UDP-*N*-acetylglucosamine:*N*-acetylmuramoyl-(pentapeptide) pyrophosphoryl undecaprenol *N*-acetylglucosamine transferase from *Escherichia coli*: overproduction, solubilization, and purification

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Abstract Plasmids for the high-level overproduction of wild-type, and C- and N-terminal His-tagged MurG *N*-acetylglucosaminyl transferase from *Escherichia coli* were constructed. In complementation tests the three forms were active in vivo. After IPTG induction, growth, spheroplast formation and lysis, overproduced MurG proteins were mainly present (90%) in the particulate fraction. Readily solubilized by CHAPS, they were purified without any detergent to over 80% purity for both His-tagged forms but only up to 20% for the wild-type form. The enzymatic activity of each purified MurG protein was determined and found to be inhibited to the same extent by ramoplanin.

Key words: Peptidoglycan; Lipid intermediate; Purification; MurG transferase; *Escherichia coli*

1. Introduction

The biosynthesis of bacterial cell wall peptidoglycan is a complex two-stage process. The first stage concerns the assembly of its monomer unit by enzymes located in the cytoplasm or at the inner side of the cytoplasmic membrane, whereas the second stage involves polymerization reactions taking place at the outer side of the cytoplasmic membrane. This implies the passage of the monomer unit from the cytoplasm to the externally located sites of polymerization through the hydrophobic environment of the membrane and is achieved by the formation of lipid intermediates [1–3]. First, a transferase (or translocase) catalyzes the transfer of the phospho-*N*-acetylmuramoyl-pentapeptide (phospho-MurNAc-pentapeptide) moiety from the cytoplasmic precursor UDP-MurNAc-pentapeptide to the membrane acceptor undecaprenyl phosphate to yield MurNAc-(pentapeptide)-pyrophosphoryl undecaprenol (lipid I). Thereafter, at least in the simple case of *Escherichia coli*, an *N*-acetylglucosaminyl transferase catalyzes the addition of *N*-acetylglucosamine (GlcNAc) to lipid I, yielding GlcNAc-MurNAc-(pentapeptide)-pyrophosphoryl undecaprenol (lipid II) which, after its transfer to the outer side of the cytoplasmic membrane, is used as the initial substrate for the polymerization reactions.

The *E. coli N*-acetylglucosaminyl transferase has been identified as the product of the *murG* gene, which is located in the *dcw* cluster at 2 min on the chromosome [4,5] and which has been cloned and sequenced [6,7]. MurG is an essential cationic protein of 354 amino acids with a molecular mass of 37 684 Da [4,8]. Moreover, it was shown to be associated with the inner face of the cytoplasmic membrane [9], thereby establishing that the entire peptidoglycan monomer unit is assembled before being transferred across the membrane. MurG is thus a key enzyme at the borderline between the two stages of peptidoglycan synthesis and can be considered as a potential target for the search of novel antibacterials. In the present paper we report the overproduction of the wild-type and His-tagged MurG proteins from *E. coli*, their solubilization, their purification, and some of their properties.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli strains JM83 (F$^-$ *ara* Δ[*lac-proAB*] *rpsL thi* [Φ80 d*lac*Δ-(*lac*Z)M15]), and JM109 (*endA1 recA1 thi gyrA96 hsdR17 relA1 supE44* Δ[*lac-proAB*], [F$^-$ *traD36 lacI*^{q} d*lac*Δ(*lac*Z)M15 *proA*$^+$*B*$^+$]) [10] were used as hosts for plasmids and for the preparation of the overproduced wild-type and mutant MurG transferases. Strain GS58 carrying an amber *murG* mutation in a thermosensitive *supF* suppressor background was previously described [8]. Plasmid vectors pQE30, pQE60, and pREP4 were purchased from Qiagen (Courtaboeuf, France). 2YT medium [11] was used and growth at 37°C was monitored by turbidity measurements at 600 nm. For strains carrying drug resistance genes ampicillin and kanamycin were used at 100 and 30 μg/ml, respectively.

2.2. Chemicals and analytical procedures

Lauryldimethylamine oxide (LDAO) was purchased from Calbiochem (La Jolla, CA, USA), sodium deoxycholate (DOC) from BDH (Poole, UK), Ni-NTA agarose from Qiagen (Courtaboeuf, France), isopropyl-β-D-thiogalactopyranoside (IPTG) from Eurogentec (Seraing, Belgium), and *t*-octyl-phenoxy-polyethoxyethanol (Triton X-100) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) from Sigma (St. Louis, MO). Ramoplanin was a gift from Lepetit (Milan, Italy). Sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) were performed as previously described [4]. Protein content was determined by the method of Bradford [12] using bovine serum albumin as a standard.

2.3. General DNA techniques and E. coli cell transformation

Small- and large-scale plasmid isolations were carried out by the alkaline lysis method [13]. Standard procedures for endonuclease digestions, ligations, and agarose electrophoresis were used [13,14]. *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich [15] or by electroporation.

2.4. Construction of plasmids carrying the murG gene

The *murG* gene [6,7] was amplified by PCR from the chromosomal DNA of *E. coli* strain JM83 and was cloned into pQE vectors which are based on the phage T5 transcription-translation system. For expression of wild-type MurG, primers were 5′-AA**GAATTC**ATTAAAGAGGAGAGGTTCACGATGAGTGGTCAAGGAA-3′ (primer A), and 5′-AAGGC**AGATCT**ACAATTACGCCCGGGCAACCCGGCTCACTTC-3′ (primer B). Primer A contained an *Eco*RI site (in bold), followed by a 21 bp sequence including the optimal ribosome-binding site found in pQE vectors, and 16 bp of the *murG* reading frame (initiation codon underlined). Primer B contained a *Bgl*II

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site (in bold) and 31 bp of anticoding sequence including the native stop codon. Amplified DNA was cut with *Eco*RI and *Bgl*II and inserted between the corresponding sites of vector pQE60, generating plasmid pDMC1. For expression of N-terminal His-tagged MurG, PCR primers were 5′-CGAGG**GGATCC**ATGAGTGGTCAAGGA-AAGCGA-3′ (primer C), and 5′-ATCG**CTGCAG**TTACGCCCGGG-CAACCC-3′ (primer D). Primer C contained a *Bam*HI site (in bold), followed by the first 21 bp of the *murG* reading frame. Primer D contained a *Pst*I site (in bold), and 17 bp of anticoding sequence including the native stop codon. Amplified DNA was cut with *Bam*HI and *Pst*I and inserted into the corresponding sites of vector pQE30, generating plasmid pDMC2. For expression of C-terminal six His-tagged MurG, PCR primers were primer A (see above) and 5′-CGCTA**AGATCT**CGCCCGGGCAACCCGGC-3′ (primer E). Primer E contained a *Bgl*II site (in bold), and 17 bp of anticoding sequence corresponding to the end of the *murG* gene sequence without the native stop codon. The amplified DNA fragment was cut with *Eco*RI and *Bgl*II, and was inserted between the corresponding sites of vector pQE60, generating plasmid pDMC3.

2.5. Preparation of crude extracts from MurG overproducing strains

Strains JM109(pDMC1), JM109(pDMC2), and JM109(pDMC3) were grown under strong aeration at 37°C. Cultures supplemented with 1 mM IPTG at OD 0.25 stopped growing after 2 h and cells were harvested in the cold at OD 0.8 1 h later. Spheroplasts, prepared as described previously [16], were lysed in 50 mM Tris-HCl, pH 8, and treated with DNase and RNase. After centrifugation at 200 000×*g* for 20 min at 4°C, the pellet (30 mg protein from 2 g bacteria) was resuspended in 1 ml of 20 mM potassium phosphate, pH 7, containing 0.1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol (buffer A) and stored at −20°C.

2.6. Solubilization of MurG by salt or detergent treatments

Spheroplast membranes were treated at 4°C for 15–60 min with either 0.2 M NaCl, 0.2 M potassium phosphate (pH 7), or 1.5 M LiCl according to Taku and Fan [17]. Solubilization by detergents was performed by treatments at 4°C for 15–60 min with either Triton X-100 (0.01, 0.1 or 0.5 M), 0.1% DOC, CHAPS (0.5, 1 or 2%) or 2.6 mM LDAO. For preparative purposes spheroplast membranes (75 mg protein) suspended in buffer A (5 ml) containing 0.5% CHAPS were gently stirred for 1 h at 4°C. After centrifugation at 200 000×*g* for 20 min at 4°C the treatment was repeated twice and supernatants pooled.

2.7. Purification of solubilized MurG proteins

CHAPS-solubilized material (75 mg protein) from wild-type MurG overproducing strain JM109(pDMC1) was applied onto a DEAE-Trisacryl M (IBF-Pharmindustrie, Villeneuve-la-Garenne, France) column (1.2×20 cm). Elutions were carried out at 4°C first with 30 ml of 20 mM Tris-HCl, pH 8, and thereafter with a 0–0.6 M NaCl gradient (90 ml) in the same buffer. Eluents were recovered in 1 ml fractions. CHAPS-solubilized material (25 mg protein) originating from His-tagged MurG overproducing strain JM109(pDMC2) or JM109(pDMC3) was gently stirred batchwise overnight at 4°C with 1 ml of Ni-NTA-agarose. Elutions were carried out on a small column at 4°C first with 50 mM sodium phosphate, pH 8, containing 20 mM imidazole (4 ml), and thereafter by stepwise increase of the imidazole concentration up to 400 mM. Purification was achieved by gel filtration on a Superose 12 (Pharmacia, Uppsala, Sweden) column (1×30 cm) eluted with 20 mM Tris-HCl, pH 8, at a flow rate of 0.5 ml/min. Purification of the highly overproduced MurG proteins was followed by SDS-PAGE.

2.8. Enzymatic assay

N-Acetylglucosaminyl transferase activity was assayed as previously described [4]. The reaction mixture 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 (9.3 GBq/mmol), *E. coli* membranes (90 μg of protein) prepared by sonication from strain JM109, and MurG protein (10 μg). All components except the radiolabeled substrate were first incubated for 10 min at 35°C in order to generate lipid I. The reaction was initiated by the addition of UDP-[^{14}C]GlcNAc. After 30 min at 35°C it was stopped by boiling for 3 min. Reaction mixtures were applied to Whatman 1 filter paper (length 50 cm) and subjected to descending chromatography for 16 h in isobutyric acid/1 M ammonium hydroxide (5/3). Radioactivity was located and counted with a scanner (Tracemaster LB285, Berthold, Bad Wilbad, Germany). Activities were expressed as nmol of transferred *N*-acetylglucosamine per min and per mg of MurG protein.

2.9. Complementation test

The extremely high transcription rate initiated at the T5 promoter of pQE vectors can only be efficiently repressed by the presence of high levels of the *lac* repressor protein. For this reason, the *murG* Ts strain GS58 was first transformed with the low-copy plasmid pREP4 which confers kanamycin resistance and constitutively expresses the LacI repressor protein. The resulting strain GS58(pREP4) was transformed by the pDMC plasmids to be tested for complementation. In each case, competent cells were mixed with plasmid DNA and the suspension was kept on ice for 3 h before being heated for 3 min at 42°C. After addition of 2YT medium cells were incubated at 30°C for at least 2 h and aliquots were plated onto two 2YT plates supplemented with ampicillin and kanamycin, one incubated at 30°C and the other at 42°C. Growth was observed after 24 h.

3. Results

3.1. Overproduction of wild-type and His-tagged MurG proteins

A plasmid suitable for the overproduction of the wild-type MurG protein, pDMC1, was constructed by cloning the *E. coli murG* gene under the control of the strong phage T5 promoter in vector pQE60. In order to discriminate between chromosomal and plasmid-originating enzymes and to allow for a rapid one-step purification procedure, plasmid vectors that expressed His-tagged forms of MurG were also constructed. Plasmids pDMC2 and pDMC3 were similar to

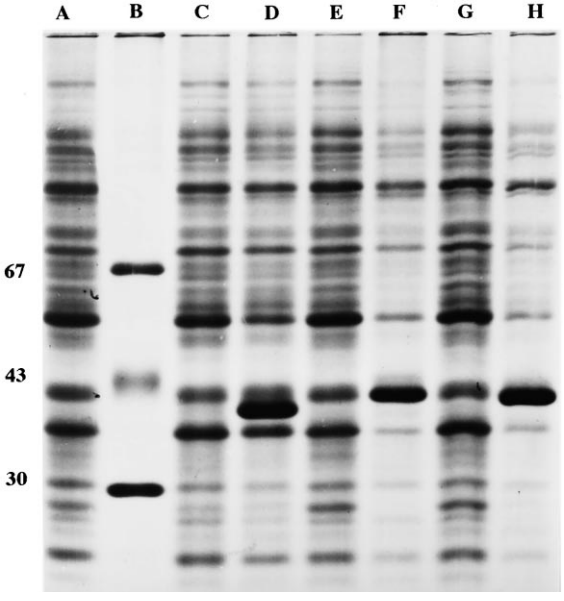

Fig. 1. Overproduction of wild-type and His-tagged MurG proteins visualized in crude extracts. Cells were grown exponentially at 37°C up to OD 0.25 before addition of IPTG (1 mM final concentration) to cultures D, F and H. Cells were harvested 3 h later at OD 0.8 and boiled prior to SDS-PAGE. Lane A, JM109; lane B, molecular mass standards (94 kDa, phosphorylase b; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase); lanes C and D: strain JM109(pDMC1) without and with IPTG; lanes E and F: strain JM109(pDMC2) without and with IPTG; lanes G and H: strain JM109(pDMC3) without and with IPTG. Staining was performed with Coomassie brilliant blue R250 (Merck, Darmstadt, Germany).

pDMC1 but expressed MurG with a 12-amino acid residue N-terminal extension (Met-Arg-Gly-Ser-His_6-Gly-Ser) and an 8-amino acid residue C-terminal extension (Arg-Ser-His_6), respectively. SDS-PAGE analysis of extracts from strains JM109(pDMC1), JM109(pDMC2) and JM109(pDMC3) clearly showed (Fig. 1) that IPTG induction led to a high-level overproduction of a 38-kDa protein corresponding to the theoretical molecular mass of the *murG* gene product [4,5]. It was mainly present (ca. 90%) in the particulate fraction from spheroplasts. Sonication led to a greater extent of solubilization (ca. 50%) but also to significant losses of enzymatic activity (data not shown). Therefore, spheroplast membranes were preferentially used as starting material for solubilization and purification of the MurG proteins.

3.2. Complementation

The different pDMC plasmids were transformed into the thermosensitive strain GS58, the only *murG* mutant characterized to date [8]. Only few transformants were obtained in each case, due probably to the unregulated and toxic expression of *murG* in the absence of IPTG in the strain. Transformation of GS58 by a first plasmid, pREP4, which expresses constitutively the *lac* repressor protein, was thus a prerequisite for complementation tests of the *murG* defect. The three pDMC plasmids restored growth of the mutant strain GS58(pREP4) at the restrictive temperature, indicating that the wild-type and His-tagged MurG proteins were active.

3.3. Solubilization and purification

Different techniques were examined to solubilize MurG from spheroplast membranes: salt (LiCl, NaCl, K phosphate) or detergent (DOC, CHAPS, LDAO, Triton X-100) treatments. CHAPS and LDAO were the most successful in terms of yield and conservation of enzymatic activity. Although SDS-PAGE analysis showed that LDAO led to a selective extraction of MurG on a 1 mg protein scale (data not shown), extraction with CHAPS was preferred since it led to more reproducible results on a 10–100 mg protein scale.

The wild-type MurG protein was partially purified by one-step DEAE chromatography. It was eluted at about 0.15 M NaCl and accounted for ca. 20% of the protein content in the main fraction recovered (Fig. 2). Attempts to further purify wild-type MurG by other classical column chromatographies remained unsuccessful. This prompted us to purify the His-tagged MurG proteins from JM109 (pDMC2) and JM109 (pDMC3) by affinity chromatography on Ni-NTA agarose. They were recovered in fractions eluting between 100 and 400 mM imidazole. After concentration, final purification and removal of imidazole were achieved by gel filtration. On SDS-PAGE the His-tagged MurG proteins accounted both for 80% of the protein contents (Fig. 2), and their migrations were consistent with their slightly increased molecular mass (39 213 and 38 881 respectively). Mass spectrometry analysis of N-terminal His-tagged MurG was in agreement with its theoretical mass (data not shown). The final products stored at −20°C remained soluble upon thawing.

The *N*-acetylglucosamine transferase activity of the three MurG proteins was determined according to the previously described assay [4] involving a coupling with the prior in situ formation of lipid I catalyzed by the MraY transferase. Values were 0.32, 1.1 and 2.2 nmol/min/mg for wild-type, N-terminal His-tagged and C-terminal His-tagged MurG proteins, respectively. Interestingly, if specific activities are based on the MurG protein contents estimated by SDS-PAGE the value for the wild-type protein is in the range of the two other ones (1.6, 1.4, and 2.8 nmol/min/mg, respectively). Moreover, they were inhibited to the same extent (70–80%) by 50 μg/ml ramoplanin, thereby confirming the mechanism of action of this lipoglycopeptide antibiotic whose target has been shown to be the conversion of lipid I into lipid II in cell-free systems from *Bacillus megaterium* and *Gaffkya homari* [18].

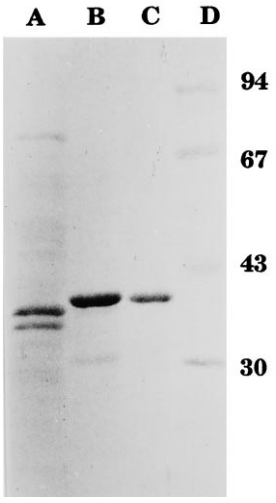

Fig. 2. Comparison of purified wild-type and His-tagged MurG proteins by SDS-PAGE. Lane A, MurG (upper band) from JM109(pDMC1); lane B, N-terminal His-tagged MurG from JM109(pDMC2); lane C, C-terminal His-tagged MurG from JM109(pDMC3); lane D, molecular mass standards as in Fig. 1. SDS-PAGE conditions, standards and staining are as in Fig. 1.

4. Discussion

E. coli MurG appears to be a peripheral membrane protein according to a number of criteria: partial solubilization by salt treatments, purification without detergent, localization on the inner side of the cytoplasmic membrane [9], a cationic theoretical p*I* value of 9.7 and a lack of significant hydrophobic regions in its amino acid sequence [6,7]. A MurG activity has been purified from *B. megaterium* after solubilization by LiCl [17,19]. Moreover, the extrinsic character of MurG appears to be a general feature as far as can be judged from the 13 ortholog sequences currently accessible in data banks. Interestingly, they all possess a specific consensus sequence (GGTGGHXXPXXA/S) near their N-terminus.

The values of the specific *N*-acetylglucosamine transferase activity of the three purified MurG proteins are very low and lead to a turnover of ca. 0.1/min, very far from the in vivo rate (50 000–100 000 units per min per cell) in growing cells [2] possessing presumably at most a few thousand MurG copies per cell. This is probably not due to an inactivation of the MurG transferases, since their specific activities based on MurG protein contents are similar, but rather to the conditions of the assay itself. The de novo formed lipid I is in limited amounts (reversibility of the MraY reaction and low pool of undecaprenyl phosphate) and located in membrane sites not easily accessible to tested soluble MurG. In vivo MurG more probably functions in a very organized environment where lipids I and II are associated with membrane-

bound MraY and MurG, and where the efficiency of the system relies in some way on the coupling between the two transferase activities [2]. There is also a possible coupling with the transport of lipid II through the membrane and its use in the polymerization reactions. Recently, the associated MurG and MraY transferases have been considered essential parts of the divisome [20].

Attempts to develop an efficient one-step MurG enzymatic assay using directly lipid I have unfortunately been hampered by the difficulty in obtaining large amounts of this intermediate [16,19,21]. Recently, direct assays have been developed using synthetic analogs of lipid I with C_{10} or C_{35} lipid chains [22,23]. Undoubtedly, the availability of reasonable amounts of such substrates will greatly facilitate further detailed studies of the MurG activity.

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