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Identification of the *mpl* Gene Encoding UDP-*N*-Acetylmuramate: L-Alanyl-γ-D-Glutamyl-*meso*-Diaminopimelate Ligase in *Escherichia coli* and Its Role in Recycling of Cell Wall Peptidoglycan

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A gene, *mpl*, encoding UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate ligase was recognized by its amino acid sequence homology with *murC* as the open reading frame *yjfG* present at 96 min on the *Escherichia coli* map. The existence of such an enzymatic activity was predicted from studies indicating that reutilization of the intact tripeptide L-alanyl-γ-D-glutamyl-*meso*-diaminopimelate occurred and accounted for well over 30% of new cell wall synthesis. Murein tripeptide ligase activity could be demonstrated in crude extracts, and greatly increased activity was produced when the gene was cloned and expressed under control of the *trc* promoter. A null mutant totally lacked activity but was viable, showing that the enzyme is not essential for growth.

The biosynthesis of bacterial cell wall peptidoglycan (murein) is a complex process involving many different cytoplasmic and membrane steps (for review, see references 11, 24, and 30). The main cytoplasmic precursors of *Escherichia coli* are a series of seven uridine nucleotides, from UDP-*N*-acetylglucosamine to UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (where A_2pm represents diaminopimelic acid). Their sequential formation is catalyzed by a set of highly specific enzymes, designated as Mur activities (MurA to MurF [Fig. 1]). Subsequent steps located in the membrane result in the synthesis of the disaccharide pentapeptide MurNAc(-pentapeptide)-GlcNAc, which is anchored in the membrane via the lipid carrier undecaprenyl-phosphate. The disaccharide pentapeptide units are then used in polymerization reactions (transglycosylation and transpeptidation) catalyzed by the well-known bifunctional penicillin-binding proteins. In order to enlarge the murein sacculus during normal cell growth and division, new peptidoglycan subunits have to be inserted into the preexisting network, a process which probably begins with a specific cleavage of some covalent bonds by peptidoglycan hydrolases (9, 29).

A large portion of the murein sacculus of *E. coli* is degraded each generation, resulting in the formation of various degradation products which can be detected in the culture medium (8). One of them, the tripeptide L-alanyl-γ-D-glutamyl-*meso*-A_2pm, is of particular interest, since it was shown to be efficiently reutilized by the cell to form new cell wall murein via a reaction sequence termed "the recycling pathway" (6–8, 25, 26). This pathway involves five different known hydrolases and a permease. However, reutilization of the tripeptide was ultimately dependent on a hypothetical ligase which could link tripeptide to UDP-*N*-acetylmuramic acid (Fig. 1). This paper demonstrates the existence of such an enzyme and presents the initial characterization of its properties as well as identification and mapping of its gene, *mpl* (which codes for murein peptide ligase).

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used in this study are listed in Table 1. Plasmid pFD12, a pBR322 derivative carrying an 18.5-kb chromosomal fragment from the *fbp* region, was a kind gift from Dan G. Fraenkel (28). Plasmid pAM1005 carrying the *murC* gene cloned into the expression vector p*Trc*99A was obtained from A. Masson (15). Cloning vectors pUC19 (33) and p*Trc*99A and the Kan^r cartridge originating from the pUC4K plasmid were purchased from Pharmacia. Unless otherwise noted, 2YT was used as a rich medium for growing cells (22). Growth was monitored at 600 nm with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). For strains carrying drug resistance genes, ampicillin and kanamycin were used at 100 and 40 $\mu g \cdot ml^{-1}$, respectively. 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 $\mu g \cdot ml^{-1}$.

General DNA techniques and *E. coli* cell transformation. Small- and large-scale plasmid isolations were carried out by the alkaline lysis method, and plasmids were further purified with cesium chloride-ethidium bromide gradients (27). Standard procedures for endonuclease digestions, ligation, filling in of 5′-protruding ends with the Klenow fragment of DNA polymerase I, and agarose electrophoresis were used (5, 27). *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (4).

Construction of plasmids. The 18.5-kb chromosomal fragment from *E. coli* which is carried by plasmid pFD12 (28) was used as the starting material for the construction of the different plasmids described in the legend to Fig. 2. The pMLD101 plasmid was constructed by insertion of the 2.9-kb *Kpn*I-*Eco*RI fragment carrying the 5′ end of the *fbp* gene and the complete *mpl* gene sequence (open reading frame *yjfG*) into the corresponding sites of the pUC19 vector (in this construction, *mpl* and the *lac* promoter from the vector are in the same orientation). A plasmid suitable for high-level overproduction of Mpl was constructed by the following procedure. PCR primers were designed to incorporate a unique *Bsp*HI site (in boldface) 5′ to the initiation codon (underlined) of *mpl* (5′-GTGAC**TCATGA**GAATTCATATTTTAGG-3′) and a unique *Pst*I site (in boldface) 3′ to the gene after the stop codon (5′-TAAG**CTGCAG**TTACTGCG CGCGGCTTCCG-3′). These primers were used to amplify the *mpl* gene from plasmid pFD12. The isolated DNA was then treated with *Bsp*HI and *Pst*I and ligated into the compatible *Nco*I and *Pst*I sites from vector p*Trc*99A. The resulting plasmid, pMLD102, allowed expression of the *mpl* gene under control of the IPTG-inducible *trc* promoter. The disruption of the *mpl* gene was obtained by

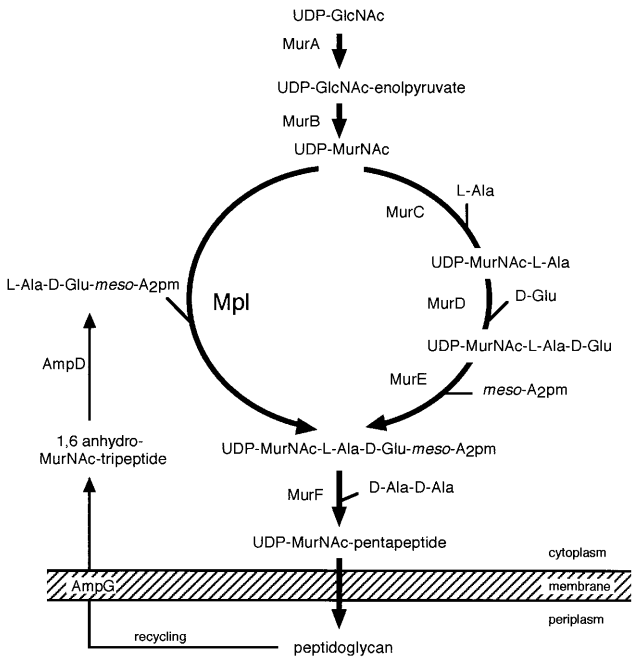

FIG. 1. Involvement of the murein tripeptide ligase activity in cytoplasmic steps of peptidoglycan biosynthesis and recycling in *E. coli.*

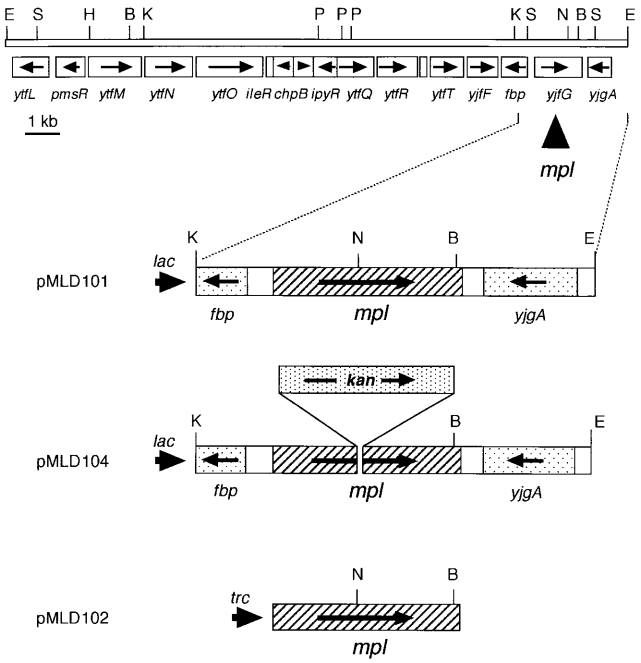

FIG. 2. Localization of the *mpl* gene at 96 min on the *E. coli* chromosome. The locations of other genes (3) present on the DNA fragment carried by plasmid pFD12 are indicated at the top, and their orientations relative to the chromosome are indicated by arrows. Bacterial DNA present in plasmid inserts is shown below. The *mpl* gene, corresponding to the open reading frame previously designated as *yjfG* or *o457* (3), is represented as a hatched region, and the positions of the *lac* or *trc* promoters relative to the insert in each plasmid are indicated by arrows. The positions of cleavage sites are shown for *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), and *Sac*I (S).

insertion of the 1.28-kb *Hin*cII *kan* cartridge originating from pUC4K into the unique *Nco*I site of plasmid pMLD101 lying within the *mpl* gene coding sequence, generating plasmid pMLD104 (Fig. 2).

Disruption of the chromosomal *mpl* gene. The wild-type chromosomal copy of *mpl* was replaced by a disrupted one by the procedure of Winans et al. (32). Plasmid pMLD104 harboring the inactivated *mpl* gene was restricted with *Kpn*I and *Eco*RI and used to transform strain JC7623, a *recB recC sbcB* strain that can be transformed by linear DNA (32). Exchange of the mutated copy of *mpl* with the chromosomal wild-type gene was selected for by plating of transformed bacteria on 2YT plates in the presence of kanamycin to obtain strain TP7623 *mpl*::*kan.*

Isolation of sacculi and quantitation of peptidoglycan. Cells of strains JC7623 and TP7623 (0.5-liter cultures) were grown exponentially at 37°C in 2YT medium to an optical density (OD) of 0.7 (250 mg [dry weight] of bacteria per liter of culture). At this time, cells were rapidly chilled to 0°C, harvested in the cold, and washed with a cold 0.85% NaCl solution. 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 (6 M HCl, 95°C, 16 h) and analyzed with a Biotronik amino acid analyzer (model LC-2000; Biotronik, Frankfurt, Germany) equipped with a DC-6A column (Dionex, Sunnyvale, Calif.) and a Spectra-Glo fluorometer (Gilson, Villiers-le-Bel, France). *o*-Phthalaldehyde–β-mercaptoethanol was used as the post-column derivatization reagent. The peptidoglycan content of the sacculi was expressed in terms of its muramic acid content (17, 19).

Pool levels of peptidoglycan precursors. Cells of JC7623 and TP7623 (1-liter cultures) were grown exponentially at 37°C in 2YT medium and harvested at an OD of 0.7. The extraction of peptidoglycan nucleotide precursors and the analytical procedure used for their quantitation were described previously (17, 18). As was the case for the nucleotide derivatives, the tripeptide L-Ala-γ-D-Glu-*meso*-A_2pm was excluded from the gel of Sephadex G-25, the first chromatographic step generally used for the analysis of cell extracts (17, 18). This property ensured its rapid separation from other small molecules and salts. The complete purification of this compound was obtained in a second step of chromatography on the DC-6A column of the amino acid analyzer. The pool of L-Ala-γ-D-Glu-*meso*-A_2pm was determined by using the analyzer under specific conditions allowing its complete separation from other cell components (elution was made with 60 mM citrate buffer [pH 3.2] containing 0.1 M NaCl).

Preparation of crude enzyme. Cells of strain DH5α harboring the different plasmids described above were grown exponentially at 37°C in 2YT-ampicillin medium (0.5-liter cultures). When needed, 1 mM IPTG was added to the culture at an OD of 0.05. In all cases, cells were harvested in the cold when the OD reached 0.7 and were washed with 40 ml of cold 0.02 M potassium phosphate buffer (pH 7.4) containing 0.3 mM $MgCl_2$ and 1 mM β-mercaptoethanol. The wet cell pellet was suspended in 5 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 ×

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
AB1133	F^- *thr-1 leuB6* Δ(*gpt-proA*)*62 hisG4 argE3 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 rpsL31 supE44*	1
JC7623	AB1133 *tsx33 recB21 recC22 sbcB15*	32
TP7623	JC7623 *mpl*::*kan*	This work
PC2453	*purE murC*(Ts) *phx lam*(*lam*) *rpsL*	Phabagen Collection[a]
AT980	λ^- *dapD2 relA1 spoT1 thi-1*	CGSC no. 4545[b]
TP980	AT980 *mpl*::*kan*	This work

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

g in a Beckman TL100 centrifuge. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same phosphate buffer, and the resulting solution (5 ml, 10 to 12 mg of protein · ml^{-1}), designated as crude enzyme, was stored at −20°C. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of proteins was performed as previously described with 13% polyacrylamide gels (14). Protein concentrations were determined by the method of Lowry et al. with bovine serum albumin as a standard (16).

Assay for tripeptide ligase activity. The standard assay mixture contained 0.1 M Tris-hydrochloride buffer (pH 8.6), 5 mM ATP, 20 mM $MgCl_2$, 0.1 mM L-Ala-γ-D-Glu-*meso*-[^{14}C]A_2pm (500 Bq), 0.5 mM UDP-MurNAc, and crude enzyme (0.2 to 10 μg of protein, depending on overexpression factor) in a final volume of 100 μl. Mixtures were incubated at 37°C for 30 min, and reactions were stopped by the addition of 10 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^{-1} with an LT36 apparatus (Savant Instruments, Hicksville, N.Y.). Two radioactive spots (free tripeptide and UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm) were detected by overnight autoradiography with type R2 films (3M, St. Paul, Minn.) or with a radioactivity scanner (Multi-Tracermaster model LB285; Berthold France, Elancourt, France). They 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). One unit of enzyme activity was defined as the amount which catalyzed the synthesis of 1 nmol of UDP-MurNAc-tripeptide in 1 min.

Complementation of the *murC*(Ts) mutation. The thermosensitive mutant PC2453 was transformed by the plasmids to be tested. Competent cells (300 μl), mixed with plasmid DNA, were kept on ice for 3 h before being heated for 3 min at 42°C. Four hundred microliters of 2YT medium was added, and the cells were incubated at 30°C for over 2 h to allow expression of plasmid-coded genes. One hundred microliter aliquots of the cell suspensions were plated onto two prewarmed 2YT-ampicillin plates, one incubated at 30°C and the other incubated at 42°C. Growth was observed after 24 h of incubation.

Chemicals. UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-[^{14}C]A_2pm and the corresponding MurNAc-peptide derivative were prepared as described by Michaud et al. (20, 21). L-Ala-γ-D-Glu-*meso*-[^{14}C]A_2pm was obtained by treatment of radioactive MurNAc-tripeptide with partially purified *N*-acetylmuramyl-L-alanine amidase according to the method of van Heijenoort et al. (31). The chemical synthesis of UDP-MurNAc was previously described (2).

RESULTS

Detection of a specific tripeptide ligase activity in *E. coli* cells. In his study of peptidoglycan recycling, Goodell (6) clearly demonstrated that the tripeptide L-Ala-γ-D-Glu-*meso*-A_2pm was reused without prior hydrolysis, suggesting the presence in *E. coli* cells of a ligase activity (Mpl) catalyzing its addition in a block on UDP-MurNAc. This role was tentatively assigned to the MurC enzyme, which normally catalyzes the addition of L-alanine on the nucleotide precursor (6) (Fig. 1). However, this hypothesis was recently negated by the demonstration that purified MurC enzyme was completely devoid of any Mpl activity (15). The Mpl activity was thus investigated again and effectively detected in significant amounts in crude extracts from *E. coli* cells. Interestingly, optimal in vitro assay conditions for Mpl were similar to those previously observed with the MurC enzyme: pH 8.6 and 20 mM $MgCl_2$. However, no increase in Mpl activity could be detected in cells overproducing MurC to high levels (cells harboring plasmid pAM1005 [Table 2]), clearly indicating that the Mpl and MurC activities were carried by two different protein species.

Identification of the *mpl* gene on the *E. coli* chromosome. In a search of the databases, it was observed that the product of an open reading frame of unknown function named *yjfG* or *o457* (3), located near *fbp* at 96 min on the chromosome (28), displayed significant homologies of amino acid sequence with MurC (28.7% identity) (3) (Fig. 3). Since homologies between YjfG and MurC could be interpreted in terms of an equivalent or similar function, we speculated that *yjfG* could be the *mpl* gene encoding UDP-MurNAc:L-Ala-γ-D-Glu-*meso*-A_2pm ligase. To test this hypothesis, strains overexpressing the *yjfG* gene, or, inversely, carrying a deletion in that gene, were constructed.

TABLE 2. Levels of murein tripeptide ligase (Mpl) in *E. coli* cells[a]

Strain	IPTG	Mpl sp act (U · mg of protein^{-1})[b]	Amplification factor
DH5α(pUC19)	+	2.4	1
DH5α(pMLD101)	+	12	5
DH5α(pMLD104)	+	2.4	1
DH5α(pMLD102)	−	17–25	7–10
DH5α(pMLD102)	+	120–200	50–80
DH5α(pAM1005)	+	1.5	0.6
JC7623	−	2.5	1
TP7623	−	ND[c]	

[a] The Mpl activity was determined with crude extracts (soluble fractions) from cells grown exponentially in the presence (+) or absence (−) of IPTG.

[b] An average value, which is probably underestimated, is reported for cells carrying plasmid pMLD102. As explained in the text, this underestimate results from the lytic phenotype of these cells and from the fact that most of the Mpl molecules formed aggregates at such a high level of expression.

[c] ND, not detectable (< 1% of the wild-type level).

Overproduction of tripeptide ligase activity in strains overexpressing the *yjfG* gene. First, a 2.9-kb *Kpn*I-*Eco*RI chromosomal fragment carrying the *yjfG* gene was cloned into the multicopy vector pUC19 (Fig. 2). Cells harboring the resulting plasmid, pMLD101, overproduced the Mpl activity by about fivefold (Table 2). This overproduction was not observed with plasmid pMLD104, in which the coding sequence of *yjfG* had been disrupted by insertion of a kanamycin resistance cartridge (Fig. 2 and Table 2). A much more efficient plasmid was constructed in which the gene was expressed under the control of the strong IPTG-inducible *trc* promoter. Curiously, strain DH5α carrying this plasmid (pMLD102 [Fig. 2]) presented an abnormal phenotype when grown in liquid medium in the absence of the inducer IPTG: cells appeared as greatly enlarged ovoids when observed by optical microscopy, and cul-

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mpl     ---------------MR----IHILGICGTFMGGLAMLARQLGHEVTGSDANVYPPMSTL   41
murC    MNTQQLAKLRSIVPEMRRVVRHIHFVGIGGAGMGGIAEVLANEGYQISGSDLAPNPVTQQL   60
                      **    ** .** *. ***.* .  . * ...***    *    *

mpl     LEKQGIELIQGYDASQLEPQPDLVIIGNAMTRGNPCVEAVLEKNIPYMSGPQWLHDPVLR   101
murC    MN-LGATIYFNHRPENVR-DASVVVVSSAISADNPEIVAAHEARIPVIRR-AEMLAELMR   117
        .   *  .  .    ..      .*..  *..  ** . *  *  ** .     .   ..*

mpl     DRWVLAVAGTHGKTTTAGMATWILEQCGYKPGFVIGGVPGNFEVSAHLGESDFFVIEADE   161
murC    FRHGIAIAGTHGKTTTTAMVSSIYAEAGLDPTFVNGGLVKAAGVHARLGHGRYLIAEADE   177
         *  .*.*********. * . *  ..*  * ** **.     * *.**    . . ****

mpl     YDCAFFDKRSKFVHYCPRTLILNNLEFDHADIFD-DLKAIQKQFHHLVRIVPGQGRIIWP   220
murC    SD-------ASFLHLQPMVAIVTNIEADHMDTYQGDFENLKQTFINFLHNLPFYGRAVMC   230
         *       . *.*  *   *..*.* ** * .  *   ... * . .. .*  ** .

mpl     END-INLKQTMAMGCWSEQELVGEQGHWQAKKLTTDASEWEVLLDGE--KVGEVKWSLVG   277
murC    VDDPVIRELLPRVGRQTTTYGFSEDADVRVEDYQQIGPQGHFTLLRQDKEPMRVTLNAPG   290
          * .       .*  .      *    .         .   *  .      *     *

mpl     EHNMHNGLMAIAAARHVGVAPADAANALGSFINARRRLELRGEA-----NG----VTVYD   328
murC    RHNALNAAAAVAVATEEGIDDEAILRALESFQGTGRRFDFLGEFPLEPVNGKSGTAMLVD   350
         **  *   *.* *   *.       ** ** .. ** .  **       **      . *

mpl     DFAHHPTAILATLAALRGKVGGTARIIAVLEPRSNTMKMGICKDDLAPSLGRADEVFLLQ   388
murC    DYGHHPTEVDATIKAARAGWP-DKNLVMLFQPHRFTRTR-DLYDDFANVLTQVDTLLMLE   408
        *. **** . **. * *         . . .*.  *       ** *  * . * . .*.

mpl     P---AHIPWQVAEVAEACVQPAHWS-------GDVDTLADMVVKTAQPGDHILVMSNGGF   438
murC    VYPAGEAPIPGADSRSLCRTIRGRGKIDPILVPDPARVAEMLAPVLTGNDLILVQGAGNI   468
              *   *.    *                 *   .*.*.      .* ***   *.

mpl     GGIHQKLLDGLAKKAEAAQ-----                                      457
murC    GKIARSLAEIKLKPQTPEEEQHD-                                      491
        * * . * .   *     .
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FIG. 3. Homology between the amino acid sequences of the *mpl* and *murC* gene products from *E. coli*. Identical amino acids are indicated by stars, and structurally related amino acids are indicated by dots. Amino acid numbers for both proteins are indicated on the right. Aligned Mpl and MurC protein sequences (referred to as P37773 and P17952 in the SwissProt library) are derived from nucleotide sequences with EMBL accession numbers U14003 and X52644, respectively.

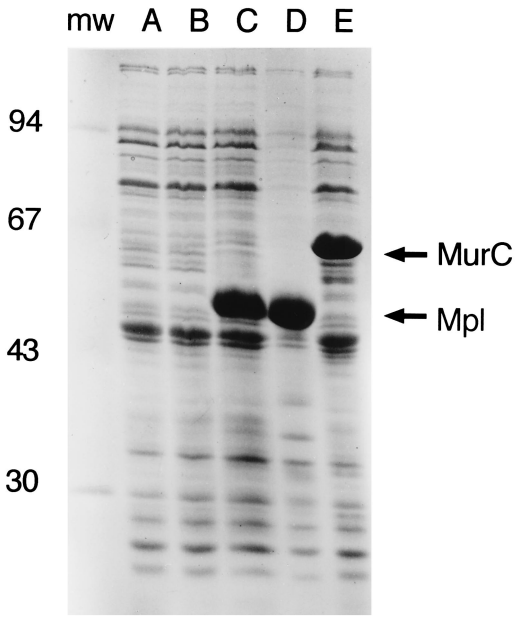

FIG. 4. Overproduction of murein tripeptide ligase in *E. coli* cells. Crude extracts from strains carrying plasmids with the *mpl* or the *murC* gene expressed under the control of the *trc* promoter were analyzed by SDS-PAGE. Lanes: A, soluble fraction from DH5α(p*Trc*99A) cells grown in the presence of IPTG; B and C, soluble fraction from DH5α(pMLD102) cells grown in the absence or presence of IPTG, respectively; D, particulate fraction from DH5α(pMLD102) cells grown in the presence of IPTG; E, soluble fraction from DH5α(pAM1005) cells grown in the presence of IPTG; mw, molecular mass standards (kilodaltons), which are indicated on the left as follows: phosphorylase b, 94; bovine serum albumin, 67; ovalbumin, 43; and carbonic anhydrase, 30.

tures were frequently lysed during exponential growth. A 7- to 10-fold increase in the specific activity of Mpl in these cells was measured (Table 2), indicating that repression of the *trc* promoter was not total in the absence of IPTG. However, for the reasons presented above, even this value was probably an underestimate because of the incomplete recovery of cells. When DH5α(pMLD102) cells were induced with 1 mM IPTG in the early exponential phase, the same abnormal phenotype was observed and the cells overproduced a very high level of the Mpl protein, as judged by SDS-PAGE analysis of the corresponding crude extracts (Fig. 4). As shown in Table 2, this was correlated with a more than 50-fold increase in the specific activity of the enzyme. An additional problem was then observed: while most (90%) of the enzyme was found in the soluble fraction in the case of wild-type or moderately overexpressing strains DH5α and DH5α(pMLD101), the Mpl protein recovered from induced DH5α(pMLD102) cells was mainly found in the particulate fraction. This was probably due to the formation of protein aggregates at this high level of expression, a finding consistent with the appearance of multiple refringent inclusion bodies in these cells. By denaturation of these inclusion bodies with 6 M urea, followed by renaturation (extensive dialysis), most of the Mpl protein could be recovered in the soluble, active form (data not shown). As mentioned above, the specific activity of Mpl was not increased in extracts from strain DH5α(pAM1005), which overproduced the MurC activity more than 2,000-fold (15) (Fig. 4). In fact, an apparent decrease (40%) in Mpl activity in this extract was observed, which may be the result of a competition between the Mpl activity and the highly overproduced MurC for their common substrate, UDP-MurNAc. Reciprocally, plasmids overexpressing the *mpl* gene (pFD12 and pMLD101) failed to complement

TABLE 3. Peptidoglycan content and pool levels of its main precursors in the parental and *mpl* mutant strains[a]

Peptidoglycan or precursor	Pool level (nmol · g [dry wt] of bacteria^{-1}) in strain:	
	JC7623	TP7623
UDP-GlcNAc	990	1,350
UDP-MurNAc-pentapeptide	1,300	680
L-Ala-γ-D-Glu-*meso*-A_2pm	90	1,150
Peptidoglycan[b]	10,500	11,500

[a] Cells were grown exponentially at 37°C in 2YT medium. At an OD of 0.7, the 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 muramic acid content (17, 19).

the defect of the thermosensitive *murC* mutant strain PC2453. All of these different results confirmed that the MurC and Mpl activities were carried by two distinct protein species and clearly characterized the open reading frame *yjfG* as the *mpl* gene coding for this specific UDP-MurNAc:L-Ala-γ-D-Glu-*meso*-A_2pm ligase.

Construction of a mutant defective in tripeptide ligase activity. As previously demonstrated with mutants defective in *ampD* or *ampG*, which are blocked in upstream steps of the recycling process, wall recycling is apparently not essential for cell growth (12, 13). Inactivation of the chromosomal *mpl* gene was therefore performed by reverse genetics by the technique of Winans et al. for mutation of nonessential genes (32). First, the *mpl* gene carried by plasmid pMLD101 was disrupted by insertion of the 1.28-kb kanamycin resistance cartridge at the unique *Nco*I site lying approximately in the middle of its coding sequence (Fig. 2). The resulting plasmid, pMLD104, was cut by *Kpn*I and *Eco*RI and used to transform strain JC7623 (32), which can be transformed with linear DNA, to kanamycin resistance. The *Kpn*I-*Eco*RI-generated fragment contained the selectable marker Kan^r inserted in *mpl*, flanked by 1.2 and 1.6 kb of chromosomal DNA. This allowed integration of the Kan^r marker into the chromosome by a double-recombination event without acquisition of ampicillin resistance (loss of plasmid pMLD104). A total of 37 clones were obtained after transformation of strain JC7623 with 1 μg of restricted pMLD104 DNA. All of them were sensitive to ampicillin. Eleven were assayed and found to lack tripeptide ligase activity (within the limits of precision of the in vitro assay—i.e., less than 1% of the wild-type activity). One of these clones used for further investigations was named TP7623. The disruption of the chromosomal *mpl* gene in that strain was confirmed by PCR analysis. With the two primers described above, which are complementary to sequences which flank the gene sequence, amplification from the JC7623 chromosome or from plasmids (pFD12 and pMLD101) carrying the intact *mpl* gene produced a fragment with a size of about 1.3 kb. When the TP7623 chromosome (or plasmid pMLD104) was analyzed, this band was absent and replaced by one with a size of about 2.6 kb, as expected for disruption of the *mpl* gene. This confirmed the inactivation of the *mpl* gene in that strain and proved that the function of its product was clearly not essential for cell viability, at least under the growth conditions used.

Characterization of the *mpl*::*kan* mutant strain TP7623. The TP7623 strain grew as normally as the parental one in rich 2YT medium, with a generation time of 35 min. No obvious morphological changes were observed, as judged by optical microscopy. The cell peptidoglycan content was not at all decreased in the *mpl* mutant (Table 3), a result implying that the work

normally done by the Mpl activity in a wild-type strain (synthesis of UDP-MurNAc-tripeptide from reused tripeptide) was now supported by the MurC, MurD, and MurE activities from the main pathway (Fig. 1). An eventual effect of the mutation on the pools of characteristic peptidoglycan precursors was also investigated. Some changes were consistently observed: the pool of UDP-GlcNAc appeared somewhat higher and the pool of UDP-MurNAc-pentapeptide was reduced by 50% compared with their values in the parental strain (Table 3). By using the chromatographic procedure developed for the complete isolation and quantification of the pool of tripeptide L-Ala-γ-D-Glu-*meso*-A_2pm in cell extracts, a significant accumulation of this compound was detected in TP7623 cells, as expected for cells lacking Mpl activity (Table 3). Another peak of increased intensity on the elution profile of the amino acid analyzer suggested that a secondary product also apparently accumulated in the mutant (data not shown). Further analyses showed that it consisted of the dipeptide L-Ala-D-Glu, a compound which probably originated from partial hydrolysis of the accumulated tripeptide by a peptidase.

Requirement of Mpl for growth of an A_2pm-requiring strain on murein tripeptide. *E. coli* AT980, which is auxotrophic for A_2pm, was transduced with P1*vir* grown on TP7623 *mpl*::*kan*. A kanamycin-resistant transductant was isolated and designated TP980 (*mpl*::*kan*). Both AT980 and TP980 grew on rich medium containing A_2pm. However, on 2YT-agar medium containing L-Ala-γ-D-Glu-*meso*-A_2pm in place of A_2pm, only the parental strain, AT980, grew. This result indicates that murein tripeptide ligase is required for utilization of murein tripeptide as a source of A_2pm.

DISCUSSION

The recycling process represents a major metabolic pathway of the cell, encompassing degradation of 30 to 40% of the cell wall peptidoglycan each generation, followed by uptake and reutilization of most of its components (6–8, 25, 26). The process begins with the action of lytic transglycosylases, together with endopeptidases and L,D-carboxypeptidases (for review, see references 9 and 29), all specific for peptidoglycan, which degrade the cell wall to the disaccharide-tripeptide *N*-acetylglucosaminyl-1,6 anhydro-*N*-acetymuramyl-L-alanyl-γ-D-glutamyl-*meso*-A_2pm. The disaccharide-tripeptide is presumably transported into the cytoplasm by AmpG, where it is cleaved into disaccharide and tripeptide by AmpD, a 1,6 anhydromuramyl-L-alanine amidase (10, 13). The disaccharide is hydrolyzed to its component monosaccharides by a known cytoplasmic β-*N*-acetylglucosaminidase (9, 34). Alternatively, the β-*N*-acetylglucosaminidase can also attack the disaccharide-tripeptide with the release of *N*-acetylglucosamine. Presumably, *N*-acetylglucosamine is reutilized, as is the tripeptide. To complete recycling of the tripeptide, a hypothetical UDP-*N*-acetylmuramate:L-alanyl-γ-D-glutamyl-*meso*-A_2pm ligase was expected to be present (6, 26).

The existence of the tripeptide ligase has now been demonstrated, and *mpl*, which codes for the structural gene, has been identified and cloned. Mpl activity was easily detected in cell extracts. Mpl was shown to be a new ligase distinct from MurC. Mpl links tripeptide to UDP-*N*-acetylmuramic acid, whereas MurC adds L-alanine instead. Each of these adding enzymes is highly specific. MurC cannot utilize tripeptide at all. Conversely, overproduction of Mpl cannot rescue a *murC* mutant. Curiously, overproduction of Mpl is detrimental to the cell and results in formation of large, oval-to-spherical cells and lysis. It is possible that since Mpl and MurC compete for UDP-*N*-acetylmuramic acid, excess Mpl reduces the flow of L-Ala into the biosynthetic pathway and hence limits the introduction of new A_2pm into the wall, leading to misshapen cells and lysis.

Construction of a null mutant demonstrated that tripeptide accumulates in such cells and that Mpl is not essential for viability. This was not unexpected, since AmpG and AmpD, which are needed for the recycling of tripeptide (Fig. 1), are also nonessential proteins (12, 13). Although murein tripeptide can also be transported into the cell via the oligopeptide permease Opp (7), recycling depends almost entirely upon AmpG and AmpD, as indicated in Fig. 1, since recycling proceeds normally in *opp* strains (25) but is blocked in strains carrying either *ampG* or *ampD* (12, 13). Although Mpl is not essential for growth under normal conditions, it is required for growth when murein tripeptide is provided instead of A_2pm as a source of A_2pm for an A_2pm-requiring strain.

The *mpl* gene was first tentatively identified by a search in databases for proteins with significant homology with MurC. One open reading frame, *yjfG*, also named *o457*, with 28.7% identity to *murC* was found (3) (Fig. 3). This was proven to be the gene for Mpl, since cloning and overproduction of the protein greatly increased Mpl activity, and, conversely, a null mutant constructed by insertion of a kanamycin resistance cassette completely eliminated the tripeptide ligase activity. As described in the SwissProt library under accession number P37773, *yjfG* was believed to code for a protein with a size of 445 amino acids and with a molecular weight of 48,500. In fact, when the DNA sequence upstream of *yjfG* was analyzed (3), another putative ATG initiation codon could be found, also preceded by a ribosome-binding site, AGGA, from which a protein with 12 additional amino acid residues could be made. Since this extension also presented a significant homology with the N-terminal amino acid sequence of MurC, we believe that Mpl (YjfG) is actually a protein of 457 amino acids with a molecular weight of 49,840 (3) (Fig. 3). A search of the protein database for *Haemophilus influenzae* revealed the probable presence of both Mpl and MurC activities in this bacterial species (under SwissProt accession numbers P43948 and P45066, respectively). It is remarkable that all 88 amino acids that are perfectly conserved between Mpl and MurC of *E. coli* (Fig. 3) are also conserved in both putative enzymes of *H. influenzae* (data not shown).

Such conservation of amino acid sequences would seem to suggest an important role for the enzymes. MurC is an essential enzyme required for cell wall synthesis. However, Mpl is not needed for cell wall synthesis or for growth, although Mpl is a critical enzyme for recycling. Recycling is a major metabolic pathway of the cell, which utilizes over 1,000,000 molecules of murein tripeptide per generation. The fact that AmpG and AmpD, essential components of the inducible β-lactamase system of many bacteria (23), are present in *E. coli*, a bacterium which lacks an inducible β-lactamase, has led to speculation that recycling may be a way in which the cell monitors the condition of its cell wall (i.e., fluctuations in the concentration of recycling intermediates may signal, for example, whether the cell should continue growth or commit to stationary phase) (26). Mpl would also be part of this hypothetical regulatory circuit. As in the case of the multiple signals determining whether bacilli continue growth or sporulate, the regulatory sensors are nonessential.

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