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Utility of Muropeptide Ligase for Identification of Inhibitors of the Cell Wall Biosynthesis Enzyme MurF

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MurF is a key enzyme in the biosynthesis of the bacterial cell wall in both gram-positive and gram-negative bacteria. This enzyme has not been extensively exploited as a drug target, possibly due to the difficulty in obtaining one of the substrates, UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-diaminopimelate, which is usually purified from bacteria. We have identified putative inhibitors of *Escherichia coli* MurF by a binding assay, thus bypassing the need for substrate. Inhibition of enzymatic activity was demonstrated in a high-performance liquid chromatography-based secondary assay with UDP-MurNAc-L-Ala-γ-D-Glu-diaminopimelate substrate prepared in a novel way by using muropeptide ligase enzyme to add UDP-MurNAc to synthetic L-Ala-γ-D-Glu-diaminopimelate; the substrate specificity of muropeptide ligase for peptides containing L-Lys in place of diaminopimelate was also investigated. Using the muropeptide ligase-generated MurF substrate, a thiazolylaminopyrimidine series of MurF enzyme inhibitors with 50% inhibitory concentration values as low as 2.5 μM was identified.

The steps involved in the synthesis of the bacterial cell wall have long been considered to be good targets for antibacterial agents, as evidenced by drugs such as β-lactams and vancomycin (18). The MurF enzyme catalyzes the last cytoplasmic step of bacterial cell wall synthesis, the ligation of D-Ala-D-Ala to UDP-MurNAc-tripeptide with the concomitant hydrolysis of ATP. In gram-negative bacteria, the tripeptide is L-Ala-γ-D-Glu-*meso*-A_2pm (where A_2pm represents diaminopimelate); in gram-positive bacteria, L-Lys replaces *meso*-A_2pm (23).

MurF is an attractive antibacterial drug target for several reasons: (i) it carries out an essential step of cell wall biosynthesis as demonstrated by the study of a temperature-sensitive lethal mutation in this gene in *Escherichia coli* (12); (ii) it is a single-copy gene in both gram-positive and gram-negative bacteria with extensive amino acid sequence conservation, raising the possibility of broad-spectrum inhibitors; and (iii) an earlier step in this pathway, MurA, is the target of the antibacterial drug fosfomycin (9), suggesting that interference with MurF function would likewise disrupt bacterial replication. In addition, normal MurF activity has been shown to be necessary for β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (20).

Despite these attractive features, MurF has not been used extensively as a target in high-throughput screening, possibly due to the difficulty in obtaining sufficient quantities of its substrate, UDP-MurNAc-tripeptide. Previous efforts to assay MurF that bypassed the need for substrate included the use of a coupled reaction containing the enzymes MurA, B, C, D, E, and F (8, 24) or permeabilized cells (2). A more direct approach would be an assay to detect compounds that bind to MurF. We have recently reported the use of capillary electrophoresis to identify compounds that bind to *E. coli* MurF. Similarly, Gu et al. (10) utilized an unspecified affinity selection screening technology to detect compounds that bind to MurF from *Streptococcus pneumoniae*.

Compounds that bind to MurF may not necessarily inhibit its enzymatic activity, and it is important to demonstrate whether inhibition of MurF occurs. This can be accomplished using a high-performance liquid chromatography (HPLC)-based secondary assay to measure the amount of the reaction product UDP-MurNAc-pentapeptide or, alternatively, the amount of ATP hydrolysis (1, 7). As discussed above, the MurF substrate UDP-MurNAc-tripeptide is not readily available and has been purified from bacteria in small quantities (7). Instead, we chose to generate substrate by taking advantage of an enzyme from the cell wall recycling pathway, muropeptide ligase (Mpl) (13). The Mpl enzyme should be able to ligate UDP-MurNAc to synthetic tripeptide to produce UDP-MurNAc-tripeptide, but to our knowledge, this method has not been previously used as a source of MurF substrate. Using appropriate controls, we demonstrated by liquid chromatography-mass spectrometry (LC-MS) that the expected products were made: UDP-MurNAc-tripeptide when Mpl enzyme was present and UDP-MurNAc-pentapeptide when both Mpl and MurF enzymes were present. This assay was used to characterize compounds that bound to MurF, and a thiazolylaminopyrimidine inhibitor series identified from this process is described.

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MATERIALS AND METHODS

Cloning and purification of Mpl and MurF. As the source of the genomic DNA template, 10 colonies of *E. coli* strain MG1655 (6) were scraped into 50 μl of sterile water with a sterile inoculating loop and boiled for 2 min. The open reading frame for *mpl* was amplified with primers mpl_up (5′-CGTCATATG

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CGCATTCATATTTTAGGAATTTGTGG-3′) and mpl_down (5′-CGTGTC GACCTGCGCGGCTTCCGCCTTCTT-3′) according to the protocol for Proof Start DNA polymerase (QIAGEN, Inc., Valencia, CA). PCR was performed with the Perkin-Elmer Cetus PCR System 9600. The expected 1.4-kb PCR product was detected by agarose gel electrophoresis and was purified using the QIAGEN QIAquick PCR purification kit, cleaved with restriction enzymes NdeI and SalI (underlined), repurified with QIAquick, and ligated into the NdeI/XhoI sites of pET23b (Novagen, Madison, WI) under T7 promoter control so that a carboxy-terminal hexahistidine tag was added from the vector. The open reading frame for *murF* was amplified and ligated in a similar fashion using primers mur_up(5′-CGTCATATGATTAGCGTAACCCTTAGCCC-3′) and mur_down (5′-CGTCTCGAGACATGTCCCATTCTCCTGTAA-3′), except that the PCR product was cleaved with NdeI and XhoI (underlined).

The ligation mixtures were transformed into *E. coli* Novablue Singles competent cells (Novagen). Plasmid from two independent ampicillin-resistant colonies for each gene was prepared using the QIAGEN Plasmid Midi kit and subjected to DNA sequence analysis (ACGT, Inc., Wheeling, IL). The DNA sequences of the cloned *mpl* isolates were identical to those reported under EMBL accession number U14003 (13). The cloned sequences of the *murF* isolates were identical to each other but had four bases that were different from those reported under GenBank accession number X55034 (1, 17), leading to amino acid substitutions A61G and R178A compared to the previously published sequence.

Plasmids were transformed into the *E. coli* expression strain BL21(pLysS) (MurF) or BL21(pLysE) (Mpl). Cultures (1 liter) were grown at 37°C to mid-log phase (A_{600} of 0.8), and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM as recommended by the manufacturer (Novagen). After 3 h of induction at 30°C, cells were pelleted by centrifugation (10 min at 10 × g), and the pellet was suspended in Bugbuster reagent containing Benzonase as recommended by the supplier (Novagen). The filtered supernatant was applied to a Pharmacia HiPrep 26/10 desalting column (Amersham Biosciences, Piscataway, NJ) and eluted in wash buffer (50 mM Tris-Cl, pH 7.5, 300 mM NaCl). The desalted sample was added to prewashed and preequilibrated Talon resin (Clontech, BD Biosciences, Palo Alto, CA) and incubated with shaking at 4°C. After washing, the Talon-protein slurry was transferred to a column according to the manufacturer's instructions, and protein was eluted by gravity flow in wash buffer containing 150 mM imidazole. The purified MurF or Mpl protein was dialyzed into 100 mM Tris, pH 8.5, containing 5% glycerol, frozen in aliquots at −70°C, and used in assays as described below. The typical yield was 40 mg of protein from a 1-liter culture.

Mpl and MurF enzymatic assays. UDP-MurNAc was purified from a coupled MurA-MurB reaction (4) using the commercially available precursors UDP-GlcNAc, phosphoenol pyruvate, and NADPH, purchased from Sigma (St. Louis, MO). The pentapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala was purchased from Sigma. The tetrapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala and the tripeptide L-Ala-γ-D-Glu-L-Lys were synthesized by SynPep (Dublin, CA). The tripeptide L-Ala-γ-D-Glu-A_2pm was synthesized by AnaSpec (San Jose, CA); the A_2pm-containing peptide is an isomeric mixture of DD-, LL-, and *meso*-A_2pm.

The Mpl reaction is based on the method described previously by Mengin-Lecreulx et al. (13), and the reaction mixture consisted of 50 μl 100 mM Tris-Cl, pH 8.5, containing 1 mM UDP-MurNAc, 2 mM peptide, 5 mM ATP, 40 mM $MgCl_2$, 2 mM dithiothreitol, and 150 ng (60 nM) Mpl. After incubation at 37°C for 1 h, the Mpl reaction, which can be prepared on a 5-ml scale, was terminated by boiling for 3 min.

For the MurF reaction (1, 7), a solution of 50 μl of 100 mM Tris-Cl, pH 8.5, containing 20 ng (8 nM) MurF, 5 mM ATP, 300 mM NaCl, and 200 μM D-Ala-D-Ala (Sigma, St. Louis, MO) was added to 50 μl of a terminated Mpl reaction mixture and incubated at 37°C for 10 min. The MurF reaction was terminated by the addition of 5 μl of 10% trifluoroacetic acid (TFA). Mpl and MurF reaction products were detected by reverse-phase HPLC by using an Agilent LiChrosphere 100 RP-18 column (catalog no. 79925OD-564-3) with a gradient of 0 to 13% acetonitrile in 0.1% trifluoroacetic acid over 8 minutes. The flow rate was 1 ml/min, and the column temperature was 22°C. Peak areas corresponding to UDP-MurNAc-peptides were measured at 260 nm; for each experiment, samples were prepared in duplicate or triplicate. The micromolar amount of UDP-MurNAc-peptide produced in the Mpl and MurF reactions was determined by comparison to a UDP-MurNAc standard curve.

The identities of all Mpl and MurF product peaks were verified by LC-MS by using an Agilent 1100 LCMSD SL single-quadrupole mass spectrometer equipped with an electrospray ionization source. The singly and doubly charged ions of each peptide were monitored using selective ion monitoring. A gradient LC method using a LiChrosphere RP-18 (5 μm; 125-mm by 4-mm internal diameter) column was employed to achieve separation. The gradient ran from 0% organic (acetonitrile containing 0.05% TFA) to 60% organic over 60 minutes. The aqueous mobile phase used in the separation was H_2O containing 0.05% TFA. The flow rate was 1.0 ml/min, with UV detection at 210 and 260 nm.

For testing of putative MurF inhibitors, MurF enzyme (20 ng) in 40 μl of 100 mM Tris-Cl, pH 8.5, 5 mM ATP, and 300 mM NaCl was preincubated with compound or dimethyl sulfoxide (2 μl) for 15 min at room temperature, followed by the addition of 10 μl of 1 mM D-Ala-D-Ala dipeptide and 50 μl of a completed Mpl reaction mixture containing L-Ala-γ-D-Glu-A_2pm substrate. After incubation for 15 min at 37°C, the reaction was terminated by the addition of 5 μl of 10% TFA and transferred to HPLC vials. Peaks were detected as described above. MurF 50% inhibitory concentration (IC_{50}) values were determined by the integration of the area of the HPLC peak corresponding to UDP-MurNAc-pentapeptide.

MurF binding assay. Capillary electrophoresis using the CE Assay was performed by Cetek Corp. (Marlborough, MA) using 1.4 μM MurF protein and 25 μM ATP to detect compounds that bind to MurF as indicated by causing a shift in protein migration.

Antibacterial assays. Inhibition of bacterial growth was assessed by CLSI (formerly NCCLS) broth microdilution assays (16).

RESULTS

Possible toxicity of Mpl in *E. coli*. The cloning of *mpl* under T7 promoter control was accomplished by standard procedures using *E. coli* strain Novablue, which lacks T7 RNA polymerase. Upon transformation of the plasmid into expression strains containing T7 polymerase, indications of toxicity of *mpl* were apparent, even in the absence of the inducing agent IPTG: no colonies were obtained from strain BL21, and only tiny colonies were obtained from BL21(pLysS), which failed to grow upon inoculation into liquid medium. The lack of growth of *E. coli* BL21 harboring *mpl* was overcome by the introduction of pLysE into the strain; this plasmid encodes higher levels of lysozyme, an inhibitor of T7 RNA polymerase, and is more effective than pLysS at repressing expression from the T7 promoter in the absence of the IPTG inducer (21). Robust expression (approximately 50 mg/liter) of Mpl protein was observed, the majority of which was found in the soluble fraction, in contrast to a previous report in which Mpl was found to partition into inclusion bodies (13). The two constructs differ at the carboxy terminus, with our construct ending in Leu-Glu-His_6, which may account for the difference in solubility. Mengin-Lecreulx et al. (13) also saw indications of Mpl toxicity, with enlarged cells prone to lysis.

Substrate specificity of Mpl. The cloned, purified Mpl enzyme was tested for enzymatic activity by incubating various substrates with the enzyme and analyzing the contents of the reaction samples by HPLC, and also by LC-MS, to determine whether the expected products were made. Mpl from *E. coli* should be able to ligate UDP-MurNAc to the amino terminus of the tripeptide L-Ala-γ-D-Glu-A_2pm; the chemical structure and the molecular mass of the expected UDP-MurNAc-L-Ala-γ-D-Glu-A_2pm Mpl reaction product are shown in Fig. 1A. HPLC analysis of the Mpl reaction (Fig. 2B) with the appropriate control reaction lacking Mpl (Fig. 2A) demonstrated the appearance of a new peak whose mass exactly corresponded to that of the expected product, UDP-MurNAc-tripeptide. Thus, the Mpl preparation was enzymatically active.

To investigate the substrate specificity of *E. coli* Mpl, purified enzyme was incubated with various peptides, and the expected products (Fig. 1) were identified by LC-MS. The amount of UDP-MurNAc-peptide product was estimated from HPLC peak areas measured at 260 nm. The absorbance of

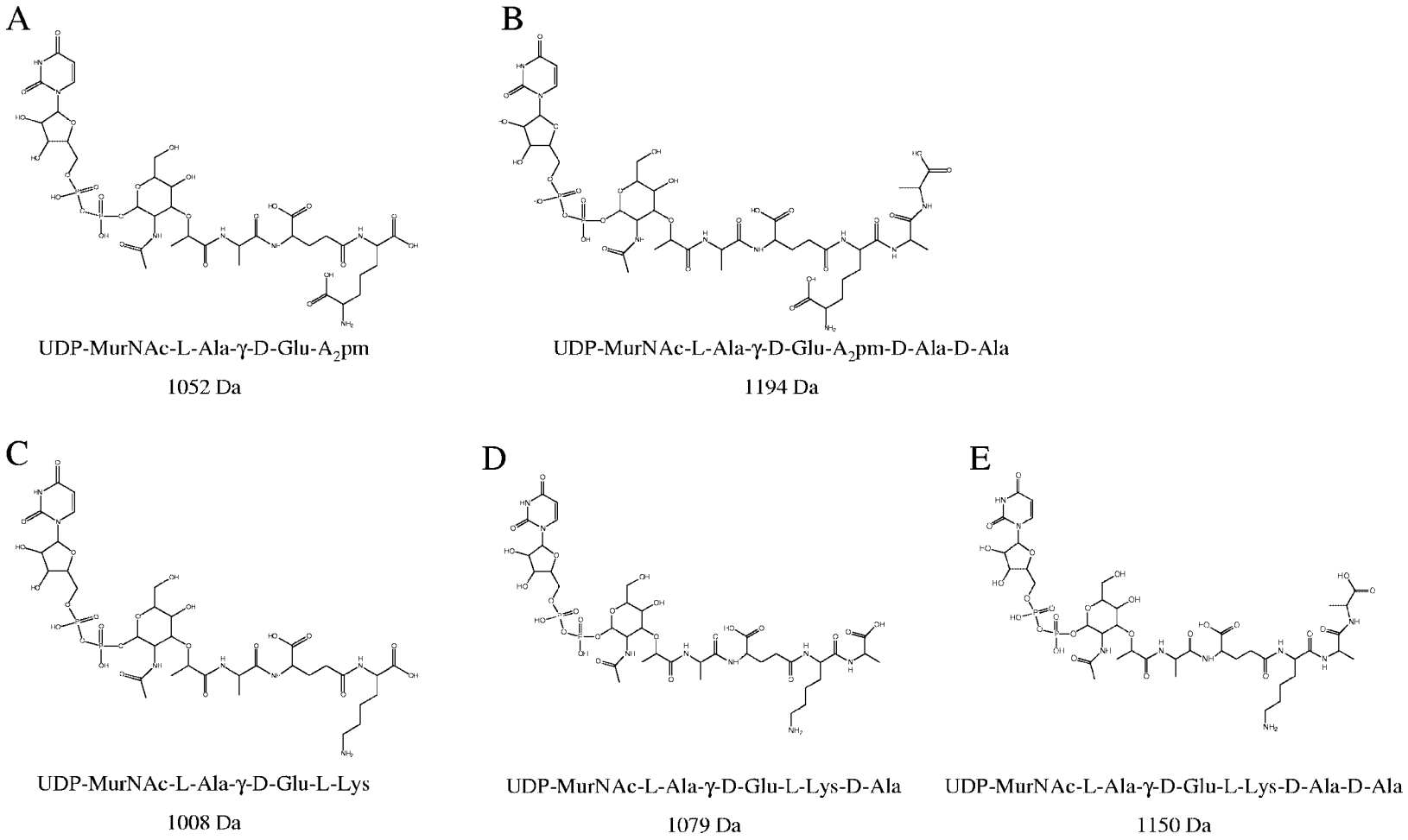


FIG. 1. Structures and molecular masses of the products of Mpl and MurF reactions using various substrates. The products of Mpl reactions using peptide substrates L-Ala-γ-D-Glu-A_2pm (A), L-Ala-γ-D-Glu-L-Lys (C), L-Ala-γ-D-Glu-L-Lys-D-Ala (D), and L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (E) are depicted. The products of MurF reactions using substrates UDP-MurNAc-L-Ala-γ-D-Glu-A_2pm (A) and UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys (C) are shown in B and E, respectively.

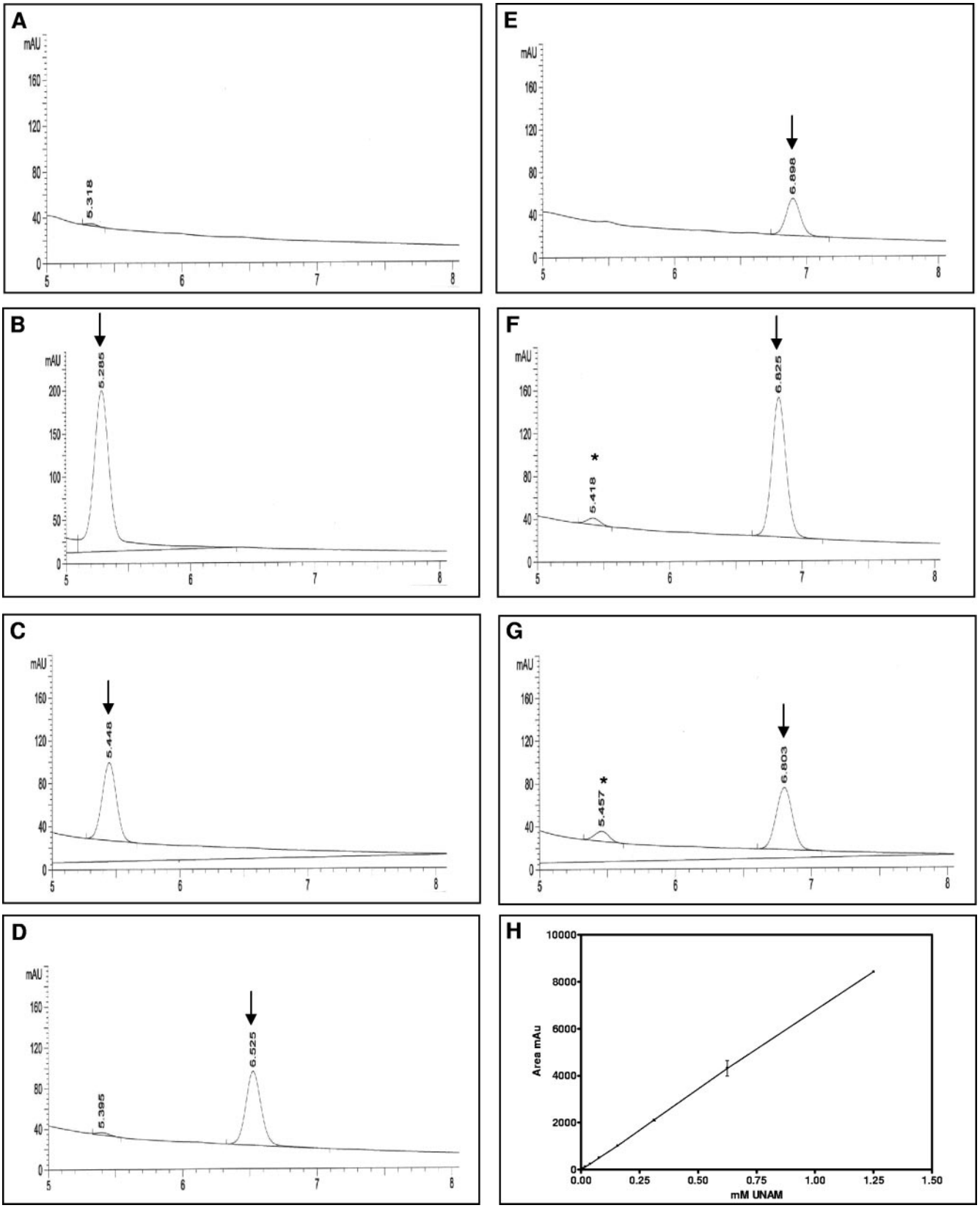


FIG. 2. HPLC chromatograms of Mpl and MurF reactions. The HPLC profiles at 260 nM are displayed for the following reactions: L-Ala-γ-D-Glu-A_2pm without enzyme (A) or incubated with Mpl (B) or with Mpl and MurF (F), L-Ala-γ-D-Glu-L-Lys incubated with Mpl (C) or with Mpl and MurF (G), L-Ala-γ-D-Glu-L-Lys-D-Ala incubated with Mpl (D), and L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala incubated with Mpl (E). Panel A is representative of the elution profile of all peptides in the absence of enzymes. Arrows indicate product peaks. For the MurF reactions (F and G), the peak corresponding to residual Mpl product is denoted by an asterisk. A standard curve of pure UDP-MurNAc (H) was analyzed by HPLC in parallel with samples A to G; the absorbance at 260 nm of the UDP-MurNAc peak detected at 4.2 min is shown.

TABLE 1. Utilization of tri-, tetra-, and pentapeptides by Mpl and MurF enzymes

Peptide	% Mpl relative activity[b] (μM)	% MurF relative activity[c] (μM)	% Conversion of Mpl product by MurF
Expt 1			
L-Ala-γ-D-Glu-A_2pm[a]	100 (376 ± 2)	100 (154 ± 9)	41
L-Ala-γ-D-Glu-L-Lys	14 (52 ± 1)	31 (48 ± 3)	92
L-Ala-γ-D-Glu-L-Lys-D-Ala	16 (59 ± 0)	NA[d]	NA
L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	31 (118 ± 3)	NA	NA
Expt 2			
L-Ala-γ-D-Glu-A_2pm[a]	100 (216 ± 59)	100 (114 ± 27)	53
L-Ala-γ-D-Glu-L-Lys	36 (77 ± 8)	61 (69 ± 5)	89
L-Ala-γ-D-Glu-L-Lys-D-Ala	41 (88 ± 1)	NA	NA
L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	18 (39 ± 1)	NA	NA
Avg % of expt 1 and 2			
L-Ala-γ-D-Glu-A_2pm[a]	100 ± 0	100 ± 0	47 ± 8
L-Ala-γ-D-Glu-L-Lys	25 ± 16	46 ± 21	91 ± 2
L-Ala-γ-D-Glu-L-Lys-D-Ala	29 ± 18	NA	NA
L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala	25 ± 9	NA	NA

[a] A mixture of DD, LL, and *meso* isomers of A_2pm.
[b] Mpl relative activity based on the amount (micromolar) of the UDP-MurNAc-peptide peak detected by HPLC. The amount of Mpl product from the substrate L-Ala-γ-D-Glu-L-A_2pm is defined as 100%.
[c] MurF relative actvity based on the amount (micromolar) of the UDP-MurNAc-peptide-D-Ala-D-Ala peak detected by HPLC. The amount of MurF product from substrate L-Ala-γ-D-Glu-L-A_2pm is defined as 100%.
[d] NA, not applicable.

these peaks results principally from the uridine component, common to each of the UDP-MurNAc-peptide molecules, and was compared to a UDP-MurNAc standard curve (Fig. 2 and Table 1). Of the substrates tested, the tripeptide L-Ala-γ-D-Glu-A_2pm was most efficiently converted to product by Mpl and is defined as "100%." The gram-positive peptide L-Ala-γ-D-Glu-L-Lys also served as a substrate for *E. coli* Mpl, albeit less efficiently, yielding on average 25% of the amount of product compared to L-Ala-γ-D-Glu-A_2pm (Fig. 2C and Table 1). Thus, this is another example of the successful in vitro utilization of gram-positive substrate by an *E. coli* cell wall synthesis or recycling enzyme, as has been shown for both MurF (1) and LdcA (3).

The ability of tetrapeptide and pentapeptide to serve as Mpl substrates was also examined (Fig. 2D and E and Table 1). The tetrapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala was utilized, on average, at 29% relative to the tripeptide L-Ala-γ-D-Glu-A_2pm. The pentapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala was utilized, on average, at 25%. These values are similar to that of the cognate tripeptide L-Ala-γ-D-Glu-L-Lys. Thus, *E. coli* Mpl is able to use both the gram-negative and gram-positive tripeptide and the gram-positive tetra- and pentapeptides as substrates in vitro.

Enzymatic activity of MurF. Anderson et al. (1) had previously shown that bona fide UDP-MurNAc-tripeptide purified from bacteria and containing either *meso*-A_2pm or L-Lys at the carboxy terminus were equally good substrates for *E. coli* MurF. We now show that the corresponding synthetic A_2pm- and L-Lys-containing tripeptides, upon the addition of UDP-MurNAc via Mpl activity, are both recognized as substrates for MurF (Fig. 2F and G and Table 1). Although the absolute amount of UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys produced in the Mpl reaction was about three- to sevenfold less than the amount of UDP-MurNAc-L-Ala-γ-D-Glu-A_2pm produced, almost all of the UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys (~90%) was converted to UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala by MurF.

Identification of MurF inhibitors. Screening of a chemical library by capillary electrophoresis yielded several compounds that appeared to bind to MurF and to inhibit its enzymatic activity. A thiazolylaminopyrimidine (compound 1) (Table 2) was one such compound. This compound bound to MurF but did not bind to two control enzymes: a serine/threonine kinase and a deacetylase/sulfotransferase. Compound 1 exhibited an IC_{50} value of 7.5 μM against MurF in the enzymatic assay. Inhibition of MurF by the thiazolylaminopyrimidine appeared to be specific, in that the compound was inactive in several dozen other assays, which included β-lactamase, receptor binding, kinase, phosphatase, and protease assays at inhibitor concentrations of ≥10 μM.

Analogs of compound 1 were identified by substructure searches and were tested in the MurF enzymatic assay (Table 2). Compounds 2 and 3 were both about threefold more potent than compound 1, displaying IC_{50} values of 2.5 μM.

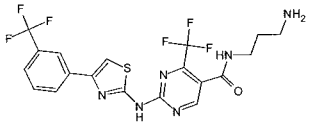
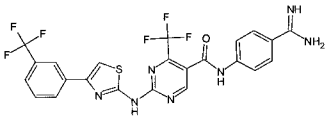
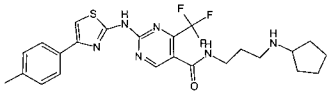
The three thiazolylaminopyrimidines were tested for antibacterial activity against wild-type *E. coli* but did not exhibit measurable MIC values (MIC ≥ 64 μg/ml).

DISCUSSION

In the current study, we have demonstrated that the MurF substrate UDP-MurNAc-L-Ala-γ-D-Glu-A_2pm can be synthesized in a new way by using the *E. coli* Mpl enzyme, synthetic tripeptide, and UDP-MurNAc, which can be synthesized either according to a method described previously by Blanot et al. (5) or as the product of a coupled MurA and MurB reaction using commercially available substrates (4).

The overproduction of Mpl appeared to be toxic to *E. coli* both in this study and as shown previously by Mengin-Lecreulx

TABLE 2. MurF IC_{50} values for members of the thiazolylaminopyrimidine series

Compound	Structure	IC_{50} (μM) in MurF assay
1		7.5
2		2.5
3		2.5

et al. (13). We speculate that several events linked to Mpl overproduction could interfere with cell wall biosynthesis and lead to cell lysis or death. Unusually high levels of the Mpl product/MurF substrate UDP-MurNAc-tripeptide would be expected to accumulate faster than it could be utilized by the MurF enzyme. This could stimulate the cell wall recycling pathway, possibly resulting in the overaccumulation of UDP-MurNAc-tetrapeptide, which is thought to be toxic upon incorporation into the cell wall (22). Mpl overproduction could also sequester much of the available UDP-MurNAc, blocking its utilization by the MurC enzyme. MurA is also negatively regulated by UDP-MurNAc (15), so MurA activity could also be compromised.

Conversely, deletion of Mpl is not lethal (13); it was suggested that MurC, D, and E can substitute for Mpl in that case.

Mpl was shown to utilize tripeptide substrates containing either A_2pm or L-Lys in vitro. A_2pm is a mixture of *meso*, LL, and DD isoforms, and in our study, it was not possible to determine which of these three isomers was utilized by Mpl. Analogous results of the utilization of L-Lys-containing substrates by *E. coli* enzymes which normally recognize *meso*-A_2pm-containing substrates have been observed for the cell wall biosynthetic enzyme MurF (1) and the cell wall recycling enzyme LdcA (3). The only difference between these amino acids is that L-Lys lacks one carboxylate group found in *meso*-A_2pm, and it appears that L-Lys is still able to bind successfully into the active sites of Mpl, MurF, and LdcA. However, the L-Lys tripeptide substrate does appear to be less efficiently utilized by Mpl, in that only 25% as much UDP-MurNAc-tripeptide product was made compared to the A_2pm tripeptide. Synthetic peptides containing A_2pm are more expensive and not as readily available commercially compared to synthetic peptides containing L-Lys. For these reasons, for some applications such as high-throughput screening, it may be preferable to use synthetic peptides containing L-Lys rather than A_2pm, despite less efficient substrate utilization. Also, synthetic peptides containing L-Lys, in contrast to A_2pm, are readily available as a single isomer, which may be an advantage for certain biochemical or biophysical studies.

The efficiency of utilization of tetrapeptide by Mpl was comparable to that of tripeptide. Tetrapeptide was hypothesized to serve as an Mpl substrate in the case of an *ldcA* deletion mutant (22). The UDP-MurNAc-tetrapeptide product is thought to be toxic to the *ldcA* mutant upon incorporation into the bacterial cell wall. We have also shown that pentapeptide can serve as a substrate for Mpl in vitro; whether the enzyme would actually encounter pentapeptide within bacteria and use it as a substrate is not known.

The search for inhibitors of MurF with antibacterial activity has employed several different assay methods, including a relatively low-throughput HPLC-based detection system to monitor the production of UDP-MurNAc-pentapeptide that is more suitable as a secondary assay method. Alternative methodologies include monitoring the production of ADP (1, 7) or inorganic phosphate (7). Perhaps of greater concern than the actual assay system is the difficulty in obtaining large amounts of UDP-MurNAc-tripeptide substrate. This substrate can be purified from bacteria (11) or, as we demonstrated here, made by the use of Mpl. Reddy et al. (19) have also reported the individual enzymatic synthesis and purification of MurA-F products. Our method would appear to have two advantages: replacing three enzymes (MurC, D, and E) with the single Mpl protein and dispensing with the purification of the resultant MurF substrate. However, obtaining sufficient amounts of MurF substrate for high-throughput screening by any of these methods still may not be feasible.

Several alternative approaches to circumvent the need for MurNAc-tripeptide substrate have been devised. Since the substrates for MurA and MurB are commercially available, coupled enzyme systems using these substrates and recombinant MurA-MurF have been employed (8, 24). A whole-cell assay that utilizes a frozen and thawed preparation of *E. coli* cells and monitors the incorporation of radioactive UDP-*N*-acetylglucosamine into peptidoglycan has also been described previously (2).

Another approach is to use a binding assay such as capillary electrophoresis, as was done in the current work. Gu et al. (10) used an unspecified affinity selection screening technique to detect compounds that bound to *S. pneumoniae* MurF. It is known that the MurF substrates bind in an ordered fashion to the enzyme, with ATP binding first, followed by UDP-MurNAc-tripeptide and finally by D-Ala-D-Ala (1). Binding of ATP apparently causes a conformational change in MurF, which aids in the binding of the other two substrates (25). Our preference was to detect compounds that bound to MurF but not at the ATP site, mainly because such compounds might be nonspecific and might inhibit other enzymes that utilize ATP. Performing the binding assay in the presence of ATP serves the dual purposes of possibly excluding, by competition, compounds that would bind to the ATP site and also producing the conformational change in MurF that would allow the binding of compounds into the UDP-MurNAc-tripeptide and/or the D-Ala-D-Ala binding sites.

Previously described inhibitors of MurF include phosphinate transition state analogs (K_i = 200 to 700 μM) (14) and the nonhydrolyzable ATP analog AMP-PCP [adenylyl 5′-(β,γ-methylenediphosphonate)] (K_{ii} = 4 μM) (1). Aside from per-

meability problems for such compounds, any antibacterial activity of ATP analogs would be expected to be nonspecific, given the wide variety of enzymes (both bacterial and mammalian) that utilize ATP. Gu et al. have previously described a series of *S. pneumoniae* MurF inhibitors with IC_{50} values as low as 22 nM (10).

The MurF inhibitors reported by Gu et al. (10) and the thiazolylaminopyrimidine series described herein lacked measurable antibacterial activity. The lack of antibacterial activity could be due to a failure of the compounds to penetrate the cell, although the possibility that MurF was inhibited, without an effect on bacterial growth, cannot be excluded. However, MurF does appear to be an essential gene in *E. coli*, as demonstrated by the existence of a conditional lethal mutant (12), suggesting that inhibition of MurF should compromise growth.

In summary, the use of the Mpl enzyme was shown to be a viable strategy to prepare MurF substrate from synthetic peptides and UDP-MurNAc. The MurF substrate prepared in this manner was the basis of a secondary assay to determine that members of a thiazolylaminopyrimidine series uncovered by a MurF binding assay were actual inhibitors of the enzyme. This approach should expedite the search for additional inhibitors of MurF with useful antibacterial properties.

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