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The *murG* Gene of *Escherichia coli* Codes for the UDP-*N*-Acetylglucosamine:*N*-Acetylmuramyl-(Pentapeptide) Pyrophosphoryl-Undecaprenol *N*-Acetylglucosamine Transferase Involved in the Membrane Steps of Peptidoglycan Synthesis

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Physiological properties of the *murG* gene product of *Escherichia coli* were investigated. The inactivation of the *murG* gene rapidly inhibits peptidoglycan synthesis in exponentially growing cells. As a result, various alterations of cell shape are observed, and cell lysis finally occurs when the peptidoglycan content is 40% lower than that of normally growing cells. Analysis of the pools of peptidoglycan precursors reveals the concomitant accumulation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylmuramyl-pentapeptide (UDP-MurNAc-pentapeptide) and, to a lesser extent, that of undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid intermediate I), indicating that inhibition of peptidoglycan synthesis occurs after formation of the cytoplasmic precursors. The relative depletion of the second lipid intermediate, undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)GlcNAc, shows that inactivation of the *murG* gene product does not prevent the formation of lipid intermediate I but inhibits the next reaction in which GlcNAc is transferred to lipid intermediate I. In vitro assays for phospho-MurNAc-pentapeptide translocase and *N*-acetylglucosaminyl transferase activities finally confirm the identification of the *murG* gene product as the transferase that catalyzes the conversion of lipid intermediate I to lipid intermediate II in the peptidoglycan synthesis pathway. Plasmids allowing for a high overproduction of the transferase and the determination of its N-terminal amino acid sequence were constructed. In cell fractionation experiments, the transferase is essentially associated with membranes when it is recovered.

The biosynthesis of bacterial cell wall peptidoglycan is a complex process involving many different cytoplasmic and membrane steps (10, 49). Conditional-lethal mutants altered at different levels of this metabolic pathway have been described previously, and most of the mutations have been mapped (5, 43, 49, 50, 52, 56). In particular, the 2-min region of the *Escherichia coli* chromosome contains a large cluster of genes from *pbpB* to *envA* that code for proteins involved in different aspects of peptidoglycan synthesis and cell division. The organization of the genes in this cluster is now completely elucidated, and the nucleotide sequence of the whole 17-kbp region has been determined (11–13, 16, 37, 39, 41; see reference 35 for references before 1990). The genes, tightly packed and overlapping in many cases, appear in the following order: *pbpB-murE-murF*-ORF-Y-*murD-ftsW-murG-murC-ddl-ftsQ-ftsA-ftsZ-envA*. The *murC*, *murD*, *murE*, *murF*, and *ddl* gene products were identified previously as L-alanine, D-glutamic acid, *meso*-2,6-diaminopimelic acid (DAP), D-alanyl–D-alanine-adding enzymes, and D-alanine:D-alanine ligase, respectively (10, 26–28, 32, 35, 43), which are involved in the synthesis of the soluble nucleotide precursors from UDP-*N*-acetylmuramic acid (UDP-MurNAc) to UDP-MurNAc-L-Ala–γ-D-Glu-*meso*-DAP-D-Ala-D-Ala (UDP-MurNAc-pentapeptide). These genes are flanked on the left by a cell division gene named *pbpB* (1, 52) or *ftsI* (44) that codes for penicillin-binding protein 3, which is involved in septum formation (17), and on the right by a group of three contiguous genes, *ftsQ*, *ftsA*, and *ftsZ* (see reference 29 for references therein), which are involved in the late steps of the cell division process but whose functions have not yet been clearly identified. Recently, the *pbpB-murG* region was investigated in more detail, and, in particular, three new genes, ORF-Y (12), *murD* (35, 39), and *ftsW* (11, 16), were identified.

The *murG* gene was discovered in this region of the chromosome 10 years ago by Salmond et al. (50). As judged from the various alterations in cell shape and the lytic thermosensitive phenotype that characterized the corresponding mutant, it was speculated that it may code for a protein involved in peptidoglycan metabolism, but its function was not further investigated. We recently localized more precisely the *murG* gene within a 1.7-kbp *Sal*I-*Kpn*I fragment (35) originating from plasmid pLC26-6 of the Clarke and Carbon collection (2, 46, 54). By studying the expression of plasmids carrying this chromosomal insert in maxicells (35) and by determining its nucleotide sequence (37), we identified the *murG* coding region as an open reading frame of 1,065 nucleotides theoretically coding for a moderately hydrophobic protein of 355 amino acids with a calculated molecular weight of 37,771.

The aim of the present study was to investigate in more detail the physiological properties of the *murG* gene product in order to determine its role in peptidoglycan synthesis or cell division. In this report, the participation of the *murG* gene product in the membrane steps of peptidoglycan synthesis is clearly established. In particular, the phospho-

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

Strain	Genotype	Source or reference
JM83	*ara* Δ(*lac-proAB*) *rpsL thi strA* φ80d*lacZ*ΔM15	58
JM109	*relA1* Δ(*lac-proAB*)*endA1 gyrA96 thiA hsdR17 supE44 recA1* (F′ *traD36 proAB lacI*^{q} ZΔM15)	58
OV2	F$^-$ *ilv his leu thyA deo ara*(Am) *lac-125* (Am) *galU42*(Am) *tyrT supF-A81*(Ts)	50
GS58	F$^-$ *ilv his thyA deo ara*(Am) *lac-125* (Am) *galU42*(Am) *galE trp*(Am) *tsx* (Am) *murG*(Am) *tyrT supF-A81*(Ts)	50
RM4102 *lysA*	*araD139* Δ(*lac*)*U169 strA thi lysA*::Km^r	C. Printz[a]
OV2 *lysA*	OV2 *lysA*::Km^r	This work[b]
GS58 *lysA*	GS58 *lysA*::Km^r	This work[b]

[a] Obtained from C. Printz, Institut de Microbiologie, Orsay, France.
[b] These strains were constructed by P1-mediated transduction of the *lysA* allele from strain RM4102 *lysA*.

MurNAc-pentapeptide translocase and the *N*-acetylglucosaminyl transferase activities involved in the translocation of the soluble precursors to the undecaprenyl-phosphate carrier lipid were investigated. Evidence is provided demonstrating that the *murG* gene codes for the *N*-acetylglucosaminyl transferase that catalyzes the formation of undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)GlcNAc (lipid intermediate II) from undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide (lipid intermediate I) and UDP-GlcNAc.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *E. coli* strains used throughout this study are shown in Table 1. Plasmid pLC26-6 from the Clarke and Carbon collection (2) was a gift of B. Bachmann, and plasmid vectors pUC18 and pUC19 were obtained from Pharmacia (Uppsala, Sweden). Plasmid pJP900, constructed by insertion of the 2.4-kbp *Xho*II-*Xho*II fragment of bacteriophage lambda that carries the p_R promoter, the *cro* ribosome-binding site, and the structural gene *c*I857 encoding a thermosensitive form of the lambda *c*I repressor into the *Bam*HI site of the pUC9 vector, was a generous gift from J. Plá (CSIC, Universidad Autónoma de Madrid, Spain). 2YT (i.e., 2× YT) medium (42) or minimal medium M63 (42) supplemented with glucose (0.4%) was used for growing cells. When required, thymine (100 μg ml^{-1}), L-amino acids (100 μg ml^{-1}), ampicillin (100 μg ml^{-1}), or kanamycin (25 μg ml^{-1}) was added. In most cases, 2-liter flasks containing 500 ml of medium were inoculated with 0.5 ml of overnight precultures and growth was monitored by measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer (model 240; Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Broth for plates was solidified with 1.5% agar. When screening plasmid inserts for the absence of α-complementation, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were added at final concentrations of 40 μg ml^{-1} and 40 μM, respectively.

Recombinant DNA techniques and *E. coli* cell transformation. Small- and large-scale plasmid isolations were carried out by the alkaline lysis method (30) and eventually further purified in cesium chloride-ethidium bromide gradients. Standard procedures for endonuclease digestions, ligation, filling-in of 5′-protruding ends with the Klenow fragment of DNA polymerase I, and agarose gel electrophoresis (30) were used. *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (3).

Chemical and analytical procedures. UDP-MurNAc-peptides, D-[^{14}C]Ala–D-[^{14}C]Ala and UDP-MurNAc–L-Ala–γ-D-Glu–*meso*-DAP–D-[^{14}C]Ala–D-[^{14}C]Ala were prepared as described previously (6, 9, 32, 33, 40). [^{3}H]DAP (888 GBq mmol^{-1}), [^{14}C]UMP (16.6 GBq mmol^{-1}), and L-[^{35}S]methionine (44 TBq mmol^{-1}) were purchased from CEA (Saclay, France), and UDP-[^{14}C]GlcNAc (9.1 GBq mmol^{-1}) was purchased from the Radiochemical Center (Amersham, England). Restriction enzymes and T4 DNA ligase were obtained from Pharmacia or from New England BioLabs, Inc. (Beverly, Mass.). Ampicillin, kanamycin, and IPTG were purchased from Serva (Heidelberg, Federal Republic of Germany), and D-cycloserine was purchased from Sigma Chemical Co. (St. Louis, Mo.).

High-pressure liquid chromatography (HPLC) was carried out with previously described devices (6, 32, 33). Detection of radioactivity in column eluents was performed with a Flo-One/DR radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, Fla.) by using the Quickszint Flow 303 scintillator (Zinsser Analytic, Maidenhead, United Kingdom) at 1 ml min^{-1}. Quantitation was carried out with an XT computer connected to the detector and equipped with FlB software (Flotec, La Queue-lez-Yvelines, France). Amino acid and amino sugar compositions were determined with an amino acid analyzer (model LC2000; Biotronik, Frankfurt am Main, Federal Republic of Germany) after hydrolysis of samples in 6 M HCl at 95°C for 16 h and by using *o*-phthalaldehyde and 2-mercaptoethanol as reagents. High-voltage electrophoresis was performed on 3469 filter paper (Schleicher & Schuell, Dassel, Federal Republic of Germany) in 2% formic acid (pH 1.9) for 1 h at 40 V cm^{-1}, by using an LT36 apparatus (Savant Instruments, Hicksville, N.Y.), and paper chromatography was performed on Whatman 3MM paper. Radioactive spots on electropherograms and chromatograms were located by autoradiography with 3M-type R2 films or with a radioactivity scanner (Multi-Tracermaster LB285; Berthold France, Elancourt, France). Radioactive material was counted in an Intertechnique SL30 liquid scintillation spectrometer with a solvent system consisting of 2 ml of water and 13 ml of Aqualyte mixture (J. T. Baker Chemicals, Deventer, The Netherlands). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli and Favre (20), by using 12% polyacrylamide gels. Experiments of electroblotting onto polyvinylidene difluoride (Immobilon-P) membranes (Millipore Corp., Bedford, Mass.) (31) were carried out with a Bio-Rad apparatus for 1 h and 40 min at 50 V; thereafter, membranes were stained with 0.1% (wt/vol) amido black 10B (Merck, Darmstadt, Federal Republic of Germany) in 45% methanol and 7% acetic acid (vol/vol). Microsequencing was performed in a gas-phase sequenator (Applied Biosystems, Warrington, United Kingdom) at the Institute of Neurophysiology of the Centre National de la Recherche Scientifique (Gif-sur-Yvette, France).

Preparation for electron microscopy. Growing cells from 1-liter cultures at a density of 3 × 10^8/ml were harvested under cold conditions and washed twice with 0.1 M sodium cacodylate buffer, pH 7.1, containing 10 mM $CaCl_2$. Cells were embedded in agar, cut into small pieces, and fixed with 2.5% (vol/vol) glutaraldehyde in sodium cacodylate buffer for 4 h at room temperature. After being thoroughly washed with the same buffer, they were postfixed, first with 1%

(wt/vol) osmium tetroxide in cacodylate buffer for 15 h at 4°C and then with 0.5% uranyl acetate for 2 h at room temperature. Specimens were slowly dehydrated in acetone and embedded in Epon 812 resin. Polymerization at 60°C was carried out for 2 days. Thin sections (30 to 50 nm) were cut out with an Ultracut Reichert microtome and were stained with uranyl acetate and lead citrate. Micrographs were taken with Philips CM10 and Zeiss EM10 microscopes operating at 80 kV.

Pools of peptidoglycan precursors. Cultures (1 liter) of exponentially growing cells were rapidly chilled to 0°C, and cells were harvested under cold conditions. The extraction of free DAP and peptidoglycan nucleotide precursors, as well as the analytical procedure used for their quantification, was previously described (32–34).

Isolation of sacculi and quantification of peptidoglycan. Cells from 1-liter cultures were rapidly chilled to 0°C and harvested in the cold. Pellets were washed with a cold aqueous 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% 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. The peptidoglycan content of the sacculi was expressed in terms of DAP content (36, 38).

Uptake of *meso*-[^{3}H]DAP and its incorporation into peptidoglycan. *meso*-[^{3}H]DAP (18.5 kBq ml^{-1}, 888 GBq mmol^{-1}) was added to 50-ml cultures of the *lysA*-carrying strains growing exponentially in minimal medium, when the OD of the medium reached the value of 0.1. After designated time intervals, 500-μl samples were removed and centrifuged for 3 min with an Eppendorf centrifuge. The supernatants were analyzed for radioactivity in order to estimate the rate of DAP uptake. In parallel, other 500-μl samples were added to 9.5 ml of ice-cold 5.5% trichloroacetic acid (TCA). Suspensions were kept at 0°C for 60 min, and the labeled peptidoglycan material (TCA insoluble) was filtered over glass fiber filters (GF/C; Whatman, Inc., Clifton, N.J.). The filters were washed with cold 5% TCA, dried, and immersed in 2 ml of 0.1 N NaOH before the counting was performed as described above.

Cellular distribution of *meso*-[^{3}H]DAP. In an attempt to identify the step in peptidoglycan synthesis which was affected by the *murG* mutation, the cellular distribution of [^{3}H]DAP incorporated in strains OV2 *lysA* and GS58 *lysA* at both permissive and nonpermissive temperatures was compared. Strains were grown at 30°C in minimal medium (50-ml cultures) supplemented with threonine and methionine in order to reduce as much as possible their internal DAP pool level (36, 55). At an OD of 0.1, cultures were divided into two portions: one portion was maintained at 30°C, and the other was transferred at 42°C. At an OD of 0.6, the alterations in cell shape characteristic of the *murG* mutant were controlled by optical microscopy, and *meso*-[^{3}H]DAP was added to all cultures (18.5 kBq ml^{-1}, 888 GBq mmol^{-1}). After different labeling times (from 5 to 30 min), cell samples were analyzed by paper chromatography as described previously (15, 18, 24, 25, 48). Briefly, cells from 10-ml culture samples were rapidly chilled to 0 to 4°C, collected by centrifugation, and suspended in 200 μl of ice-cold distilled water. Aliquots were applied to Whatman 3MM filter paper (50 cm long) and subjected to descending chromatography for 16 h in isobutyric acid–1 M NH_4OH (5:3). Under such conditions, peptidoglycan remains at the origin and UDP-MurNAc-peptides, DAP, and the mixture of lipid intermediates migrate with R_f values of 0.15, 0.4, and 0.9, respectively. As described previously (19, 48), the determination of the relative amounts of UDP-MurNAc-peptides, lipid I, and lipid II required their selective extraction from the cell contents and the utilization of appropriate chromatographic procedures allowing their complete isolation and quantification. For this purpose, cells from 25 ml of culture labeled as described above were harvested, resuspended in 2 ml of boiling water, and maintained at 100°C for 15 min. After centrifugation of this suspension at 200,000 × *g* for 20 min, the pellet was extracted again with 2 ml of boiling water for 15 min. After centrifugation, both supernatants were pooled and the pellet was resuspended in 800 μl of water. As demonstrated previously by Ramey and Ishiguro (48), the pellet fraction contains peptidoglycan and lipid intermediates I and II and the supernatant contains DAP and the UDP-MurNAc-peptides as labeled compounds. To determine the relative amounts of UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide in this latter fraction, aliquots were analyzed as described previously (6) by HPLC by using a μBondapak C_{18} column and isocratic elution with a 0.05 M ammonium formate buffer, pH 3.70, at a flow rate of 0.5 ml min^{-1}. Under these conditions, DAP, UDP-MurNAc-tripeptide, and UDP-MurNAc-pentapeptide were eluted in 10, 16, and 45 min, respectively. To determine the relative amounts of lipid I and lipid II in the pellet fraction, previously described procedures (19, 48) that involve a mild-acid hydrolysis of the extracted lipophilic compounds and a separation of the resulting MurNAc-pentapeptide and disaccharide-pentapeptide products by appropriate chromatographic procedures were essentially followed. In our case, the material was hydrolyzed in 10% acetic acid for 60 min at 105°C in sealed tubes (48). The samples were lyophilized and, prior to chromatography, the muramyl residues of the muropeptides were reduced to their muramicitol derivatives (8) after 30 min of incubation at room temperature in 0.25 M sodium borate buffer, pH 9, containing 1 mg of sodium borohydride ml^{-1}. The reaction was stopped by adjusting the pH to 3 with phosphoric acid, and HPLC of aliquots was done under the same conditions as those described above to separate the nucleotide precursors. Under these conditions, MurNAc-pentapeptide and GlcNAc-MurNAc-pentapeptide were eluted at 19 and 35 min, respectively.

Preparation of crude envelopes. Plasmid-containing strains were grown under vigorous aeration in 2YT-ampicillin broth (50 ml) to an OD of 0.7 (250 mg of bacteria [dry weight] per liter of culture). Cells rapidly chilled in ice to 0 to 4°C were harvested under cold conditions, washed with 0.02 M potassium phosphate buffer (pH 7.0) containing 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol, and centrifuged again. Final suspensions in 2.5 ml of the same buffer were sonicated in the cold until complete disruption of the cells. Cell debris was removed by centrifugation at 6,000 × *g* for 15 min, and the supernatant was centrifuged at 200,000 × *g* for 20 min in a TL-100 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The resulting supernatants and pellets (particulate fractions) were analyzed for protein contents by the method of Lowry et al. (23) with bovine serum albumin as the standard.

Preparation of membranes from spheroplasts. Cells from 1-liter cultures were resuspended in 8 ml of cold 0.02 M Tris-HCl, pH 8.0 (buffer A), containing 0.75 M saccharose and were kept at 4°C for 10 min. Then, 80 μl of a solution of 20 mg of lysozyme per ml (in buffer A) was added, and 10 min afterwards, 400 μl of a 0.4 M EDTA solution (pH 8.0) was

also added. After 1 h at 4°C, complete spheroplast formation was verified by measuring the decrease in the OD_{600} that followed dilution of aliquots in water. Spheroplasts were recovered by centrifugation at 30,000 × *g* for 20 min in a TL-100 Beckman centrifuge. After resuspension in 15 ml of 0.05 M Tris-HCl, pH 7.5, containing 0.1 mM $MgCl_2$, 1 mM 2-mercaptoethanol (buffer B), and desoxyribonuclease and ribonuclease, both at 20 μg ml^{-1}, spheroplasts were allowed to lyse for 2 h at 4°C. Finally, membrane fractions were recovered by centrifugation at 200,000 × *g* for 30 min, resuspended in 2 ml of buffer B, and stored at −20°C.

Enzymatic assays. (i) Transfer of UDP-linked peptidoglycan precursors to membranes. The synthesis of lipid intermediates from the cytoplasmic UDP-linked precursors was followed in a reaction mixture containing, in a final volume of 25 μl, 0.2 M Tris-HCl (pH 7.5), 40 mM $MgCl_2$, 30 mM ATP, 5 μM UDP-MurNAc–L-Ala–γ-D-Glu–*meso*-DAP–D-[^{14}C]Ala–D-[^{14}C]Ala (0.4 GBq $mmol^{-1}$), 0.5 mM UDP-GlcNAc, and membranes (90 μg of protein). Reaction mixtures were incubated for 5 to 60 min at 35°C. After incubation, samples were placed in a boiling water bath for 2 min, applied to Whatman 3MM filter paper, and subjected to descending chromatography for 16 h in isobutyric acid–1 M NH_4OH (5:3, vol/vol). Areas corresponding to peptidoglycan, UDP-MurNAc-pentapeptide, and lipid intermediates were cut out and counted.

(ii) Transfer assay of phospho-MurNAc-pentapeptide translocase. The standard assay was performed essentially as described above with the exception that UDP-GlcNAc was omitted from the reaction mixture.

(iii) Exchange assay of phospho-MurNAc-pentapeptide translocase. As previously established (7, 45), the translocase also catalyzes the exchange of [^{14}C]UMP with the unlabeled UMP moiety of UDP-MurNAc-pentapeptide. The reaction mixture (7) contained, in a final volume of 40 μl, 25 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, 5 μM [^{14}C]UMP (16.6 GBq $mmol^{-1}$), 0.45 mM UDP-MurNAc-pentapeptide, and membranes (20 μg of protein). After 30 min of incubation at 35°C, the reaction was stopped by the addition of 10 μl of glacial acetic acid and the incubation mixture was analyzed by high-voltage electrophoresis on paper in 2% formic acid (pH 1.9) for 1 h at 40 V cm^{-1}. Spots corresponding to radioactive UDP-MurNAc-pentapeptide and UMP, which migrate towards the anode at 5 and 10 cm, respectively, were cut out and counted.

(iv) Assay of *N*-acetylglucosaminyl transferase. Reaction mixtures contained, in a final volume of 25 μl, 0.1 M Tris-HCl (pH 7.5), 40 mM $MgCl_2$, 30 mM ATP, 0.7 mM UDP-MurNAc-pentapeptide, 4 μM UDP-[^{14}C]GlcNAc (9.1 GBq $mmol^{-1}$), and membranes (90 μg of protein). Membranes were incubated with UDP-MurNAc-pentapeptide for 10 min at 35°C to generate undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide before the addition of the radiolabeled substrate for 30 min at 35°C. Reaction mixtures were analyzed by paper chromatography as described above.

Labeling of proteins directed by plasmids. The maxicell method described by Sancar et al. (51) of labeling the proteins encoded by plasmids was used. In all cases, strain JM109 was used as the host for the plasmid to be tested and the procedure followed was as previously described (35).

Complementation of mutations. The thermosensitive GS58 mutant was made competent as described previously (3) 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 2YT medium was added and cells were incubated at 30°C for over 1 h for expression of plasmid genes. Cells were recovered by centrifugation, and a final suspension made in 200 μl of 2YT medium was separated into two parts which were plated onto two prewarmed 2YT-ampicillin plates, one incubated at 30 and the other at 42°C. Growth was observed after 24 or 48 h of incubation.

RESULTS

Phenotypic properties of the *murG* mutant. The *murG* amber mutation studied throughout this work was originally discovered in a *supF*(Ts) background by Salmond et al. (50). When cultures of a *murG* mutant strain (GS58) growing exponentially at 30°C are shifted to the nonpermissive temperature of 42°C, cells progressively lose their rod shape, become ovoid with a greatly increased volume (Fig. 1), and finally lyse after a period that depends on the growth conditions used: 2 or 4 generation times after the temperature shift in 2YT medium or minimal medium, respectively (Fig. 2 and 3).

At 42°C, the thermosensitive mutation suppressor is inactivated and the newly made MurG protein molecules are consequently truncated. The fact that the alterations of cell shape and cell lysis only occur after a few hours could thus be explained by the time required for the inactivation of the suppressor as well as for the disappearance (progressive dilution or inactivation) of the functional MurG protein molecules still present at the time of the temperature shift.

***murG* codes for a 38K protein.** The *murG* gene was recently more precisely located between the genes *ftsW* and *murC* (16, 35) within the left part of a 1.7-kbp *Sal*I-*Kpn*I fragment originating from the synthetic ColE1 plasmid pLC26-6 from the Clarke and Carbon collection (2, 46, 54). Different plasmids carrying chromosomal inserts derived from this *Sal*I-*Kpn*I fragment were constructed (Fig. 4) and analyzed for their capability to still complement the *murG* mutation of strain GS58 as well as to direct the formation of radioactive proteins in maxicell experiments. Plasmids pDML7, pDML8, pDML19, and pDML20 were previously described (35). pLTG1 was constructed from pDML19 by an *Mlu*I-*Pst*I deletion, full filling-in of the resulting ends, and ligation. Plasmid pLTG7 was obtained by partial *Sma*I digestion of plasmid pDML7, and pMLD49 was obtained from pDML8 by internal *Sma*I deletion.

We have previously shown (35) that all the plasmids that carry in common the *Sal*I-*Kpn*I fragment express a radioactive protein with a molecular weight of 38,000 (38K protein) in maxicells. In addition, a 28K protein species could be observed, but only when this latter chromosomal insert was inserted in the wrong orientation, causing the minus strand to be under the vector *lac* promoter (*lacZp*) control. The results shown in Fig. 4 indicate that the *murG* gene begins between the *Mlu*I site and the leftmost *Sma*I site of the insert. Furthermore, it is clearly shown that the complementation of the *murG* mutation is always associated with the presence of only the 38K protein.

As suggested previously (35), the 28K protein may correspond to a fusion protein resulting from reading through the vector into the sequence of the truncated *murC* gene, also present at the distal end of the *Sal*I-*Kpn*I insert (13). It was effectively observed that the 28K protein band was lost during expression in maxicells of plasmids in which the frame at this *Kpn*I site, which is the junction between the insert and the pUC19 vector sequence, has been modified.

We have recently determined the nucleotide sequence of the 1.7-kbp *Sal*I-*Kpn*I fragment (37). It revealed an open

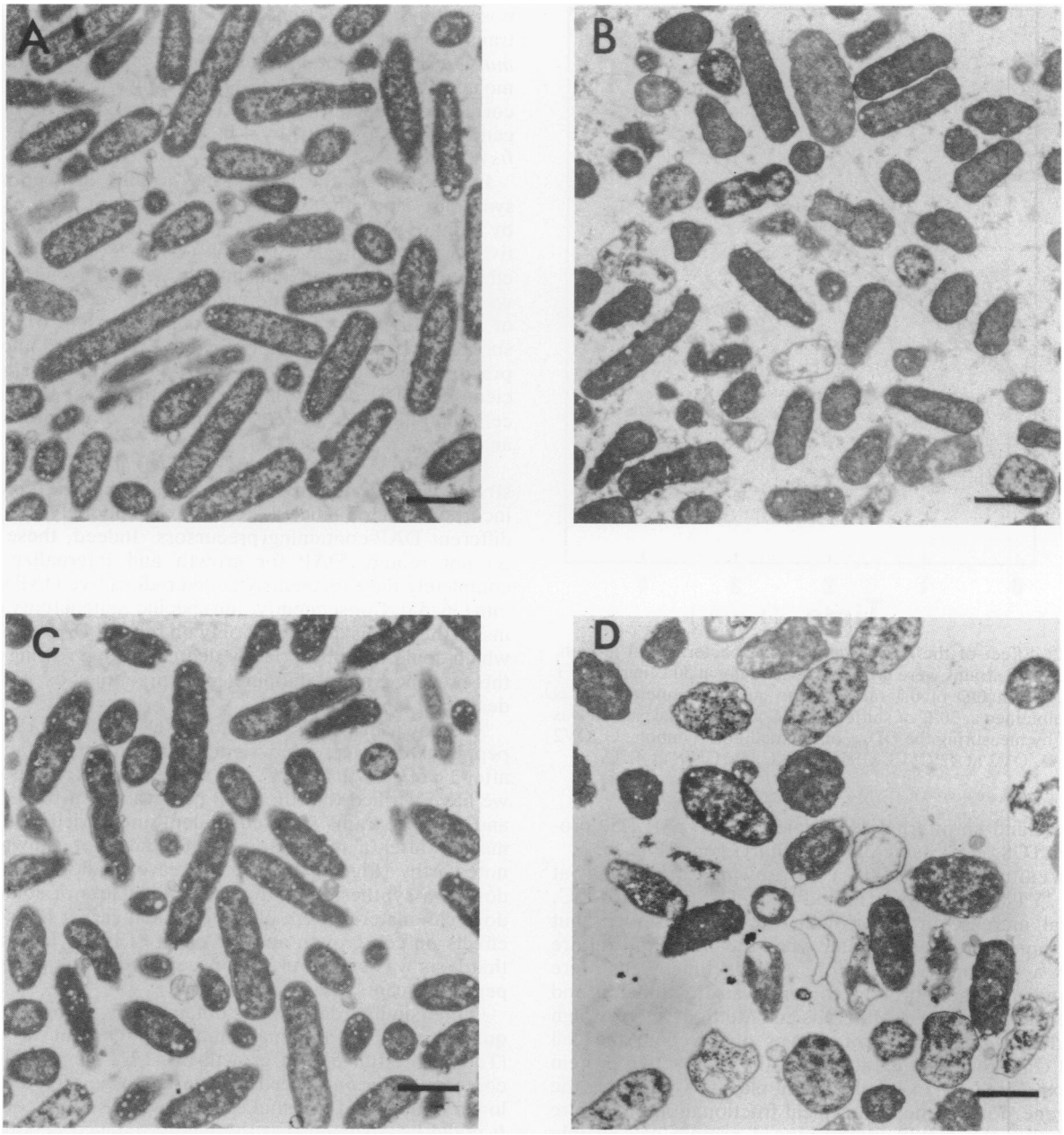

FIG. 1. Alterations of cell shape related to the *murG* mutation. OV2 and GS58 strains were grown at 30°C in minimal medium. At an OD of 0.1, the culture temperature was either maintained at 30°C or shifted to 42°C. Cells were harvested 5 h later and were observed by electron microscopy after the fixation procedure detailed in the text. (A and C) OV2 cells grown at 30 and 42°C, respectively; (B and D) GS58 cells grown at 30 and 42°C, respectively. Bars = 1 μm.

reading frame of 1,065 nucleotides, initiated effectively between the *Mlu*I and *Sma*I sites at positions 41 and 141, respectively, and theoretically coding for a 355-amino-acid protein with a molecular weight of 37,771. The nucleotide sequence of the *murG* gene was also more recently confirmed by Ikeda et al. (13).

Subcellular localization and purification of the *murG* gene product. In order to purify to some extent the *murG* gene product as well as to determine more easily its subcellular localization, we constructed plasmids overproducing it to a high level. The strategy used was originally described by Leplatois and Danchin (22) and consists of expressing the considered gene under the control of the lambda p_R promoter. This was performed by inserting upstream from the *murG* gene in a pUC19-derived plasmid (pMLD37 [Fig. 5]) a 2.4-kbp *Xho*II-*Xho*II lambda fragment carrying the lambda p_R promoter, the lambda *cro* ribosome-binding site, and the structural gene *c*I857, encoding a thermosensitive form of the lambda *c*I repressor. When the temperature is shifted to 42°C, the *c*I857 repressor is inactivated, allowing the expression of genes downstream from p_R and leading to a high overproduction of the corresponding proteins.

First, a pMLD50 plasmid was obtained, allowing expression of genes *murD-ftsW-murG* under the control of the p_R

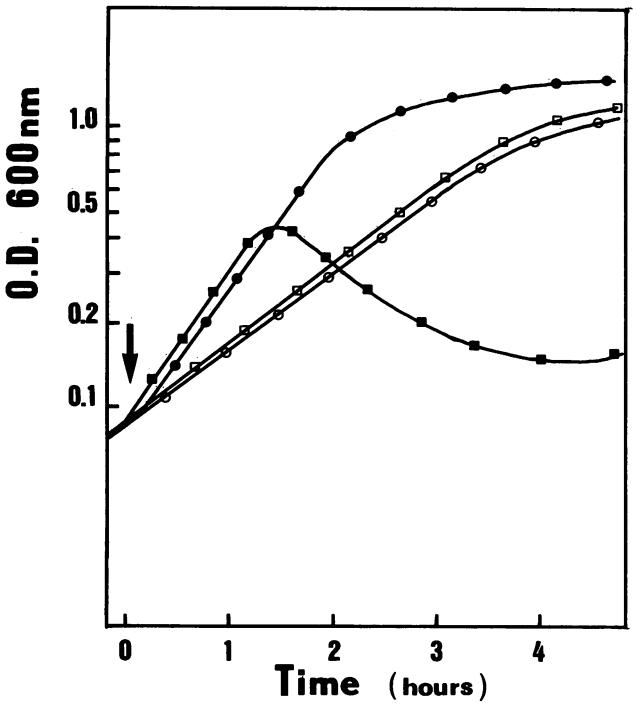

FIG. 2. Effect of the *murG* mutation on bacterial cell growth. OV2 and GS58 strains were grown exponentially at 30°C in rich 2YT medium. At an OD of 0.1 (arrow), the culture temperature was either maintained at 30°C or shifted to 42°C and bacterial growth was followed by measuring the OD_{600} of the medium. Symbols: ○, OV2 at 30°C; ●, OV2 at 42°C; □, GS58 at 30°C; ■, GS58 at 42°C.

promoter, and an internal *Mlu*I deletion into pMLD50 provided pLTG8, expressing only the *murG* gene (Fig. 5).

When cultures of JM109(pLTG8) growing exponentially at 30°C in 2YT medium were shifted (at an OD of 0.1) to 42°C, an initial increase in the growth rate was observed, but growth rapidly stopped 2 h later when the OD of the culture reached a plateau value of 0.7. At this time, cells were recovered and their protein content was fractionated and analyzed as described in Materials and Methods. A very high accumulation of a 38K protein was observed in the cell content (Fig. 6). It comigrated with the radioactive protein band expressed in maxicells from plasmids carrying the *murG* gene. Furthermore, a typical fractionation procedure showed that the highly overproduced protein was mainly found in the particulate fraction, with only some traces in the soluble fraction (Fig. 6). It was verified that the behavior of the protein in the fractionation experiment was normal and independent of its overproduction, by doing a similar fractionation protocol on maxicells of JM109(pDML19) expressing the radioactive *murG* gene product. The same results were obtained (data not shown).

Analysis of the particulate fraction by gel electrophoresis showed that the overproduced protein was migrating at a position where no other major membrane-associated protein was observed in extracts of control JM109(pJP900) cells grown at 30 or 42°C or in extracts of JM109(pLTG8) cells grown at 30°C (Fig. 6). In particular, this finding allowed us to directly recover the protein band from the gel and to determine its N-terminal amino acid sequence without any further purification step. The sequence obtained was S-G-Q-G-K-?-L-M-V-M-A-G-G-T-G-G-H, which is in complete agreement with the 5′ end of the nucleotide sequence proposed for the *murG* gene (37), taking into account a post-translational loss of the terminal methionine residue. The *murG*-encoded protein thus has 354 amino acids and a molecular weight of 37,640. This result thus confirmed the correct assignment of the ATG initiation codon of the *murG* gene (37) that overlaps by 4 bases the end of the preceding *ftsW* gene (11).

Involvement of the *murG* gene product in peptidoglycan synthesis. Since the temperature-dependent lysis exhibited by strain GS58 resembled the phenotypes of mutants defective in peptidoglycan synthesis (4, 26–28, 43, 53, 56), the effect of a temperature upshift on peptidoglycan synthesis was determined. Two different methods to estimate the rate of synthesis of this macromolecule were used: (i) the measure of incorporation of radioactive DAP into the TCA-precipitable material, by using *lysA* derivative strains (deficient in DAP-decarboxylase) and (ii) the direct estimation of cell peptidoglycan content by amino acid and amino sugar analyses of isolated sacculi.

It was previously shown (36, 55) that the *lysA*-carrying strains were very convenient for following the kinetics of incorporation of labeled DAP into peptidoglycan and its different DAP-containing precursors. Indeed, these strains do not require DAP for growth and internalize almost completely the exogenously added radioactive DAP within 5 min (36, 55). Consequently, the specific radioactivity of DAP inside the cells is dependent only on the cell DAP pool value, which could be appropriately decreased by growing cells in the presence of methionine and threonine, as previously described (36).

As shown in Fig. 3, the rate of DAP incorporation into peptidoglycan decreased significantly in the mutant strain after 2 h of growth at 42°C. For the reasons expressed above, we have verified that the DAP pool values of both parental and mutant strains were equivalent and varied in a similar manner after a temperature shift (data not shown). It is noteworthy (Fig. 3) that the decrease in the rate of peptidoglycan synthesis and the subsequent loss of some peptidoglycan material were observed much earlier than the first effects on cell growth and the onset of lysis. This suggests that lysis was effectively a consequence of the inhibition of peptidoglycan synthesis.

Quite similar data were obtained by using the direct quantification of the macromolecule content in the cell (Table 2). After 5 h of growth at 42°C, the peptidoglycan content of the *murG* cells appeared to be effectively 40% lower than that of parental cells grown in similar conditions. It could be assumed that the highly reduced peptidoglycan content determined in the mutant cells just before cell lysis occurs probably represents the lowest physiological value compatible with cell integrity.

Finally, since inactivation of the function of the *murG* gene product had the effect of decreasing the rate of peptidoglycan synthesis, we also investigated the effect of its overproduction in exponentially growing cells. In particular, the peptidoglycan content of cells harboring plasmid pDML19, in which the *murG* gene is under the control of the *lac* promoter (which overproduced fourfold the *murG* gene product [see Table 5]), was identical to that of control cells (data not shown), a result indicating that the *murG* gene product was probably not a limiting factor in the peptidoglycan synthesis pathway.

Site of the mutational block. Different experiments were carried out to determine which step in peptidoglycan synthesis was affected by the *murG* mutation. We first examined the possible function of the *murG* gene product in some early

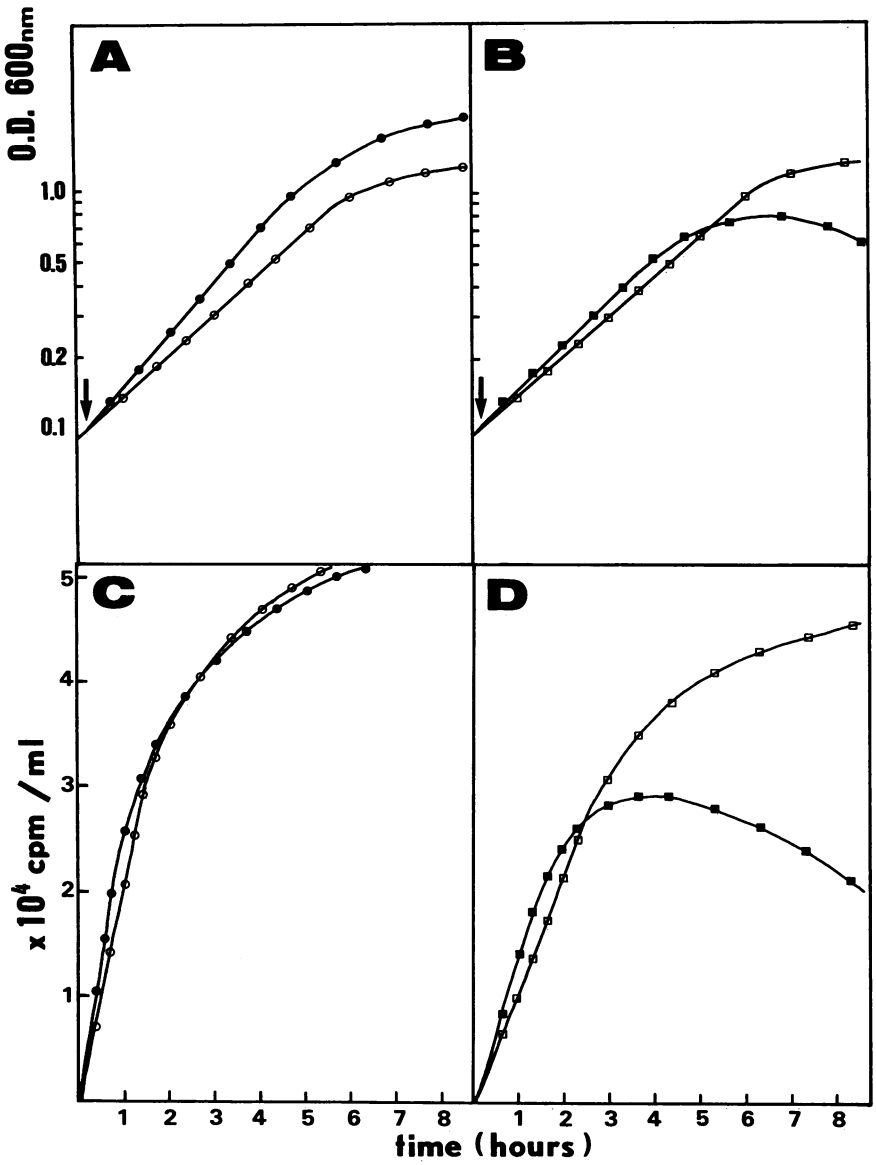

FIG. 3. Effect of the *murG* mutation on peptidoglycan synthesis. OV2 *lysA* and GS58 *lysA* strains were grown exponentially at 30°C in minimal medium supplemented with lysine. At an OD of 0.1 (arrow), the culture temperature was either maintained at 30°C or shifted to 42°C. At the same time, radioactive *meso*-[^{3}H]DAP was added, and its incorporation into peptidoglycan (expressed in counts per minute per milliliter of culture) was measured as described in Materials and Methods. Bacterial growth was followed in parallel by measuring the A_{600} of the culture. (A and C) OV2 *lysA*; (B and D) GS58 *lysA*. Symbols: ○, OV2 *lysA* at 30°C; ●, OV2 *lysA* at 42°C; □, GS58 *lysA* at 30°C; ■, GS58 *lysA* at 42°C.

cytoplasmic step by determining the effect of its inactivation on the pool levels of the different cytoplasmic nucleotide precursors characteristic of this metabolic pathway. As shown in Table 3, an arrest in the de novo synthesis of functional MurG protein molecules led to an increase of the pool levels of the two main cytoplasmic precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide. No variation was observed in the pool of the other intermediate precursors from UDP-GlcNAc-enolpyruvate to UDP-MurNAc-tripeptide, which are always detected at a much lower pool level, as previously described (32, 33, 38).

It was noteworthy that both accumulated compounds, UDP-GlcNAc and UDP-MurNAc-pentapeptide, were the substrates of the subsequent membrane steps of peptidoglycan synthesis. This result suggested that the mutational block was located downstream from UDP-MurNAc-pentapeptide in this metabolic pathway and consequently that the *murG* gene product was involved in a membrane step rather than in some early cytoplasmic step. This finding was in agreement with the subcellular localization of the corresponding protein recovered almost exclusively as a membrane-associated protein in the experiments described above.

To further localize the step blocked in the *murG* mutant, the cellular distribution of *meso*-[^{3}H]DAP was determined in strains OV2 *lysA* and GS58 *lysA* grown at both permissive and nonpermissive temperatures. As shown in Table 4, UDP-MurNAc-pentapeptide was in all cases detected as the main labeled compound, accompanied by very small amounts of lipid intermediates. This finding was consistent

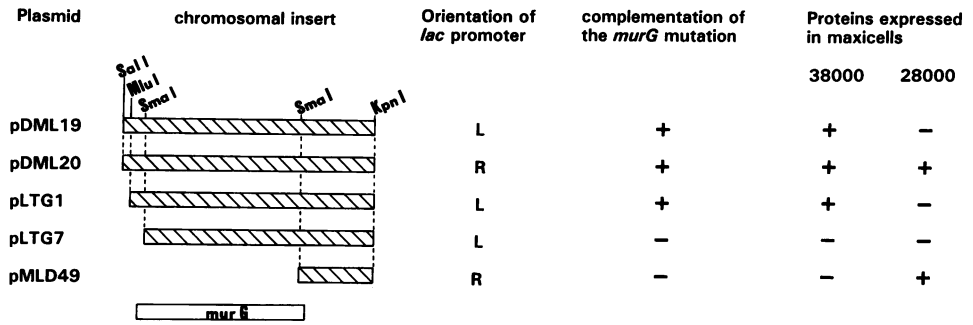

FIG. 4. Identification of the *murG* gene product as a 38K protein. Different plasmids carrying inserts derived from the 1.7-kbp *SalI-KpnI* fragment that contains the *murG* gene were constructed and analyzed for their capability to complement the *murG* mutation and to express the radioactive 38K and 28K protein species previously observed with this latter fragment in maxicell experiments (35). L (left) and R (right) indicate the positions of the *lac* promoter relative to the chromosomal inserts.

with previous data from other laboratories (19, 48) and indicated that the pool level of the lipid intermediates was very low compared with that of the cytoplasmic nucleotide precursor. When looking at the differences observed between the two strains, it was noteworthy that the *murG* mutant strain grown at 42°C accumulated significant amounts of UDP-MurNAc-pentapeptide and to a lesser extent a lipid compound as labeled DAP-containing precursors (Table 4). This was in agreement with the increase in the pool level of the two major nucleotide precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, of this metabolic pathway observed in this strain during growth at 42°C (see above). The accumulation of labeling in lipid intermediates in this strain was very interesting, since it could be interpreted as a block either at the level of the second membrane reaction that catalyzes the synthesis of lipid II from lipid I or at the level of the utilization of lipid II by polymerization reactions. In all cases, it excluded the hypothesis of a block at the level of the first membrane reaction catalyzed by the phospho-MurNAc-pentapeptide translocase that would have had the effect of depleting the pool of both lipid intermediates. To differentiate between the two other hypotheses, the relative amounts of the two lipid intermediates in the respective labeled lipid fractions were determined by previously described procedures (19, 48). As shown in Table 4 and Fig. 7, a value of 0.37:1 was determined for the ratio between lipid I and lipid II in the parental strain OV2 *lysA* grown either at 30 or at 42°C or in the mutant strain GS58 *lysA* grown at 30°C. This finding was in agreement with the value of 0.33:1 recently estimated in strain MC4100 *lysA* by Kohlrausch et al. (19) or 0.3:1 to 0.6:1 determined previously for other *lysA*-carrying strains by Ramey and Ishiguro (48). However, in considering the mutant strain grown at 42°C, this ratio was reversed and reached the considerably high value of 8.2:1 (Table 4 and Fig. 7), a result indicating clearly that it was the step involved in the formation of lipid II from lipid I which was altered in the mutant at 42°C. Consequently, it could be deduced that the *murG* gene product was the UDP-GlcNAc:MurNAc-(pentapeptide)pyrophosphoryl-undecaprenol-GlcNAc-transferase which catalyzed the formation of lipid II from lipid I.

It was also noteworthy that the concentration ratio between UDP-MurNAc-pentapeptide and lipid I was found to be more or less constant, whatever the strain and the growth temperature used (from 110:1 to 140:1 [Table 4]). Since it was established previously (45) that the phospho-MurNAc-pentapeptide translocase also catalyzed the reverse reaction,

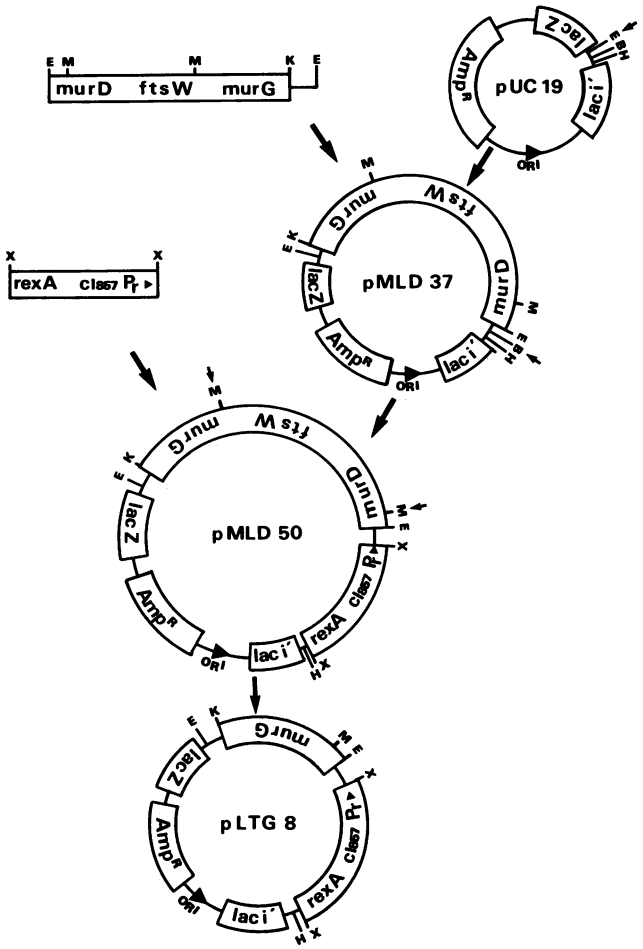

FIG. 5. Construction of plasmids carrying the *murG* gene. The 4.5-kbp *Eco*RI-*Kpn*I chromosomal fragment carrying the series of genes *murD-ftsW-murG* was isolated as an *Eco*RI-*Eco*RI sequence from the already described pDML7 plasmid (35) and inserted in the right orientation into the *Eco*RI site of pUC19 to yield pMLD37. Plasmid pMLD50 was constructed by inserting the 2.4-kbp *Xho*II-*Xho*II lambda fragment that carries the p_R promoter into the *Bam*HI site of pMLD37. Plasmid pLTG8 was obtained from pMLD50 by internal *Mlu*I-*Mlu*I deletion. Symbols for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; X, *Xho*II. Small arrowheads indicate sites used in plasmid construction.

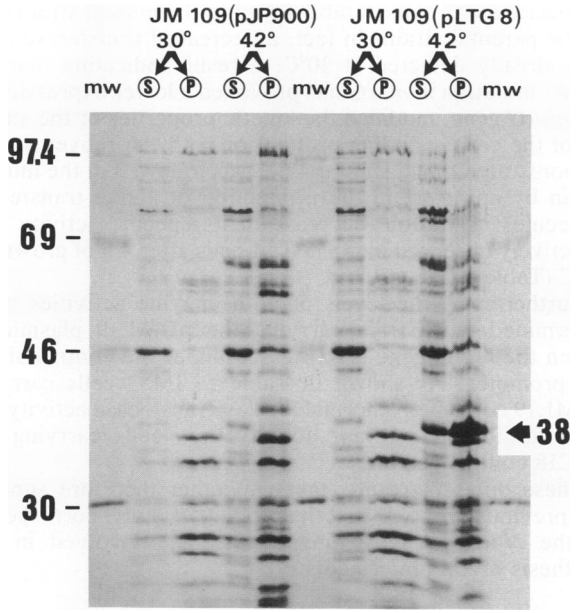

FIG. 6. Overproduction of a 38K protein in crude extracts from strain JM109(pLTG8). Strains JM109(pLTG8) and JM109 harboring the control plasmid pJP900 that does not contain the *murG* gene were grown exponentially at 30°C in rich 2YT medium. At an OD of 0.1, the culture temperature was either maintained at 30°C or shifted to 42°C. Cells were harvested 2 h later and were disrupted by sonication. As described in Materials and Methods, the protein contents of the soluble (S) and particulate (P) fractions obtained after high-speed centrifugation of the corresponding crude extracts were analyzed by SDS-PAGE. Molecular weight (mw) standards (thousands) indicated on the left are as follows: phosphorylase *b* (97.4), bovine serum albumin (69), ovalbumin (46), and carbonic anhydrase (30). The 38K protein, highly overproduced in the particulate fraction from JM109(pLTG8) after growth at 42°C, is indicated by the arrow on the right.

such a constant ratio probably indicated that both the transfer and the reverse reactions were equilibrated in all cases.

Levels of translocase and transferase activities in membranes. To further substantiate these results, we determined the specific activities of the phospho-MurNAc-pentapeptide translocase and *N*-acetylglucosaminyl transferase in crude extracts of strains OV2 *lysA* and GS58 *lysA*.

TABLE 2. Peptidoglycan content of the parental and *murG* mutant strains as a function of growth temperature[a]

Strain and growth temp (°C)	Amt (nmol/g of bacteria [dry wt]) of isolated peptidoglycan
OV2 *lysA*	
30	9,250
42	9,500
GS58 *lysA*	
30	8,900
42	5,750

[a] Cells were grown exponentially at 30°C in minimal medium supplemented with lysine. At an OD of 0.1, the culture temperature was either maintained at 30°C or shifted to 42°C and the cultures were stopped approximately 5 h later (the time at which the growth rate of the mutant strain at 42°C begins to decrease). Cells were harvested, and their peptidoglycan was isolated and quantified as detailed in Materials and Methods. The peptidoglycan content of sacculi is expressed in terms of the DAP content.

TABLE 3. Pool levels of the main cytoplasmic peptidoglycan precursors in the parental and *murG* mutant strains as a function of growth temperature[a]

Strain and growth temp (°C)	Pool level (nmol/g of bacteria [dry wt])	
	UDP-GlcNAc	UDP-MurNAc-pentapeptide
OV2 *lysA*		
30	510	390
42	410	520
GS58 *lysA*		
30	580	690
42	660	910

[a] Cells were grown exponentially in rich 2YT medium at 30°C. At an OD of 0.1, the culture temperature was either maintained at 30°C or shifted to 42°C and the cultures were stopped approximately 90 min later (the time at which the growth rate of the mutant strain at 42°C begins to decrease). Cells were harvested, and their peptidoglycan precursors were extracted and quantified as described in the text.

As previously established (45), the translocase activity catalyzes the following: (i) the transfer of phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to the membrane acceptor according to the reaction UDP-MurNAc-pentapeptide + undecaprenyl-phosphate $\rightleftharpoons$ undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide + UMP and (ii) an exchange according to the reaction [^{14}C]UMP + UDP-MurNAc-pentapeptide $\rightleftharpoons$ [^{14}C]UMP-phosphoryl-MurNAc-pentapeptide + UMP. Its activity was thus assayed by these two methods. The exchange assay was performed essentially as described previously (7, 45), with the exception that final separation and quantification of UMP and UDP-MurNAc-pentapeptide were facilitated by using a rapid electrophoretic procedure.

N-Acetylglucosaminyl transferase was assayed by following the incorporation of radioactivity from UDP-[^{14}C]GlcNAc into lipid II. Almost no incorporation was observed when UDP-MurNAc-pentapeptide was omitted from the reaction mixture, a control indicating that the labeled lipid component quantified in this assay mainly consists of the lipid II specific for peptidoglycan synthesis, with only traces of other eventual [^{14}C]GlcNAc-containing lipid intermediates involved in the biosynthesis of other cell envelope components (lipopolysaccharides and enterobacterial common antigen).

The specific activities of both enzymes were determined in crude membrane preparations from spheroplasts of OV2

TABLE 4. Relative concentrations of the DAP-containing peptidoglycan precursors in strains OV2 *lysA* and GS58 *lysA*

Strain and growth temp (°C)	Distribution of *meso*-[^{3}H]DAP as measured by radioactivity (cpm)[a]			
	UDP-MurNAc-tripeptide	UDP-MurNAc-pentapeptide	Lipid I	Lipid II
OV2 *lysA*				
30	11,900	58,500	430	1,160
42	28,700	67,000	490	1,340
GS58 *lysA*				
30	10,010	101,300	720	1,930
42	85,400	448,300	4,150	510

[a] Amount of radioactivity incorporated in these different precursors corresponds to that found in 2.5 ml of cell culture labeled as described in Materials and Methods for 15 min with 18.5 kBq ml^{-1} of *meso*-[^{3}H]DAP.

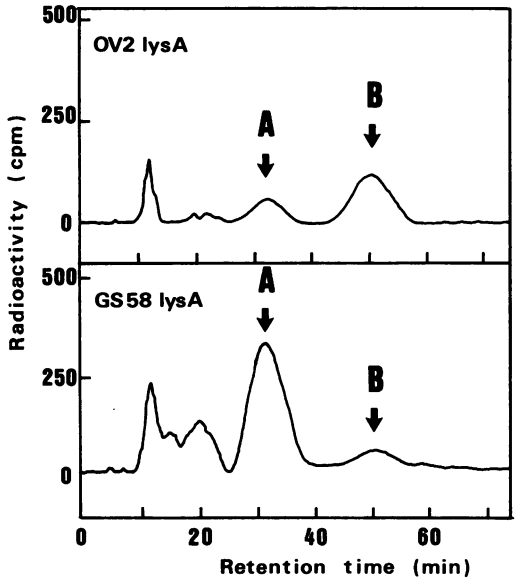

FIG. 7. Relative amounts of lipid intermediates I and II in the parental and *murG* mutant strains after growth at 42°C. Strains OV2 *lysA* and GS58 *lysA* were grown in minimal medium for 4 h at 30 or 42°C before cells were labeled for 15 min with *meso*-[^{3}H]DAP. Lipids were extracted and hydrolyzed, and the resulting labeled MurNAc-pentapeptide and disaccharide-pentapeptide were reduced prior to their separation by reverse-phase HPLC. Operating conditions were as follows: isocratic elution at room temperature on a μ-Bondapak C_{18} column with a 0.05 M ammonium formate buffer (pH 3.7) at a flow rate of 0.5 ml min^{-1}. Peak A, MurNAc-pentapeptide; peak B, GlcNAc-MurNAc-pentapeptide (identified by running authentic standards). With cells grown at 30°C (data not shown), the elution profiles for the lipid extracts of both strains OV2 *lysA* and GS58 *lysA* were quite similar to that of strain OV2 *lysA* at 42°C.

lysA and GS58 *lysA* cells harvested after 4 h of exponential growth at 30 or 42°C. It is clear, from the data shown in Table 5, that the level of translocase was not at all affected by an arrest in *murG* gene expression. Inversely, the level of transferase was considerably lower in the mutant strain than in the parental strain. In fact, a decreased transferase level was already detected at 30°C, a result indicating that the amber mutation in *murG* has provoked a lower expression of the *murG* gene, modified the kinetic properties or the stability of the corresponding protein, or both. Moreover, it was demonstrated that a shift at 42°C was followed in the mutant strain by an arrest in the production of active transferase molecules: a 3.5-fold decrease in its specific activity was effectively observed in GS58 *lysA* cells after 4 h of growth at 42°C (Table 5).

TABLE 5. Levels of translocase and transferase in *E. coli* cells

Strain and growth temp (°C)	Sp act of enzymes in membranes[a]		
	Translocase activity		Transferase activity
	Exchange assay	Transfer assay	
OV2 *lysA*			
30	1.0	1.0	1.0
42	0.9	1.2	1.25
GS58 *lysA*			
30	0.9	0.9	0.21
42	1.1	1.3	0.06
JM83(pUC18), 37	1.1	1.2	1.1
JM83(pDML19), 37	1.2	1.3	4.0

[a] Enzymatic assays were carried out as described in the text. For the exchange assay of translocase activity, a value of 1.0 corresponds to 50 pmol of [^{14}C]UMP incorporated per min per mg of protein in UDP-MurNAc-pentapeptide; for the transfer assay of translocase, a value of 1.0 corresponds to 4 pmol of lipid I synthesized per min per mg of protein; and for the transferase assay, a value of 1.0 corresponds to 1.6 pmol of lipid II synthesized per min per mg of protein.

Furthermore, the levels of both enzyme activities were determined in a strain carrying the pDML19 plasmid in which the *murG* gene is expressed under the control of the *lac* promoter. As shown in Table 5, JM83 cells carrying pDML19 contained a normal level of translocase activity but fourfold-more transferase activity than cells carrying the pUC18 control vector.

These different results taken together therefore support our previous proposal that the product of *murG* corresponds to the *N*-acetylglucosaminyl transferase involved in the synthesis of lipid intermediate II.

DISCUSSION

The *murG* gene of *E. coli* was discovered previously (50) at 2 min on the chromosome within the cluster of genes from *pbpB* to *envA* that code for proteins involved in different aspects of peptidoglycan synthesis and cell division. Its involvement in a cell envelope-related function was deduced from the various alterations of cell shape that characterize the thermosensitive GS58 mutant strain under restrictive growth conditions. The absence of filamentation and the fact that cells apparently divide normally (50) further excluded its participation in the cell division process.

It is shown in the present work that the *murG* mutant strain GS58 resembles other previously described mutants with defects in peptidoglycan synthesis in terms of the lysis phenotype it exhibits at the nonpermissive temperature. This defect was unequivocally established by demonstrating the inhibition of peptidoglycan synthesis at 42°C in the *murG* mutant strain either by measuring the peptidoglycan cell content itself or by following the incorporation of *meso*-[^{3}H]DAP into the macromolecule. In particular, we observed that lysis of *murG* mutant cells growing exponentially at 42°C finally occurred when their peptidoglycan content was reduced to a value representing only 50 to 60% of its normal cell level. It was reported previously that a DAP-requiring *E. coli* mutant was still able to grow and divide normally with a 50% reduced amount of peptidoglycan, when grown in the presence of limited amounts of DAP (47). Furthermore, Leduc et al. (21) previously determined a 6.6-nm thickness for the peptidoglycan of exponential-phase cells and showed that this value was consistent with a two- to three-layer structure. These different data are thus in agreement if we assume that under normal growth conditions *E. coli* cells contain about twice as much murein per unit of surface area as would be strictly required to maintain the physical integrity, morphology, and growth rate of the cell.

To determine the function of the *murG* gene product in *E. coli* cells, we analyzed the effect of inactivating the *murG* gene on the pool levels of the different cytoplasmic precursors characteristic of this pathway as well as on the cellular distribution of *meso*-[^{3}H]DAP in a *lysA* derivative strain. The mutant was shown to accumulate UDP-GlcNAc, UDP-MurNAc-pentapeptide, and, to a lesser extent, lipid inter-

mediate I after a shift to the nonpermissive temperature. Furthermore, the ratio of 0.4:1 that normally exists between the in vivo concentrations of lipid intermediates I and II in wild-type cells or in the mutant cells at 30°C was completely reversed (around 8.0:1) in the mutant cells grown at 42°C. This result was interpreted as an inhibition of the conversion of lipid I to lipid II, which is catalyzed by *N*-acetylglucosaminyl transferase activity. This was further confirmed by enzymatic assays that demonstrated a normal phospho-MurNAc-pentapeptide translocase level but a considerably lower level of *N*-acetylglucosaminyl transferase in the membranes from *murG* mutant cells grown at 42°C.

It is noteworthy that the extent of accumulation of lipid I in the membrane was very poor compared with that of the cytoplasmic precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide located upstream in this metabolic pathway. This probably represents the maximum level at which this lipid intermediate could be accumulated in the membrane. Different hypotheses to explain this limited accumulation could be made. (i) It should be remembered that the biosynthesis of lipid I is dependent on the availability in the membrane of the undecaprenyl-phosphate carrier lipid, the level of which has never been determined in *E. coli* cells but which is generally assumed to be low (4, 19). Moreover, this carrier lipid is not only utilized for peptidoglycan synthesis, since it is supposed to also participate in the synthesis of other cell envelope components such as lipopolysaccharides and enterobacterial common antigen (49). (ii) Since the phospho-MurNAc translocase also catalyzes the reverse reaction (45), the accumulation of lipid I is thus theoretically limited to a value defined by the equilibrium constant of both the transfer and reverse reactions, a hypothesis in total agreement with our finding that the ratio of concentrations existing between UDP-MurNAc-pentapeptide and lipid I remained more or less constant, whatever the strain and the growth conditions used.

The accumulation of UDP-MurNAc-pentapeptide previously observed (24, 25, 36) during treatments of *E. coli* and other bacteria by antibiotics (moenomycin, vancomycin, etc.) that block the utilization of lipid II by the subsequent polymerization reactions at a site located three steps downstream from this cytoplasmic nucleotide precursor in the peptidoglycan synthesis pathway is thus also consistent with a limited pool of lipid I.

The *murG* gene coding for the *N*-acetylglucosaminyl transferase involved in a membrane step of this metabolic pathway thus belongs to the same cluster as the genes involved in the synthesis of soluble precursors from UDP-MurNAc to UDP-MurNAc-pentapeptide. It is noteworthy that the gene coding for the phospho-MurNAc-pentapeptide translocase activity has also been mapped recently within this particular chromosomal region between *murF* and *murD* and has been named ORF-Y (12) or *mraY* (14).

As previously discussed (35), all the genes from the *pbpB-envA* cluster that code for proteins involved in different cell envelope-related functions appear to be organized into many different transcriptional units, and the reason for the clustering of these genes is thus not immediately apparent. However, a clustering of the genes coding for peptidoglycan synthetases in the 2-min region is not a general feature: the genes *murA* (57), coding for the phosphoenolpyruvate:UDP-GlcNAc-enolpyruvyl transferase, and *murB* (26, 49), also named *mrbA* (1, 43), coding for the UDP-GlcNAc-enolpyruvate reductase, have been mapped in a completely separated region around 90 min.

ACKNOWLEDGMENTS

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