Characterization of the Essential Gene glmM Encoding Phosphoglucomucose Mutase in Escherichia coli*

(Rceived for publication, July 26, 1995, and in revised form, October 10, 1995)

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Two different approaches to identify the gene encoding the phosphoglucomucose mutase in Escherichia coli were used: (i) the purification to near homogeneity of this enzyme from a wild type strain and the determination of its N-terminal amino acid sequence; (ii) the search in data bases of an E. coli protein of unknown function showing sequence similarities with other hexosephosphate mutase activities. Both investigations revealed the same open reading frame named yhbF located within the leuD-dacB region at 69.5 min on the chromosome (Dallas, W. S., Dev, I. K., and Ray, P. H. (1993) J. Bacteriol. 175, 7743-7744). The predicted 445-residue protein with a calculated mass of 47.5 kDa contained in particular a short region GIVISASHNP with high similarity to the putative active site of hexosephosphate mutases. In vitro assays showed that the overexpression of this gene in E. coli cells led to a significant overproduction of the 15-fold (phosphoglucomucose) amine mutase activity. A hexose 1,6-diphosphate-dependent phosphorylation of the enzyme, which probably involves the serine residue at position 102, is apparently required for its catalytic action. As expected, the inactivation of this gene, which is essential for bacterial growth, led to the progressive depletion of the pools of precursors located downstream from glucosamine 1-phosphate in the pathway for peptidoglycan synthesis. This was followed by various alterations of cell shape and finally cells were lysed when their peptidoglycan content decreased to a critical value corresponding to about 60% of its normal level. The gene for this enzyme, which is essential for bacterial growth, led to the formation of UDP-GlcNac has been poorly investigated. Considering that it was a potential site for the regulation of the flow of metabolites going through the peptidoglycan and lipopolysaccharide pathways, the latter reaction sequence has recently been investigated in more detail (11, 12). Four successive steps are required for the synthesis of UDP-GlcNac from fructose 6-phosphate (13, 14) (Fig. 1). The first of these reactions is catalyzed by the l-glutamine-s-fructose-6-phosphate amidotransferase (also named glucosamine-6-P synthase) (15). Mutants altered in this activity are characterized by an auxotrophy for GlcN or GlcNac (16, 17), and the corresponding glmS gene has been located at 84 min on the E. coli map (15, 18). The subsequent steps from GlcN-6-P to UDP-GlcNac are via GlcN-1-P (Fig. 1). We have previously shown that the gene of unknown function preceding glmS on the E. coli chromosome codes in fact for the GlcNac-1-P uridylyltransferase, and the gene for this last step leading to UDP-GlcNac (Fig. 1) was named glmU (11). More recently, we demonstrated that the glmU gene product also catalyzes the preceding step of acetylation of GlcN-1-P and thus appears as a bifunctional enzyme catalyzing two subsequent steps in the same pathway (12) (Fig. 1). Here we describe the partial purification and some properties of the phosphoglucomucose mutase which catalyzes the interconversion of GlcN-6-P and GlcN-1-P isomers, as well as the identification of the corresponding glmM gene on the chromosome of E. coli.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—E. coli strain JM83 [ara D lac-proAB] rpsL thi 980 gluC2 D M15) (19) was used as the host strain for plasmids and for the purification of the glmM gene product. Strain W1485 pGPM1 tet and plasmid pML14 carrying the E. coli pgm (phosphoglucometase) gene (20) were kindly provided by S. Slater. pJP900, a pUC9-derived plasmid carrying the 2.4-kb XhoI fragment of bacteriophage λ with the pml promoter inserted into its BamHI site, was obtained from J. Pli (CSIC, Universidad Autonoma de Madrid, Spain). Cloning vector pUC18 and the Kan’ cartridge originating from the pUC4K plasmid were purchased from Pharmacia Biotech Inc. Plasmid pMAK705 bearing a thermosensitive replicon was obtained from S. R. Kushner (21).

Growth Conditions—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, antibiotics were used at the following concentrations (in μg/ml): ampicillin (100), kanamycin (30), and chloramphenicol (25).

General DNA Techniques and E. coli Cell Transformation—Small and large-scale plasmid isolations were carried out by the alkaline lysis method (23). Standard procedures for endonuclease digestions, ligation, filling-in of protruding ends by using the Klenow fragment of DNA polymerase I, and agarose electrophoresis were used (23, 24). E. coli cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (25).

Construction of Plasmids—The 17-kb chromosomal fragment from E. coli which is carried by phage 14F1 (clone 521) from the bank of Kohara et al. (26) was used as the starting material for the construction...
The pMLD90 plasmid was constructed by inserting the 3.1-kb BamHI fragment carrying the glmM gene (yhbbII) into the BamHI site of the pUC18 vector (with glmM in the opposite orientation as compared to the lac promoter from the vector). The pMLD96 plasmid was constructed by inserting the 2.1-kb PstI fragment from pMLD90 into the corresponding site of the pUC18 vector (with glmM and lac promoter in the same orientation). An internal Accl-ClaI deletion in pMLD06 produced the plasmid pMLD99 (Fig. 2).

For the expression of glmM under the control of the lac promoter, plasmid pMLD100 was constructed by inserting the 2.4-kb XhoII λ fragment (carrying the structural gene c857 encoding a thermosensitive form of the λ cI repressor, the strong p85 promoter, and the ribosome-binding site of cro) (27) into the unique BamHI site of pMLD99. The disruption of the glmM gene was obtained by inserting the 1.28-kb HindII kan cartridge originating from pUC4K into the unique BssII site of plasmid pMLD90 lying within the glmM gene coding sequence, generating plasmid pMLD94 (Fig. 2). The pGMMkan plasmid was constructed by inserting the 4.5-kb BamHI fragment from pMLD94 (harboring the disrupted glmM gene) into the BamHI site of pMAK705 (21). The pGMM plasmid was isolated as described below during the excision process that followed integration of the pGMMkan plasmid at the chromosomal glmM locus.

Disruption of the Chromosomal glmM Gene—The wild-type chromosomal copy of glmM was replaced by a disrupted one by following the procedure of Hamilton et al. (21) which uses pMAK705, a plasmid bearing a thermosensitive replicon, pGMMkan, a pMAK705 derivative carrying the disrupted glmM gene, was transformed into JM83. Integration of the plasmid into the chromosome was selected for by plating 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 in 5 ml of water were homogenized by brief sonication. Aliquots were hydrolyzed and analyzed as described previously, and the pepidoglycan content of the sacculi was expressed in terms of its DAP content (6, 28).

Preparation of Crude Enzyme—Cells of JM83 harboring the different plasmids described above (excepting pMLD100) were grown exponentially at 37 °C in 2YT-ampicillin medium (1-liter cultures). Cells were harvested in the cold when the optical density of the cultures reached 0.7. A different protocol was used with strain JM83(pMLD100): cells were grown first at 30°C and at an absorbance of 0.01 (5 × 10^6 cells/ml), cultures were shifted to 42°C for 4 h during which time the absorbance reached a value of 0.7. Strains JM83 and GPM83 were grown at 30°C or 43°C as described above for the extraction of peptidoglycan precursors. In all cases, harvested cells were washed with 40 ml of cold 0.02 M potassium phosphate buffer (pH 7) containing 1 mM β-mercaptoethanol. The wet cell pellet was suspended in 9 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. The supernatant was dialyzed overnight at 4°C against 100 volumes of the same phosphate buffer, and the resulting solution (10 ml, 10 to 12 mg of protein/ml) designated as crude enzyme was stored at −20°C. SDS-PAGE analysis of proteins was performed as described previously using 13% polyacrylamide gels (29). Protein concentrations were determined by the method of Lowry, using bovine serum albumin as standard (30).

Assay for Phosphoglucomutase Mutase Activity—A coupled assay was routinely used in which the GlcN-1-P-synthesized from GlcN-6-P by the mutase was quantitatively converted to UDP-GlcNAc in the presence of purified bifunctional GlmU enzyme (12). The standard assay mixture contained 50 mM Tris-HCl buffer (pH 8.0), 3 mM MgCl₂, 1 mM GlcN-6-P, 0.4 mM [14C]acetyl-CoA (700 Bq), 10 mM UTP, 0.7 mM GlcN-1,6-diP, pure GlmU enzyme (1 μg), and enzyme (0.1 to 10 μg of protein, depending on overexpression or purification factors) in a final volume of 100 μl. Mixtures were incubated at 37°C for 30 min, and reactions were terminated by the addition of 10 μl of acetic acid. Reaction products were separated by high-voltage electrophoresis on Whatman No. 3MM filter paper in 2% formic acid (pH 1.9) for 90 min at 40 V/cm using an.
TABLE I

| Step                  | Total protein | Total activity | Specific activity | Purification factor |
|-----------------------|---------------|----------------|-------------------|---------------------|
| Crude extract         | 5000 mg       | 240 units      | 0.048 units·mg⁻¹   | 1 100               |
| DEAE-Trisacryl        | 420 mg        | 96 units       | 0.228 units·mg⁻¹   | 4.8 40              |
| Hydroxylapatite-Ultrafuge | 41 mg       | 50 units       | 1.22 units·mg⁻¹    | 25 21               |
| Ultrogel-AcA 44       | 32 mg         | 48 units       | 1.5 units·mg⁻¹     | 31 20               |
| Carboxymethyl-cellulose | 7.7           | 20 units       | 2.59 units·mg⁻¹    | 54 8.3              |

* The purest fraction eluted from this column of CM32 and used for electrophoretic concentration and protein sequencing contained 0.5 mg of protein with a specific activity of 7.2 units · mg⁻¹, corresponding to a purification factor of 150.

**RESULTS**

Purification and Amino Acid Sequencing of Phosphoglucomutase—Starting from a 54-liter culture of wild-type strain JM83 which yielded 5 g of total soluble proteins, the phosphoglucomutase activity was purified approximately 150-fold to a final specific activity of 7.2 μmol·min⁻¹·mg⁻¹ (Table I). The purest fractions eluted from the last column contained a major protein species migrating after denaturing polyacrylamide gel electrophoresis as a protein of 45–50 kDa (Fig. 3). It was the only one coeluting perfectly in all purification steps with the phosphoglucomutase activity. The N-terminal amino acid sequence of this protein species was thus determined after electrophoretic Immobilon-P and appeared as follows: SNKKYPGTX.

Search for the glmM Gene Using Data Bases—The alignment of this sequence with those of proteins from the Swissprot and Genpro libraries revealed that it matched perfectly with the N-terminal sequence MSNKKYPGTDGI predicted for the product of an E. coli open reading frame of unknown function named ybfF (31) or mrsA (GenBank accession number U01376) which was located near folP at 69.5 min on the chromosome. Several discrepancies were observed when comparing the two reported sequences of this gene. Using plasmid pMLD96 (Fig. 2) as the template and appropriate synthetic oligonucleotides, the complete 1335-bp coding sequence was thus verified again and appeared similar to that published first by Yamada et al. Taking into account the post-translational removal of the N-terminal methionine (revealed by protein sequencing), the mature product of this gene is theoretically a 444-amino acid protein with a molecular mass of 47,380.

In fact, this gene was identified at the same time by searching in data bases for a protein exhibiting sequence similarities with other known hexosephosphate mutases. The sequence of the phosphomannomutase from E. coli (the cpsG gene product) was used in the search which yielded all phosphoglucomutase and phosphomannomutase sequences characterized to date, as well as the YhbF putative peptide (26% identity in 433 overlaps). The alignment of YhbF with the same libraries gave as best scores two other proteins of unknown function containing 445 and 463 amino acids, respectively: UreC from Helicobacter pylori (44.1% identity on 425 residues) (32) and a putative protein from Mycobacterium leprae (Genpro accession number U00209, PE23, 44% identity on 440 residues). All these sequences contain in particular a short region previously characterized as the putative active site of hexosephosphate mutases. This motif which is described as (GA)(LIVM)(ST)(PGA)S*H in the PROSITE data base (PS00710) appeared as GIV VishNPDGNG in YhbF, where S* represents the active-site serine residue. It was generally assumed that the serine residue was phosphorylated during the catalytic action of the hexosephosphate mutases, a reaction requiring the presence of the corresponding hexose 1,6-diphosphate (33–35). This serine residue was located at position 102 in the sequence of YhbF. All these data clearly showed that the...
By similarity with the reaction phosphoglucosamine Mutase—from the same pathway (11, 17). Menclature previously adopted for the thus named taken together supported the initial proposal that the pUC18 control vector (Table II). All these different results phosphoglucosamine mutase activity than cells carrying the at 42°C, JM83(pMLD100) cells contained about 50-fold more optical microscopy. Finally, we showed that after 3 h of growth at 42 °C, JM83(pMLD100) cells contained about 50-fold more phosphoglucosamine mutase activity than cells carrying the yhbF gene was expressed under the control of the strong \(\lambda\) promoter was constructed. When cells of JM83(pMLD100) were grown exponentially at 30 °C and then shifted to 42 °C, an initial increase in the growth rate was observed, but growth progressively slowed down and the culture finally reached a plateau value. As soon as 1 h after the temperature shift, a large accumulation of the 47K protein was observed, which further increased to finally account for more than 20–30% of the cell proteins after a 3-h incubation period (Fig. 4). It comigrated with the protein overproduced from the other plasmid construction as well as with the phosphoglucomutase purified from wild-type strain JM83 (Fig. 4). A typical fractionation procedure of cell extracts showed that the highly overproduced protein was mainly found in the soluble fraction, but that significant amounts (10 to 20%) remained associated with the particulate fraction. This finding was certainly due to the formation of aggregates at this high level of protein expression, as confirmed by the presence of multiple inclusion bodies in induced cells when observed by optical microscopy. Finally, we showed that after 3 h of growth at 42 °C, JM83(pMLD100) cells contained about 50-fold more phosphoglucosamine mutase activity than cells carrying the pUC18 control vector (Table II). All these different results taken together supported the initial proposal that the yhbF gene coded for the phosphoglucosamine mutase. This gene was thus named glmM (for glucosamine mutase) following the nomenclature previously adopted for the glmS and glmU genes from the same pathway (11, 17).

Hexose 1,6-Diphosphate-dependent Phosphorylation of Phosphoglucomutase—By similarity with the reaction mechanism of other well characterized hexose-phosphate mu-
tases (33–37), the phosphoglucomutase was assumed to be active only in a phosphorylated form. As explained before, the serine residue at position 102 was the putative phosphorylated site and GlcN-1,6-diP was expected to be the phosphorylating agent. Since the latter compound was not commercially available, we used instead Glc-1,6-diP in our enzymatic assays, as generally was done with hexosephosphates mutases other than the phosphoglucomutase species (35, 37). The apparent GlmM activity that could be detected in the extract from wild-type strain JM83 was very low when Glc-1,6-diP was omitted from the reaction mixture (Table III). This basal level was greatly enhanced (up to 20-fold) by increasing the concentration of this compound (Table III), according to a hyperbolic saturation curve from which a constant of 150 \(\mu\)M could be calculated (data not shown). Interestingly, the overproduction of the enzyme activity was no longer detected in crude extracts from strains JM83(pMLD96) and JM83(pMLD100) when assays were performed in the absence of Glc-1,6-diP. Under these conditions, the apparent activity of GlmM was similar or curiously lower in these extracts than that detected in the wild-type extract (Table III). The addition of a saturating concentration of Glc-1,6-diP was accompanied by a very large increase (500- or 2000-fold) of the activity, which was consistent with the relative amounts of GlmM known to be present in these various extracts (see above). These different results first indicated that only a small part of the enzyme extracted from the cell content was present in the active phosphorylated form (5%), as judged from results obtained with the extract of strain JM3(pUC18)). Furthermore, the total amount of phosphorylated enzyme was clearly not increased in strains overproducing this protein to high levels, suggesting that the level of enzyme phosphorylation could be tightly regulated.

Inactivation of the Chromosomal glmM Gene and Its Effect on Bacterial Growth—To inactivate the glmM gene on the E. coli chromosome, we used the procedure described by Hamilton et al. (21) that is particularly well adapted for the disruption of essential genes, as recently shown for the construction of mutants altered in the glmU and murI genes which are essential for peptidoglycan synthesis (11, 38). First, the glmM gene coding sequence carried by the pMLD90 plasmid was disrupted by inserting at the unique BstEII site the 1.28-kb kanamycin resistance gene from pUC4K, generating plasmid pMLD94 (Fig. 2). The complete insert from the pMLD94 plasmid was inserted into the pMAK705 vector which bears a thermosensitive replicon. The resulting plasmid, pGMM::kan, was used to construct strain GPM83 (JM83 glmM::kan [pGMM]) having the inactivated glmM gene on the chromosome and the wild-type allele on the pMAK705 vector. At the restrictive temperature for plasmid replication (43 to 44 °C), GPM83 failed to grow on 2YT plates, indicating that this strain with a disrupted chromosomal copy of the glmM gene was viable only in the presence of a plasmid carrying the wild-type gene. The failure to transduce by phage P1 the glmM::kan marker from GPM83 to other E. coli K12 strains further confirmed that glmM was an essential gene.

Since the pGMM plasmid bears a thermosensitive replicon, the effects of the specific inactivation of the glmM gene were observed by shifting exponentially growing cells of GPM83 and JM83 from 30 °C to 43 °C. Both strains showed an identical growth rate and cell morphology when grown at 30 °C. However, after 5 to 6 h at 43 °C, the growth rate of GPM83 rapidly slowed down and cells apparently entered a stationary phase at a lower cell mass (Fig. 5). In addition, GPM83 cells progressively changed from rods to greatly enlarged ovoids when observed by phase-contrast microscopy, whereas the morphology of the parental strain was unaltered (data not shown). Cells
finally lysed after prolonged incubation at the restrictive temperature, as judged by a progressive decrease of turbidity of the culture (Fig. 5) and the presence of many ghosts within the cell population. This was consistent with the involvement of the glmM gene product in the biosynthesis of a cell-envelope component. The fact that these different effects were observed only after a few hours was explained by the time required for the progressive dilution or inactivation of the low-copy number pGMM plasmid and of the functional GlmM enzyme molecules present at the time of the temperature shift. As previously observed with a \( \text{glmU} \) mutant, these morphological changes were amplified when using a growth medium deprived of NaCl, and the precocious stationary phase which characterized GPM83 cells grown at 43°C was no longer observed when either 2% NaCl or 20% sucrose was added to the growth medium.

Biochemical Effects of the glmM Mutation—

The effects of the mutation on bacterial growth were clearly associated with the depletion of the phosphoglucosamine mutase activity. GPM83 cells effectively contained 50-fold less phosphoglucosamine mutase activity than the parental strain after growth for 5 to 6 h at the restrictive temperature (Table II). As shown in Table IV, an arrest in the \( \text{de novo} \) synthesis of functional GlmM enzyme molecules rapidly led to the depletion of the pools of the two main nucleotide precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide (Fig. 1). The pools of the other intermediates from UDP-GlcNAc-enolpyruvate to UDP-MurNAc-tripeptide, which are always detected at a much lower intracellular concentration (28), were also depleted (data not shown). This finding confirmed that the mutational block was in one of the early steps located upstream from UDP-GlcNAc, and the precocious stationary phase which characterized GPM83 cells grown at 43°C was no longer observed when either 2% NaCl or 20% sucrose was added to the growth medium.

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### Table II

| Strain         | Growth temperature | Specific activity | Amplification factor |
|---------------|--------------------|------------------|---------------------|
| JM83(pUC18)   | 37                 | 0.04             | 1                   |
| JM83(pMLD98)  | 37                 | 0.05             | 1                   |
| JM83(pMLD96)  | 37                 | 0.72             | 18                  |
| JM83(pMLD99)  | 37                 | 0.53             | 13                  |
| JM83(pMLD100) | 42                 | 2.12             | 53                  |
| JM83          | 30                 | 0.04             | 1                   |
| GPM83         | 42                 | 0.09             | 2                   |
| GPM83         | 30                 | 0.001            | 0.02                |

### Table III

| Strain    | Glucose 1,6-diphosphate | Phosphoglucosamine mutase activity (arbitrary units) |
|-----------|-------------------------|------------------------------------------------------|
| JM83(pUC18) | 0                      | 1                                                     |
| JM83(pUC18) | 30                     | 6                                                     |
| JM83(pUC18) | 120                    | 14                                                    |
| JM83(pUC18) | 700                    | 20                                                    |
| JM83(pMLD96) | 0                      | 0.7                                                  |
| JM83(pMLD96) | 700                    | 360                                                  |
| JM83(pMLD100) | 0                      | 0.5                                                  |
| JM83(pMLD100) | 700                    | 1060                                                 |

*The value of 1 for the activity detected in the absence of added glucose 1,6-diphosphate in the extract from the wild-type strain corresponded to an activity of \( 2 \times 10^{-3} \) units · mg of protein \(^{-1}\).*
representing about 60% of its normal level (Table IV). The highly reduced peptidoglycan content determined in the mutant cells most probably represented the lowest physiological value compatible with cell integrity.

We failed to detect any significant accumulation of GlcN-6-P (the substrate of GlmS) in GPM83 cells grown at the restrictive temperature (data not shown). This unexpected finding probably resulted from its permanent and rapid conversion by the GlcN-6-P deaminase (the nagB gene product) back to fructose 6-phosphate (Fig. 1) (14, 39).

**Capability of Different Plasmids to Complement the glmM::kan Mutation**—The different pUC18-derived plasmids which carry the glmM gene were tested for their capability to complement the thermosensitivity of strain GPM83. Interestingly, only those carrying inserts with glmM in the same orientation as the lac vector promoter (pMLD96, pMLD99) could restore the growth of GPM83 at 43 °C. The pMLD90 plasmid carrying the BamHI fragment where glmM and most (800 bp) of the preceding folP gene were inserted in the orientation opposite to that of the lac promoter (Fig. 2) failed to complement the mutation. As described in Table II, no overproduction of phosphoglucomutase mutase could be detected in cells carrying the pMLD90 plasmid. These results implied that transcription of the chromosomal glmM gene may occur from a promoter located far upstream from its initiation codon, suggesting that glmM is cotranscribed with the proximal folP gene.

Owing to the fact that the phosphoglucomutase and phosphoglucomutase mutase activities catalyzed similar reactions and used substrates which only differ by the presence of the amino group at position 2 of the sugar, a plasmid pML14 (20) carrying the pgm gene from *E. coli* was also tested but it failed to restore growth of GPM83 cells at 43 °C. Reciprocally, the pMLD96 plasmid was assayed for complementation of a strain deficient in phosphoglucomutase activity. W1485 pgm::tet (20) appeared as pink colonies of good size on Mac Conkey-galactose plates, when transformed with this plasmid. As compared to the large red colonies obtained with the pML14 plasmid and to the very small white colonies obtained with the control vector pUC18, this result could be interpreted as a partial complementation. It suggested that GlmS could catalyze at least to some extent the interconversion of the glucose-phosphate isomers. This side activity of phosphoglucomutase mutase was not investigated further.

As described above, two proteins of unknown function from *Helicobacter pylori* and *Mycobacterium leprae* showing more than 40% sequence identity with GlmM were found in data bases. It was tempting to speculate that these proteins were also phosphoglucomutase mutases. The observation that a plasmid carrying the ureC gene from *H. pylori* (pILL594 in Ref. 32) fully complemented the GPM83 mutant apparently confirmed this hypothesis.

**DISCUSSION**

Recently, we showed that both glucosamine-1-phosphate acetyltransferase and *N*-acetylglucosamine-1-phosphate uridylyltransferase activities from *E. coli* were carried by the glmU gene product which thus acted as a bifunctional enzyme catalyzing two contiguous steps in this pathway (11, 12). The actual characterization of a phosphoglucomutase mutase activity in crude extracts of *E. coli* and the demonstration that it is essential for growth is a final confirmation that the steps leading from GlcN-6-P to UDP-GlcNAc are via GlcN-1-P and bacteria (Fig. 1). This finding is consistent with the previous demonstration that exogenously supplied GlcNAc had to be deacetylated before it could be incorporated into cell walls (13). It suggests that any isomerase converting GlcN-6-P to GlcNAc-1-P has insignificant activity and that the only (or major) flux goes via GlcN-1-P which has to be reacylated before formation of the nucleotide. Interestingly, a different reaction sequence is encountered in yeast where synthesis of GlcNAc-1-P from GlcN-6-P occurs by the successive actions of glucosamine-6-P acetylase and *N*-acetylglucosamine-phosphate mutase activities (37, 40).

The glmM gene encoding phosphoglucomutase mutase was the last gene of the pathway for UDP-GlcNAc synthesis to be identified. Evidence is here provided that it corresponded to the previously sequenced open reading frame *yhhF* located at 69.5 min on the *E. coli* map. *glmM* is thus not linked to the related *glmS* and *glmU* genes previously identified at 84 min (11, 18) and which are probably cotranscribed (39, 41). The presence of other genes involved in peptidoglycan metabolism in the vicinity of *glmM* is noteworthy: in particular, the *dabC* and *murZ* genes encoding penicillin-binding protein 4 (42) and phosphoenolpyruvate:UDP-GlcNAc enolpyruvyl transferase (43), respectively (Fig. 2). However, it is clear that these genes of related function do not belong to a cluster of tightly packed genes as observed for the *mur* genes in the 2-min region (8). The experiments of complementation described in this work and the lack of an obvious promoter sequence on the DNA upstream of *glmM* (31, 44) suggested that this gene could be cotranscribed with the proximal folP gene. This is quite surprising when considering that the function of the folP gene product (dihydropteroate synthase involved in tetrahydrofolic acid synthesis) has no apparent relationship with cell-envelope metabolism (44). In fact, the same was observed in *H. pylori* where the gene for phosphoglucomutase mutase appears inserted within the urease operon, the reason for its initial designation as *ureC* (32). The transcription in this chromosomal region and a possible regulation of the *glmM* gene expression now has to be examined.

The effects of inactivating the *glmM* gene on the growth and cell morphology of *E. coli* were reminiscent of those observed with a *glmU* mutant altered in the next step from the same pathway (11, 12). Cells progressively lost their rod shape to become greatly enlarged ovoids and growth stopped early at a lower cell density. Interestingly, this was not followed by an abrupt decrease of culture absorbance indicative of cell lysis, as generally observed with mutants defective in peptidoglycan synthesis (8, 9, 38, 45). This was most probably due to the fact that the *glmM* mutation not only affects peptidoglycan but also

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**TABLE IV**

Peptidoglycan content and pool levels of its main precursors in the parental and glmM mutant strains

| Strain | Growth conditions | UDP-GlcNAc | UDP-MurNAc-pentapeptide | Peptidoglycan |
|--------|------------------|------------|-------------------------|---------------|
|        | °C               | nmol/g bacteria (dry wt) | pmol/g bacteria (dry wt) | nmol/g bacteria (dry wt) |
| JM83  | 30               | 975        | 605                     | 8600          |
| JM83  | 43               | 740        | 700                     | 9050          |
| GPM83 | 30               | 850        | 700                     | 9200          |
| GPM83 | 43               | 45         | 95                      | 5500          |
lipopolysaccharide synthesis. Effectively, thermosensitive mutations altered in the lipopolysaccharide pathway and in particular in the essential steps leading from UDP-GlcNAc to lipid A are characterized by an arrest of growth at the restrictive temperature (4, 5). The effects observed here with the glmM mutation are probably those expected from a simultaneous depletion of both cell-envelope components.

In the present paper, the first characterization and purification to near-homogeneity of a bacterial phosphoglucomannose mutase is described. The final preparation had a specific activity of 7.2 units/mg of protein where that of the crude extract from a wild-type strain was approximately 0.05 (Table I). Assuming that there was no loss of activity in this crude extract from $4 \times 10^{13}$ cells (5 g of protein) and that the purified enzyme contained only active GlmM molecules, a copy number of about 10,000 per cell could be estimated for this enzyme in a plasmid-free parental strain. This significant cellular abundance (0.5 to 1% of cell proteins) has certainly facilitated the successful purification in milligram quantities of this protein from a wild-type strain. It also explains why a 50-fold overproduction factor is enough to make it represent more than 20–30% of total cell proteins.

The amino acid sequence of the phosphoglucomannose mutase contains the characteristic signature of hexosephosphate mutases. This motif includes the putative serine residue (S102) whose phosphorylation is a prerequisite for enzyme activity. By similarity with other mutase activities and in particular the well-characterized phosphoglucomutase species (33–35), the reaction catalyzed by GlmM is thought to proceed in two subsequent steps as follows:

$$\text{GlcN-1-P} + \text{phosphorylated enzyme}$$
$$\text{GlcN-1,6-diP} + \text{dephosphorylated enzyme}$$
$$\text{GlcN-1,6-diP} + \text{phosphorylated enzyme}$$

**Scheme 1**

GlcN-1,6-diP which appears as an intermediate in the catalytic process could also be considered as the compound required for the initial activation (phosphorylation) of the enzyme. However, this is not yet clearly established as Glc-1,6-diP itself could efficiently phosphorylate GlmM. An extension of this work will be to characterize a putative enzyme involved in the specific synthesis of GlcN-1,6-diP in E. coli.

The fact that the phosphoglucomannose mutase is active only in a phosphorylated form is of great interest when considering the regulation of the flow of metabolites in this pathway. When assayed in the absence of added hexose-1,6-diphosphate, the apparent GlmM activity that could be measured after cell extraction theoretically reflects the total amount of phosphorylated enzyme present in the cell. As described in this work, this basal activity in a wild-type strain was enhanced about 20-fold in the presence of saturating concentrations of Glc-1,6-diP, suggesting that most of the GlmM molecules were phosphorylated and thus active in vitro. From the specific activity of 7.2 units/mg determined for the purified enzyme in the presence of a saturating concentration of Glc-1,6-diP and assuming a molecular weight of 47,380, a turnover of 340 min$^{-1}$ could be calculated. Under the in vitro conditions used, the phosphoglucomannose mutase can catalyze the formation of approximately $10^8$ molecules of GlnN-1-P in each cell during a 35-min generation time ($10,000 \times 340 \times 30$). Even if a 50% turnover of peptidoglycan material is taken into consideration (46), this value is much higher than that required for the formation of the average peptidoglycan content of exponentially growing cells previously estimated in the range from 3.5 to $5.5 \times 10^8$, depending on growth conditions (6, 28). The requirements for GlcN-1-P (UDP-GlcNAc) molecules of the lipopolysaccharide pathway have not been precisely determined but seem to be more or less equivalent (4, 47). This relative excess of enzyme is consistent with the observation that only a small number of enzyme molecules are apparently phosphorylated and thus active in vitro. It was also noteworthy that this basal activity was clearly not increased in strains overproducing as much as 50-fold the GlnM protein, a result indicating that the total amount of phosphorylated enzyme present in cells was unchanged and thus probably tightly regulated. Any specific regulation of the activity of this enzyme which catalyzes the first step in this reaction sequence could adjust in some way the synthesis of GlcN-1-P molecules to the specific requirements of the peptidoglycan and lipopolysaccharide pathways. The extent of enzyme phosphorylation could therefore be an important factor in the control of enzyme activity which should be investigated in detail now. Taking advantage of the plasmids constructed in this work, the enzyme is now being purified in large amounts for more precise investigations of its kinetic parameters and structure.

**Acknowledgments**—We wish to thank J. Plumberg and B. Badet for helpful discussions and critical reading of the manuscript, and N. Kleckner, A. Labigne, Y. Kohara, Y. Mechulam, and S. Slater for the generous gift of phages, plasmids, and bacterial strains. M. Nicaise and C. Ghels are greatly acknowledged for the sequencing of the purified GlnM enzyme.

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