could be interpreted in two ways. (i) The *murI* mutation was effectively transferred to the other genetic backgrounds, but without any effect on D-glutamic acid biosynthesis, a result indicative of the presence of another gene on the *E. coli* chromosome compensating for the *murI* mutation. However, this hypothesis seemed unlikely, since our previous experiments of complementation of the WM335 defect by various DNA sources allowed us to identify only

the *murI* gene. (ii) The *murI* mutation could not be transferred to strains other than the WM335 mutant, probably as a result of an additional mutation in the latter strain that is a prerequisite to the isolation of mutants altered in the *murI* gene. This second hypothesis was corroborated by studying the cotransduction frequencies of three markers equidistant from this region (Fig. 1): *argE*, *murI*, and *murB*, which codes for the UDP-GlcNAc-enolpyruvate reductase (12, 36). A thermosensitive ST5 strain of *E. coli* K-12 (32) altered in the *murB* gene was transduced to tetracycline resistance by using two different phage stocks: one specifying *argE*::Tn*10* *murI*$^+$ *murB*$^+$ and one specifying *argE*::Tn*10* *murI-335* *murB*$^+$. Arginine-requiring clones were selected at 30°C on 2YT plates supplemented with tetracycline and D-glutamic acid. Although in the first case, 30% of the Tet^r ST5 derivatives isolated also became thermoresistant (*murB*$^+$), none of the Tet^r clones selected in the other experiment could grow at 42°C, a result suggesting that it was impossible to transfer the *murB*$^+$ gene together with a mutated copy of the *murI* gene.

A second mutation is present in strain WM335 that is essential for the isolation of a *murI* mutation. To confirm the involvement of the sole *murI* gene in the synthesis of D-glutamic acid in *E. coli*, we tried to inactivate this gene on the chromosome by using the procedure described by Winans et al. (45). Cartridges expressing resistance to chloramphenicol or kanamycin were inserted into various *murI*-carrying plasmids at the *Nae*I site located in a middle position within its sequence. The resulting plasmids with a disrupted copy of *murI* were linearized with enzymes that cut only in the polylinker of the vectors and were used to transform strain JC7623 of *E. coli* K-12, a *recB recC sbcB* strain that could be transformed with linear DNA (45). All of the Cm^r of Kan^r clones selected in the presence of D-glutamic acid were in fact also ampicillin resistant and thus contained the original intact plasmid. By this method, we were unable to exchange the chromosomal copy of the *murI* gene with a disrupted one, whatever the lengths of the homologous DNAs present in these plasmids on both sides of the inserted cartridge and in spite of the presence of a high concentration of D-glutamic acid in the growth medium.

The results from the experiments described above were that the altered *murI* gene from strain WM335 was untransferable to other *E. coli* strains and that the chromosomal *murI* gene could not be disrupted without affecting cell viability, although D-glutamic acid was present at a high concentration in the growth medium. This suggested the presence in strain WM335 of a second mutation that was essential for the expression of a phenotype requiring D-glutamic acid. The most likely hypothesis was that wild-type *E. coli* strains did not efficiently internalize D-glutamic acid, i.e., at a rate that is sufficient to fulfill the specific requirements of peptidoglycan synthesis. Transductants that acquired a disrupted copy of the *murI* gene in the chromosome probably lysed during the expression period, in spite of the presence of the D-amino acid in the growth medium.

We observed that labeled [DL-^{14}C]glutamic acid was internalized very poorly by both WM335 and WM301 strains when added to exponential-phase cells growing in rich medium (data not shown). However, a slightly higher rate of uptake was observed in the mutant cells. This probably explains the capability of the D-amino acid to complement the *murI* defect in this strain. It was not the aim of the present study to analyze in more detail the additional mutation that increased the uptake of D-glutamic acid in strain WM335. Likely candidates are the permeases specific for L-glutamic acid and in particular the permease encoded by the *gltS* gene which was previously shown to also transport the D-isomer (10, 19, 42).

Inactivation of the chromosomal *murI* gene and its effect on peptidoglycan metabolism. To inactivate the *murI* gene on the chromosome, we used the procedure described by Hamilton et al. (15) that is particularly well adapted in the case of essential genes. First, the *murI* gene-coding sequence carried by the pPDM27 plasmid was disrupted by the insertion at the *Nae*I site of the 1.24-kb kanamycin resistance gene from pUC4K, generating plasmid pPDM31 (Fig. 1). Then, the total insert from the pPDM31 plasmid was inserted into the pMAK705 vector which bears a thermosensitive replicon. The resulting plasmid, pMAKI*kan*, was used as described in Materials and Methods in the construction of a strain derived from JM83, GRA83 (JM83 *murI*::*kan* [pMAKI]), having the inactivated *murI* gene on the chromosome and the wild-type allele on the pMAK705 vector. At the nonpermissive temperature for plasmid replication (43 to 44°C), GRA83 failed to grow on 2YT plates, indicating that this strain with a disrupted copy of the *murI* gene on the chromosome was viable only in the presence in *trans* of a plasmid carrying the *murI*$^+$ gene. As a control, GRA83 was infected with P1 for transduction in strains having one (JC7623) or two (NFB366 [JC7623 λ*darg13*]) copies of the *murI* gene. When the selected marker was Kan^r, only the strain with two copies of *murI* yielded transductants, and it did so at a high frequency, a result confirming that *murI* is an essential gene.

Since the plasmid pMAKI bears a thermosensitive replicon, the effects of the inactivation of the *murI* gene were observed by shifting cultures of GRA83 cells growing at 30°C in 2YT medium to the nonpermissive temperature of 43°C. As shown in Fig. 2A, cells grew normally at 43°C for about 3 h and then rapidly lysed. The delay preceding cell lysis (about 9 generation times) corresponds to the time needed for the few copies of the pMAKI plasmid to be cured, followed by the time for the preexisting MurI molecules to be progressively diluted and for the remaining internal pool of D-glutamic acid to be consumed. To demonstrate that lysis occurred as a result of a depletion of the pool of D-glutamic acid, cells were harvested just before the onset of cell lysis, and their contents in peptidoglycan and nucleotide precursors were estimated. As previously shown with the WM335 mutant deprived of D-glutamic acid (11), GRA83 cells accumulated at 43°C large amounts of UDP–MurNAc–L-Ala, while pools of the precursors located downstream in the pathway were progressively depleted (data not shown). As a result, cell lysis was observed when the cell peptidoglycan content was reduced to a critical value representing 70% of its normal cell content.

Furthermore, D-glutamic acid was shown to restore the normal phenotype of GRA83 cells at 43°C when growth was performed in minimal medium (Fig. 2B). This result further indicated that the uptake of the D-amino acid by wild-type strains was blocked only when complex growth media were used. It was finally proved that this phenomenon was related to the presence in rich medium of a high concentration of L-glutamic acid which competes with the D-isomer for the same permease system (data not shown).

Overproduction of the *murI* gene product. Previous attempts to determine the biochemical mechanism by which the *murI* gene product synthesizes D-glutamic acid in *E. coli* had all been unsuccessful (11, 22, 33, 40). The overproduction of the *murI* gene product was therefore considered a prerequisite for a facilitated identification of this activity.

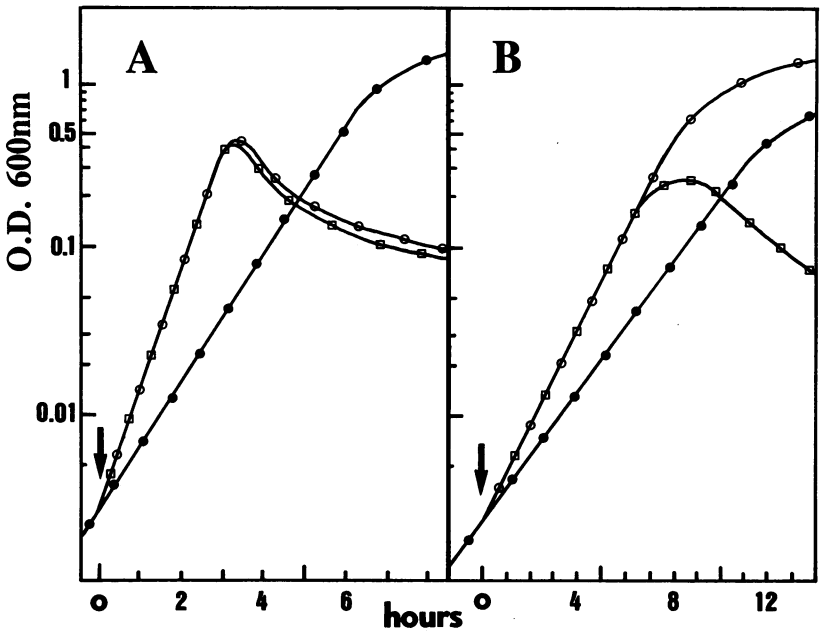

FIG. 2. Effect of an inactivation of the *murI* gene on the growth of *E. coli* K-12. GRA83 cells were grown exponentially at 30°C in either 2YT (A) or minimal medium supplemented with 0.4% glucose (B). At the time indicated by an arrow, the temperature of the culture was either maintained at 30°C or shifted to 43°C. Symbols: ●, growth at 30°C in the absence of D-glutamic acid; ○, growth at 43°C in the presence of D-glutamic acid; □, growth at 43°C in the absence of D-glutamic acid. O.D., optical density.

For this purpose, plasmids expressing the gene under the control of strong promoters were constructed. A first plasmid, pPDM33, contained the *Bst*EII-*Dra*I fragment expressed under the control of the lambda p_R promoter. However, when exponentially growing cells of JM83 (pPDM33) were shifted to 42°C (for derepression of the p_R promoter), no overproduction of a protein could be detected in SDS-PAGE analysis of the corresponding extracts. Baliko et al. (4) previously tried to determine the biological function of this gene, supposed to be related to the downstream *rrnB* operon of rRNA genes (5, 14). In particular, they reported that their attempts to achieve a significant overproduction of the protein by merely exchanging the promoter of the ORF1 gene (our *murI* gene) with a stronger promoter (in their case, the p_2 promoter of *rrnB*) failed completely, and they succeeded only in constructing an overproducing plasmid by exploiting the principle of translational coupling (4).

We thus constructed a plasmid expressing the *murI* gene directly under the control of the strong *tac* promoter (Fig. 3). In this construct, all of the chromosomal DNA sequence preceding the ATG initiation codon was eliminated. We took advantage of the fact that a unique *Pst*I site lies within the gene sequence at only 25 bp from the putative start codon (Fig. 1). The 1.3-kb *Pst*I-*Hin*dIII fragment that contains the truncated *murI* gene was purified and ligated to the pBTac1 vector (cut by *Eco*RI and *Hin*dIII), in the presence of a synthetic phosphorylated double-strand linker corresponding to the beginning of the gene (from the ATG initiation codon to the *Pst*I site) preceded by an *Eco*RI site (Fig. 3). The D-glutamic-acid-requiring strain WM355 was transformed with this ligation mixture by electroporation. By selecting directly for complementation, we isolated 25 clones that all carried the expected plasmid, called pTACI2 (Fig. 3).

Recombination-deficient strain JM109 carrying one copy of the *lacI*^{q} repressor gene on the F′ was then transformed with this plasmid. Exponential-phase JM109 cells harboring

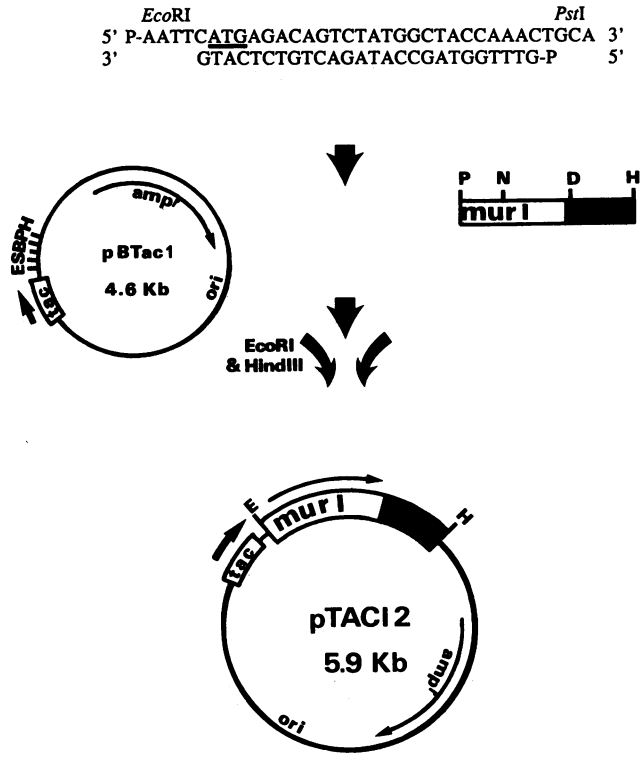

FIG. 3. Construction of plasmid pTACI2. The 1.3-kb *Pst*I-*Hin*dIII fragment that contains the *murI* gene truncated of its first 25 bases was ligated to the pBTac1 vector cut by *Eco*RI and *Hin*dIII in the presence of two phosphorylated synthetic oligonucleotides whose sequences are shown at the top. When hybridized together, these oligonucleotides reconstitute the 5′ end of the gene, from the initiation codon (underlined) to the *Pst*I site, preceded by an *Eco*RI site. Positions of cleavage sites are indicated for *Bam*HI (B), *Dra*I (D), *Eco*RI (E), *Hin*dIII (H), *Nae*I (N), *Pst*I (P), and *Sma*I (S).

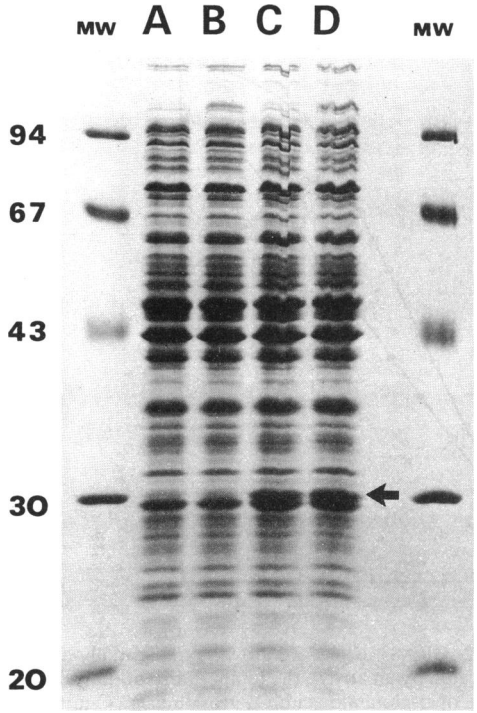

FIG. 4. Overproduction of a 31K (31,000-molecular-weight) protein in crude extracts from strain JM109(pTACI2). Strains JM109 (pTACI2) and JM109 harboring the control vector pBTac1 that does not contain the *murI* gene were grown exponentially at 37°C in LB-ampicillin medium. When necessary, IPTG was added at a 1 mM final concentration when the optical density of the culture reached 0.1. Cells were harvested 3 h later and were disrupted by sonication. As described in Materials and Methods, the protein contents of the soluble and particulate fractions obtained after high-speed centrifugation of the corresponding crude extracts were analyzed by SDS-PAGE. Molecular weight (MW) standards (in thousands) indicated on the left are as follows: phosphorylase *b* (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and soybean trypsin inhibitor (20). Lanes: A and B, analysis of the soluble fractions from JM109(pBTac1) cells grown in the absence or in the presence of IPTG, respectively; C and D, analysis of the soluble fractions from JM109(pTACI2) cells grown in the absence or in the presence of IPTG, respectively. The 31K protein corresponding to the *murI* gene product, highly overproduced in the extract from strain JM109(pTACI2), is indicated by the arrow on the right. This band was completely absent in the corresponding particulate fractions (data not shown).

this plasmid overproduced to relatively high levels the *murI* gene product (Fig. 4), detected by SDS-PAGE as a band migrating as a 31- to 32-kDa species, a finding in agreement with the value of 31,500 calculated from the DNA sequence (6). This band was detected exclusively in the soluble fraction of the extract. A high-level overproduction of the protein was already observed in the absence of IPTG (Fig. 4), probably because a single copy of *lacI*^{q} did not produce enough repressor molecules to efficiently repress the transcription of the *murI* gene from this high-copy-number plasmid.

Identification of the *murI* gene product as a glutamate racemase activity. The apparent overproduction of the *murI* gene product prompted us to assay the corresponding enzymatic extracts for glutamate racemase and D-amino acid transaminase activities. To detect an eventual racemase activity in crude extracts, we first used a convenient coupled assay in which the D-glutamic acid produced from the L-isomer was converted to UDP-MurNAc-L-Ala-D-Glu, taking advantage of the activity of the D-glutamic-acid-adding enzyme also present in the extracts. This procedure allowed us to detect a high level of glutamate racemase activity in extracts from strain JM109(pTACI2), compared with the practically undetectable level in extracts from strain JM109(pBTac1) or from wild-type strains previously investigated (11). The specific activities of glutamate racemase (in nanomoles of UDP-MurNAc-L-Ala-[D-^{14}C]Glu synthesized per hour per milligram of protein) were as follows for crude extracts from cells grown in LB medium: JM109(pBTac1) with or without IPTG, 0.02; JM109(pTACI2) without IPTG, 3.8 (overproduction factor, 190); and JM109(pTACI2) with IPTG, 4.6 (overproduction factor, 230). Evidence that [D-^{14}C]glutamic acid was synthesized by direct racemization of the L-isomer and not by the action of a D-alanine:D-glutamic acid transaminase was provided by different experiments. (i) The racemase activity was still detected after an extensive dialysis of the extract which eliminated the cosubstrates of putative transaminase activities. It should be noted that dialysis was accompanied by a significant loss of racemase activity (more than 50%), but we verified that it was related to the instability of the enzyme rather than to the loss of eventual cofactors or substrates. (ii) Addition of α-ketoglutarate (1 mM) in the reaction mixture did not significantly affect the transfer of radioactivity between isomers of glutamic acid, a result indicating clearly that α-ketoglutarate was not an intermediate in the process. (iii) Addition of D-cycloserine (1 mM) only slightly inhibited the reaction, while it was previously shown that the D-alanine:D-glutamate transaminase from *Bacillus* species (24) as well as other enzymes with D-alanine as substrate were completely inhibited by micromolar concentrations of this antibiotic.

When a direct assay was carried out without UDP-MurNAc-L-Ala, no [D-^{14}C]glutamic acid was detectable by HPLC analysis. The reaction mixture was devoid of ATP and Mg^{2+}, which are required for the conversion of D-glutamic acid to the nucleotide UDP-MurNAc-L-Ala-D-Glu. We hypothesized that D-glutamic acid could be a very good substrate of the racemase, with a K_m value lower than that of the L-isomer, implying that any molecule of D-glutamic acid produced in the assay had been rapidly reused for the reverse synthesis of the L-isomer, if not immediately incorporated into the nucleotide precursor. In the presence of very low concentrations of UDP-MurNAc-L-Ala (5 μM), at least 30% of the radioactivity from L-glutamic acid was recovered under the D-isomer form. This effect was specific, since no other nucleotide precursor from UDP-GlcNAc to UDP-MurNAc-pentapeptide was an effector when added at 0.1 mM. A purification of the glutamate racemase is now required to determine the kinetic parameters of both forward and reverse reactions catalyzed by this enzyme and to investigate the UDP-MurNAc-L-Ala effect in more detail.

Effects of an overproduction of glutamate racemase on the pool of D-glutamic acid. The overexpression of glutamate racemase activity in JM109(pTACI2) cells was without apparent effect on cell growth and bacterial shape. We assumed that an overproduction of this enzymatic activity in the cell content could have resulted in a modification of the pool levels of both isomers of glutamic acid. It was effectively observed that the pool of glutamic acid (L plus D) in cells growing exponentially in 2YT medium was significantly increased in the presence of the plasmid (from 19,000 to 27,000 nmol when expressed per g of bacterial dry weight). Moreover, the D-isomer, which normally represents a small

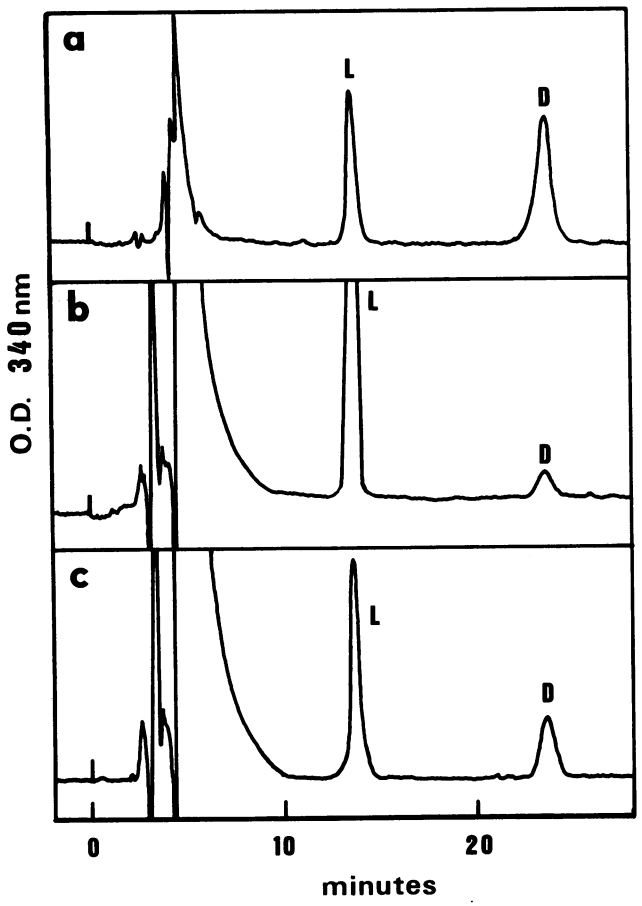

FIG. 5. Separation of L- and D-isomers (L and D, respectively) of glutamic acid by HPLC after derivatization with Marfey's reagent. Diastereoisomers obtained after reaction with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide were separated by HPLC on a LiChrospher 100CH 18/2 column (Merck). Elution was with 12 mM ammonium phosphate (pH 5.6) containing 10% acetonitrile. The flow rate was 1 ml · min^{-1}, and the column temperature was 37°C. Elution was monitored at 340 nm at a sensitivity of 0.04 absorbance units (full scale). (a) Analysis of a standard mixture containing 0.5 nmol of each isomer of glutamic acid; (b and c) analysis under the same conditions of the pool of free glutamic acid isolated from JM109(pBTac1) and JM109(pTACI2) cells, respectively. O.D., optical density.

percentage (3% in that case) of this pool, was now detected as more than 20% of the total glutamic acid (Fig. 5).

DISCUSSION

D-Glutamic acid is an essential component of bacterial cell wall peptidoglycan and is practically exclusively encountered therein. Identification of the enzymatic process by which this D-amino acid is synthesized in bacteria is therefore of the greatest interest, since it could be considered a potential target in the search for new antibacterial compounds. In early studies, the biosynthesis of D-glutamic acid was investigated in *Bacillus*, *Lactobacillus*, and *Pediococcus* species, but only little attention had been paid to this problem in enteric bacteria such as *E. coli* and other medically important bacterial species.

The genetics of D-glutamic acid biosynthesis in *E. coli* was recently investigated in detail with the only mutant requiring D-glutamic acid for growth that had been described to date: strain WM335 of *E. coli* B/r (17, 22). The altered gene, named *murI*, was identified as a previously sequenced open reading frame, ORF1 (6), lying in the 90-min region of the chromosome, between the *btuB* gene encoding the vitamin B_{12} receptor protein (16) and the *rrsB* gene coding for the 16S rRNA (6). It was noteworthy that the *btuB* and *murI* genes were in a considerable 65-bp overlap (1, 6). Interestingly, several mutations affecting peptidoglycan metabolism were earlier located in this region of the chromosome near *argH* (32, 41), which was consequently named *mrb*, for murein region b, including, in particular, a mutation conferring resistance to fosfomycin (41), which was interpreted as an alteration of the *murA* gene coding for a PEP:UDP-GlcNAc-enolpyruvyl transferase, as well as a mutation characterized by a thermosensitive lytic phenotype and the accumulation of the pool of UDP-GlcNAc-enolpyruvate at the restrictive growth temperature (strain ST5 in reference 32). In this latter case, the corresponding altered gene *murB* that encodes the UDP-GlcNAc-enolpyruvate reductase was recently identified (12, 36), 6 kb clockwise with respect to the *murI* gene, between the *rrnB* operon and the *birA* gene required for biotin synthesis (Fig. 1). The cellular functions of all of the other genes in this particular region from *btuB* to *rpoBC* have now been established. The *murA* gene has not been identified to date, but it is now clear that the *murA*, *murB*, and *murI* genes do not belong to a cluster of tightly packed genes with related functions as is the case for the other *mur* genes in the 2-min region of the *E. coli* chromosome (28).

The *murI* gene was the only *E. coli* gene that was selected for complementation of the specific defect of strain WM335. Evidence is now provided that it is an essential gene whose inactivation results in D-glutamic acid auxotrophy. We were able to construct a strain, GRA83, with its chromosomal *murI* gene disrupted that was viable only in the presence of a plasmid carrying the wild-type gene. Since the plasmid bears a thermosensitive replicon, the effects of this inactivation were easily visualized by shifting exponentially growing cells to the nonpermissive temperature. Cells were shown to accumulate UDP-MurNAc-L-Ala while pools of the nucleotide precursors located downstream in the pathway were rapidly depleted. As a result, peptidoglycan synthesis was inhibited and cell lysis finally occurred, as previously observed with the initial WM335 mutant when depleted of D-glutamic acid (11). It was thus clear that inactivation of the sole *murI* gene was sufficient for expression of a D-glutamic-acid-requiring phenotype.

Furthermore, our data show that it was not possible to transduce the altered *murI* gene from either WM335 or GRA83 species into other genetic backgrounds, in spite of the presence of a high concentration of D-glutamic acid in the growth medium. Only strains harboring two copies of the *murI* gene were efficient as recipients in such experiments. The reason for this discrepancy was that wild-type *E. coli* strains very poorly take up D-glutamic acid, i.e., at a rate which is apparently not sufficient to fulfill the specific requirements of peptidoglycan synthesis. Although D-glutamic acid was unable to complement the *murI* defect of GRA83 cells growing at 43°C in rich medium, it no longer failed to do so in minimal medium. This was due to the absence in synthetic minimal medium of compounds such as L-glutamic acid which could compete with D-glutamic acid for its permeation system. As a control, it was shown that the *murI* defect could be transduced in every genetic background chosen, as soon as transductants were selected in minimal medium supplemented with D-glutamic acid. These

results further confirmed the assumption that the original WM335 mutant, which was initially selected in rich medium, necessarily carries an additional mutation in a specific glutamate permease gene. Recently, Dougherty et al. (13) sequenced the *gltS* gene from strain WM335 and showed that it was effectively altered by two missense mutations. The isolation of such a double mutant was thus very unfavorable, and this could explain why it was the only known D-glutamic-acid-requiring strain that has been described to date.

Attempts to detect either a glutamate racemase or a D-alanine:D-glutamate transaminase in wild-type *E. coli* strains had all previously met with failure (11, 22, 33, 40). It was assumed that the enzyme was probably expressed at a very low level in *E. coli* cells, and this was confirmed by data from Baliko et al. (4) which showed that the ORF1-encoded protein was observed at the limit of detectability when cells were labeled with [L-^{35}S]methionine. Evidence is provided in the present study that the *murI* gene encodes a glutamate racemase activity. This was made possible by constructing strains that overproduce the *murI* gene product to a high level and by developing a very efficient and sensitive assay for glutamate racemase activity. The enzyme seems to be greatly unstable, since overnight dialysis of the crude extract was accompanied by an important loss of activity. In all of the purification steps of the glutamate racemase from *Pediococcus pentosaceus*, Soda and coworkers (7, 33, 34) used a standard buffer containing 1 mM DL-glutamic acid, 10% glycerol, and 0.1% β-mercaptoethanol for stabilization of the enzymatic activity. These authors recently reported (7) that the glutamate racemase from *P. pentosaceus* is composed of 265 amino acids with a molecular weight of 29,140. It should be noted that these values are very similar to those calculated for the *E. coli murI* gene product. The search of an eventual homology between the amino acid sequences of both racemase species required the DNA sequence of the *Pediococcus* gene, which has not been published to date.

D-Glutamic acid is a specific component of the peptidoglycan structure, a macromolecule which normally accounts for 1 to 2% of the dry cell weight (30). This is a minor cellular utilization compared with that of the L-isomer, an important metabolite used in several pathways and in particular in protein synthesis. The pool level of D-glutamic acid is always very low compared with that of the L-isomer, except in the case of cells overproducing the glutamate racemase activity. It was tempting to imagine that a physiological mechanism exists that controls the activity of the glutamate racemase to avoid an excessive conversion of L-glutamic acid to the D-isomer. An adjustment of the synthesis of D-glutamic acid to the requirements of peptidoglycan synthesis, and in particular to the flow of the UDP-MurNAc derivatives in the main reaction sequence of this pathway, was therefore considered. In fact, the activity of the glutamate racemase was detectable in direct-assay conditions only if UDP-MurNAc-L-Ala was added to the reaction mixture. Interestingly, this effect was specific to this nucleotide, since no other precursor from UDP-GlcNAc to UDP-MurNAc-pentapeptide was an effector. This last result is very important, since it suggests that the activity of the glutamate racemase could be modulated in vivo by the concentration of the other substrate of the D-glutamic-acid-adding enzyme which catalyzes the subsequent step in the pathway for peptidoglycan synthesis. A purification of the enzyme is in progress to control the phenomena observed with crude enzyme preparations.

ACKNOWLEDGMENTS

We thank B. Bachmann for supplying most of the *E. coli* strains used in this work and R. Aufrère for helpful discussions.

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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