An assay for exogenous sources of purified MurG, enabled by the complementation of Escherichia coli murG(Ts) by the Mycobacterium tuberculosis homologue

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Abstract

The Mycobacterium tuberculosis murG gene, Rv2153, was expressed in Escherichia coli murG(Ts) strain OV58 on a plasmid under the control of the arabinose-inducible araBAD promoter. Mycobacterium tuberculosis murG rescued the growth of E. coli murG(Ts) at the nonpermissive temperature: transformants were only obtained in the presence of 0.2% arabinose at 42 °C, and their growth rate was dependent on arabinose concentrations. However, no MurG activity was detected in membranes from the transformant grown in arabinose at 42 °C, while MraY activity was normal. This observation led to the development of a membrane-based scintillation proximity assay for exogenous sources of MurG. Addition of purified E. coli MurG resulted in the reconstitution of MurG and peptidoglycan synthesis in these membranes. MurG is an attractive target for drug discovery, but assays to measure the activity of purified MurG are challenging. This presents an easy method to measure the activity of exogenous sources of MurG for structure–activity studies of mutant MurG proteins. It can also be used to compare the activity of, or effect of inhibitors on, MurG from other bacterial species.

Introduction

There is an urgent need for new antibacterial agents to treat resistant bacterial infections (Boucher et al., 2009). Many successful drugs, for example the β-lactams and vancomycin, target enzymes in the peptidoglycan pathway. MurG, which catalyses an essential step of peptidoglycan synthesis, is an attractive target for both target- and structure-based drug discovery, because crystal structures of MurG have been determined (Ha et al., 2000; Hu et al., 2003).

MurG catalyses (Fig. 1a) the transfer of N-acetylglucosamine (GlcNac) from UDP-GlcNac to MurNac-(pentapeptide)-pyrophosphoryl undecaprenol (lipid I) yielding GlcNac-MurNac(pentapeptide)-pyrophosphoryl undecaprenol (lipid II). However, measuring the activity of MurG during purification is challenging, as its substrate, lipid I, is not water soluble and is difficult to synthesize or isolate from bacteria in large quantities. The enzyme can either be assayed in its natural membrane environment (Mengin-Lecreulx et al., 1991) or in solution (Auger et al., 1997, 2003; Ha et al., 1999, 2000; Chen et al., 2002), although the synthetic substrates (Men et al., 1998; Auger et al., 1997, 2003; Ha et al., 1999, 2000; Chen et al., 2002) need considerable expertise to synthesize. MurG can be
assayed using the natural lipid substrate present in membranes, but the presence of endogenous MurG makes it difficult to measure purified MurG.

Cross-linked peptidoglycan synthesis has been monitored in *Escherichia coli* (Eco) membranes by incubation with the two sugar precursors UDP-N-acetyl-muramylpentapeptide [UDP-MurNAc(pp)] and UDP-GlcNAc, one of which is radiolabelled (Chandrakala et al., 2001). In the membranes, the disaccharide unit of peptidoglycan is synthesized on a lipid carrier by the MraY and MurG enzymes and subsequently polymerized by the transglycosylase and cross-linked to pre-existing peptidoglycan by the transpeptidase (Fig. 1). The radiolabelled, newly synthesized cross-linked peptidoglycan formed can be monitored by paper chromatography or a microplate scintillation proximity assay (SPA) using wheat germ agglutinin (WGA)-coated SPA beads (Chandrakala et al., 2001, 2004).

To monitor MurG activity, the pathway of reactions must be stopped at lipid II (Mengin-Lecreulx et al., 1991) (Fig. 1a) using an inhibitor of the transglycosylase (Ravishankar et al., 2005). Typically, in a first step, the MurG substrate is synthesized in situ in a second step, transfer of radiolabelled GlcNAc by MurG occurs (Fig. 1b). The product lipid II can be separated from UDP-GlcNAc by paper chromatography (Mengin-Lecreulx et al., 1991) or by an SPA (Ravishankar et al., 2005) (Fig. 1b).

We intended setting up an assay for *Mycobacterium tuberculosis* (Mtu) MurG by introducing it into an *E. coli* background, so that an established SPA (Ravishankar et al., 2005) could be used. Strain OVS58 has an amber mutation in murG and a temperature-sensitive amber suppressor, so that practically no *E. coli* protein is made at 42 °C (Salmond et al., 1980; Mengin-Lecreulx et al., 1991). A key question was whether the Mtu murG would functionally replace the *E. coli* homologue.

### Materials and methods

#### Materials

Wheat germ agglutinin-coated (WGA) beads for the SPAs were from Amersham International plc. U.K. UDP-[3H]-N-acetyl glucosamine was from NEN Dupont, USA. Moenomycin was gifted by Hoechst India. Ni-NTA resin was from Qiagen, USA. Other chemicals were from Sigma-Aldrich. UDP-N-acetyl muramyl pentapeptide [UDP-MurNAc(pp)] was purified from *Bacillus cereus* 6A1 (Chandrakala et al., 2001) and radiolabelled by incubation with [3H]-NHS-propionate (Solapure et al., 2005).

#### Bacterial strains, plasmids and growth media

*Escherichia coli* murG(Ts) (Salmond et al., 1980) was a gift from W.D. Donachie. pRSETA and *E. coli* BL21(DE3) were from Novagen; pBAD/Myc-HisA and PMOSBlue were from Stratagene. L-broth (LB) was used for bacterial growth medium, and ampicillin was added at 50 or 100 μg mL⁻¹ when required (LB-amp).

#### Cloning of Mtu murG (Rv2153) in an arabinose-inducible vector

The *murG* gene was PCR-amplified from *Mtu* genomic DNA with forward (5′- AAG GAC ACG GTC AGC CAG CC -3′) and reverse primers (5′- TCT AAA GCT TCG TCG TTG TCC TGG CAC CGG -3′) and cloned into pBAD/Myc-His A (Guzman et al., 1995) between the NcoI and HindIII sites. The resulting plasmid pAZI8952 has *Mtu* murG gene under the control of BAD promoter. The recombinant MurG has an additional 25 amino acids at the C-terminus compared with the native protein.
Expression of *M. tuberculosis* MurG in an *E. coli* murG(Ts) strain

pAZ18952 was transformed into *E. coli* murG(Ts) using heat shock (Sambrook *et al.*, 1989) but resuscitation was at 30 °C for 2 h. Cells were plated on LB-amp agar containing 0%, 0.02% and 0.2% arabinose. Two sets of plates were incubated at 30 and 42 °C. The transformants are referred to as *E. coli* murG(Ts);pAZ18952.

For studying the growth kinetics, *E. coli* murG(Ts);pAZ18952 was grown overnight in LB-amp, 0.2% arabinose (LB-amp-ara) at 42 °C. The cells were washed twice and used to inoculate fresh prewarmed LB-amp (initial A_{600 nm} ~ 0.1) containing different concentrations of arabinose or glucose; growth at 42 °C was monitored by the A_{600 nm}.

Membrane preparation from *E. coli* murG(Ts); pAZ18952 grown at 42 °C

pAZ18952 was transformed into *E. coli* murG(Ts), and transformants were selected on LB-amp-ara plates at 42 °C. Freshly grown transformants were inoculated into LB-amp-ara (A_{600} 0.01) and grown on a shaker till A_{600} of 1.6. Membranes were isolated (Chandrakala *et al.*, 2001) and will be referred to as *Eco*(Ts) ΔMurG.

Cloning, expression and purification of *E. coli* MurG

*Escherichia coli* murG was PCR-amplified using forward (5′-GCC GGA TCC ATG AGT GGT CAA CGA AA- 3′) and reverse (5′-GTC AAGC TTA CGCCCG GGC AAC CCG G-3′) primers and cloned into vector pRSETA between the BamHI and HindIII sites. The resulting plasmid, pARC0359, encoded *E. coli* MurG with an N-terminal His-tag, which, along with other epitopes, contributed an extra 35 amino acids compared with the native sequence, giving a calculated molecular weight of 42 kDa.

*Escherichia coli* BL21(DE3) transformed with pARC0359 was inoculated into LB-amp (A_{600 nm} 0.01) and grown at 37 °C on a shaker till A_{600 nm} 0.6. IPTG (1 mM) was added and the cells were harvested after 3 h. All further processing was carried out at 4 °C. The cells were washed in 20 mM Tris–HCl pH 7.5, 0.1 mM MgCl₂, resuspended in the same buffer and lysed in a French Press. The lysate was centrifuged at 6000 g for 10 min, and the supernatant was centrifuged at 200 000 g for 40 min. This membrane pellet was resuspended in 50 mM Tris–HCl, pH 7.5, 0.1 mM MgCl₂ and 1% CHAPS for solubilization. After 1 h, the solubilized material was centrifuged at 200 000 g. The supernatant was filtered through a 0.45-μm syringe filter, and the filtrate was stirred overnight with 1 mL Ni-NTA-agarose. Stepwise batch elution was carried out in a column with 1 mL of 50 mM Tris–HCl, pH 7.5 containing 100, 300 and 400 mM imidazole. The purified fractions were dialysed and concentrated for further analysis.

Enzyme assays

All enzyme assays were performed in duplicate in flexible 96-well microplates (1450-401) from Wallac, Finland, and the radioactivity was read in a Microbeta Trilux. For paper chromatography analysis, 2 μCi UDP-[³H]GlcNAc was used, and reactions were stopped by the addition of 5 μL of 90 mM EDTA instead of the SPA beads (Chandrakala *et al.*, 2001).

MraY assay

This was performed as earlier described (Solapure *et al.*, 2005). Briefly, *E. coli* membranes (source of MraY) were incubated with UDP-[³H]MurNAc(pp). The product, lipid 1, was captured by WGA-SPA beads.

MurG assay

MurG was assayed by the two-step SPA method (Ravishankar *et al.*, 2005). Briefly, in a first step, lipid I was formed by incubating membranes with UDP-MurNAc (pp) and moenomycin (1 μM) to prevent the conversion of lipid II to peptidoglycan. In the second step, MurG was assayed by adding UDP-[³H]GlcNAc (1.2 μCi, 2.5 μM) and DMSO, bringing the reaction volume to 25 μL. Lipid II was monitored using WGA-SPA beads. The ‘blank’ had no UDP-MurNAc(pp), and this reading was subtracted from the complete reaction for MurG ‘activity’.

Reconstituted MurG assay

This was performed as the MurG assay with the following modifications. *Eco*(Ts) ΔMurG membranes were used, and in the second step, 10 ng of purified *E. coli* MurG (an exogenous source of MurG) and Triton X-100 [to 0.05% (v/v)] was added along with UDP-[³H]GlcNAc. The enzyme blank had no exogenous MurG in step 2; the cpm obtained were similar to a blank where no UDP-MurNAc(pp) was added in the first step.

Peptidoglycan synthesis assay

This assay was performed as described earlier (Chandrakala *et al.*, 2001). Membranes were incubated with UDP-MurNAc(pp) (15 μM) and UDP-[³H]GlcNAc (0.5 μCi, 2.5 μM) in HEPES ammonia pH 7.5 at 37 °C for 90 min, and the cross-linked peptidoglycan was captured by
Results and discussion

Complementation of E. coli murG(Ts) with M. tuberculosis murG

The E. coli murG(Ts) (OV58) strain grows at 30 °C but not at 42 °C (Salmond et al., 1980). When this strain was transformed with 10 ng pAZI8952, containing Mtu murG under the control of an arabinose promotor, transformants were obtained at 30 °C (1.4 × 10³ CFU). However, at 42 °C, transformants were only obtained when 0.2% arabinose was included in the medium (1.5 × 10³ CFU). No transformants were obtained at 42 °C in the absence of arabinose or in 0.02% arabinose. The vector plasmid (10 ng) was used as control for transformation, and as expected, transformants appeared only at 30 °C (9.6 × 10³ CFU) but not at 42 °C.

Growth of the Mtu murG complemented E. coli murG (Ts) strain was dependent on arabinose. It was slow in the absence of arabinose, increasing steadily from 0.05% and saturating at 0.2% arabinose (Fig. 2). The initial growth in the absence of arabinose is probably due to Mtu MurG accumulated during the overnight growth at 42 °C in arabinose. Similarly, cells grew in 2% glucose (which represses expression from the arabinose promotor) initially but after 2 h, no further growth was observed (Fig. 2). The inhibition of growth in the presence of glucose (Fig. 2) is confirmation that no reversion of the mutation had occurred.

These data demonstrate that the Mtu murG gene can functionally complement the E. coli homologue to maintain cell viability, despite the fact that there is only 37% identity between the Mtu and E. coli MurG proteins. Additionally, Mtu MurG appears to be quite promiscuous in its substrate recognition (Auger et al., 1997) because it recognizes the C55-undecaprenyl lipid carrier in E. coli vs. its natural substrate of C-50 lipid linked to N-glycolylmuramylpentapeptide.

Lack of MurG activity in membranes of E. coli murG(Ts); pAZI8952

Membranes were prepared from E. coli murG(Ts); pAZI8952 grown at 42 °C in 0.2% arabinose to assay Mtu MurG. Unfortunately, no MurG activity was detected in these membranes (see data below and Table 2). Activity was undetectable even in the membranes of transformants grown in 2% arabinose to obtain higher levels of Mtu MurG.

The lack of MurG activity was surprising given that the Mtu murG complemented the E. coli (Ts) homologue and must have been functional. Activity was checked in the peptidoglycan synthesis assay in case the specific activity of the Mtu MurG protein was very low, because this assay is more sensitive than the MurG assay (Chandrakala et al., 2001; Ravishankar et al., 2005). No cross-linked peptidoglycan synthesis was detected in these membranes (Table 2), whereas the expected level of activity was observed in the membranes of wild-type E. coli grown at 37 °C. The assay time, temperature and quantity of protein were varied in an attempt to improve the sensitivity but peptidoglycan synthesis remained undetectable.

Both MurG and peptidoglycan synthesis assays are dependent on having a functional MraY (Fig. 1a). However, the MraY enzyme was active in the membranes of

Table 1. Assay of peptidoglycan synthesis and MraY in the membranes of wild-type Escherichia coli (AMA1004) and E. coli murG (Ts); pAZI8952 grown in 0.2% arabinose at 42 °C (Eco(Ts) ΔMurG). Cross-linked peptidoglycan synthesis or MraY was assayed as described at 37 °C and room temperature, respectively. Values represent activity (i.e. cpm after the subtraction of the blank value); the blank values are represented in italics below the ‘activity’. Data represent the mean ± SD (n = 2); the experiments were performed more than three times, and one representative experiment is shown.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Type</th>
<th>Result</th>
<th>E. coli</th>
<th>E. coli murG(Ts); pAZI8952–42 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan synthesis</td>
<td>Activity</td>
<td>4567 ± 20</td>
<td>Nil (−70)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>664 ± 27</td>
<td>412 ± 1</td>
<td></td>
</tr>
<tr>
<td>MraY</td>
<td>Activity</td>
<td>463 ± 69</td>
<td>469 ± 78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>367 ± 25</td>
<td>390 ± 12</td>
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</tbody>
</table>
the transformant, and the activity was similar to that in membranes from wild-type *E. coli* (strain AMA1004) grown at 37 °C (Table 1). This indicated that the block in peptidoglycan synthesis was downstream of the MraY and was probably due to the lack of MurG activity in these membranes. Either the *Mtu* MurG protein was unstable under the conditions of membrane preparation and storage, or the specific activity of the *Mtu* MurG protein was below the limit of detection or the assay conditions were not appropriate for *Mtu* MurG.

It is not obvious how membranes devoid of MurG can be made under normal circumstances, as *murG* is an essential enzyme. This result, while unexpected, offered an opportunity. Because the membranes contained the lipid carrier and all enzymes involved in peptidoglycan synthesis other than MurG, they provided a powerful assay system for MurG, provided that the addition of the pure enzyme could reconstitute the system. For ease of description, these membranes are referred to as *E. coli* (Ts) ∆MurG membranes. Membranes from wild-type *E. coli* or the Ts mutant cannot be used to assay exogenous MurG because the endogenous MurG activity would mask the activity.

**Reconstitution of peptidoglycan synthesis in E. coli(Ts) ∆MurG membranes**

Solubilized, purified *E. coli* MurG (2 µg) was added to the membranes of *E. coli(Ts) ∆MurG* incubated with the two UDP-linked sugar precursors under conditions for peptidoglycan synthesis. Considerable cross-linked peptidoglycan was synthesized (Table 2). This indicates that the exogenous *E. coli* MurG protein was not only able to access the lipid carrier in the membrane, but also able to interact with other membrane and enzyme components to reconstitute peptidoglycan synthesis in these membranes. That the product was peptidoglycan was confirmed by paper chromatography analysis, as the counts in the complete reaction (13 270 cpm) vs. the blank (179 cpm) were at the origin (Fig. 3a). This result confirmed that it was the MurG activity of the membrane preparation that was deficient.

MurG was assayed under similar conditions. Lipid II was synthesized when 10 ng of pure *E. coli* MurG was added to these membranes along with Triton X-100 (Table 2). The identity of the product was confirmed by paper chromatography analysis (Fig. 3b) where radioactivity was detected at the solvent front (Rf ~ 0.9) where lipid II migrates. Thus, the MurG activity in the MurG-deficient membranes could be reconstituted, and this assay for convenience is further referred to as the ‘reconstituted MurG assay’.

| Table 2. Reconstitution of peptidoglycan synthesis and MurG activity in the membranes of *Escherichia coli murG(Ts); pBAD8952, 42 °C*. For peptidoglycan synthesis, *E. coli* ∆MurG membranes were incubated with 2 µg purified *E. coli* MurG and 2 µCi UDP-[3H]GlcNAc as described, and the synthesis of cross-linked peptidoglycan was monitored by captured with WGA-SPA beads. In parallel, reactions were analysed by paper chromatography (Fig. 4). MurG activity: membranes were incubated with 0.2 µg *E. coli* MurG, TritonX-100 (0.05%), and 2 µCi UDP-[3H]GlcNAc under conditions for MurG activity. Lipid II was captured by WGA-SPA beads. Values represent the mean ± SD (n = 2); activity is the average blank subtracted from the cpm in the complete reaction. |
| --- | --- | --- |
| Peptidoglycan synthesis | Activity (complete – blank) |
| Nil | 1121 ± 6 | −57 ± 50 |
| MurG (2 µg) | 1306 ± 11 | 11 509 ± 995 |
| MurG Triton X-100 | 885 ± 31 | 85 ± 59 |
| Triton X-100+ MurG (0.2 µg) | 1776 ± 18 | 10 514 ± 475 |

**Dependence of activity on protein concentration and time**

In the reconstituted MurG assay, the product formed was dependent on the quantity of MurG added and the time of the reaction (Fig. 4). Using 10 ng of MurG, the reaction was linear up to ~ 30 min. Synthesis of lipid II was linear to ~ 20 ng and saturated above 100 ng.

In membrane-based assays of MurG, both the quantity of the substrate, lipid I, and the quantity of enzyme are undefined (Mengin-Lecreulx et al., 1991; Ravishankar et al., 2005). However, in the reconstituted MurG assay, the quantity of enzyme is defined, allowing the specific activity of MurG with the natural substrate to be defined for the first time. In the SPA, the efficiency of counting and capture is difficult to estimate, and hence, results are reported in cpm and not nmols. However, using the paper chromatography analysis, presuming the efficiency of counting of lipid II on the paper is similar to that of UDP-[3H]GlcNAc (~ 10%), and the specific activity of *E. coli* MurG was 1.4 nmol min⁻¹ mg⁻¹; some batches had activity five times higher than this. Interestingly, the specific activity appears similar to that reported (Ha et al., 2000), despite the fact that the published assay used a synthetic lipid analogue and MurG was in solution.

**Effect of detergent in the ‘reconstituted MurG’ assay**

MurG activity in the reconstituted MurG assay was 60- to 100-fold higher in the presence of Triton X-100 than in
In contrast, peptidoglycan synthesis activity of the MurG-reconstituted membranes was inhibited by Triton X-100. This is not unexpected, because peptidoglycan synthesis in wild-type membranes was inhibited 50% by 0.05% Triton X-100, most likely due to the inhibition of the transglycosylase (Branstrom et al., 2000; Chandra-kala et al., 2001). Triton X-100 did not stimulate MurG in wild-type membranes, so it is likely that the detergent improved accessibility of the purified soluble MurG to the lipid substrate and other components present in the membranes.

**Effect of inhibitors**

Nisin and vancomycin inhibited the reconstituted MurG assay with IC₅₀s of 3.5 μg mL⁻¹ and 32 μM, respectively; these were similar to the IC₅₀s for MurG in wild-type membranes (nisin:10 μg mL⁻¹ and vancomycin: 30 μM). Thus, the reconstituted MurG assay closely resembles the assay of MurG in wild-type membranes.

The assay can potentially be used to assay purified MurG from different bacterial species and would obviate the need to set up MurG assays in membranes from each bacterial species. It could also be used to compare the effect of inhibitors on MurG from different bacteria, especially as all other membrane components of the system and nonspecific effects would be similar. An added advantage is that the assay described measures MurG activity in its natural lipid environment. The assay is easy to perform and reagents can be bought or easily prepared, unlike the reported solution-based assays (Auger et al., 2003). A solution assay is not the natural environment for MurG: the natural lipid substrate is less preferred than a short-chain synthetic substrate and unusual assay conditions may be required, for example 35% DMSO (Auger et al., 2003) or 15% methanol (Chen et al., 2002). Hence, it is possible that compounds that inhibit MurG in solution may be ineffective in the natural environment (Silva et al., 2000), misleading the structure–activity relationship and running the risk that enzyme inhibition may be divergent from whole-cell antibacterial activity. Impon-
tantly, the reconstituted MurG assay can be used to monitor the specific activity of the protein during purification or that of mutant MurG proteins to elucidate structure–activity relationships.

In summary, the Mtu murG gene can support the growth of an E. coli strain, which is devoid of the murG gene product. The surprising lack of MurG activity in the membranes of this strain enabled a novel microplate assay to measure the activity of external sources of MurG in an E. coli membrane background.

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To measure the activity of external sources of MurG in an E. coli strain. K.D. designed research and wrote the gene complementation part of this manuscript.

References


