The Lysis Protein E of φX174 Is a Specific Inhibitor of the MraY-catalyzed Step in Peptidoglycan Synthesis*

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Coliphage φX174 encodes a single lysis protein, E, a 91-amino acid membrane protein. Dominant mutations have been isolated in the host gene mraY that confer E resistance. mraY encodes translocase I, which catalyzes the formation of the first lipid intermediate in bacterial cell wall synthesis, suggesting a model in which E inhibits MraY and promotes cell lysis in a manner analogous to cell wall synthesis inhibitors like penicillin. To test this model biochemically, we monitored the effect of E on cell wall synthesis in vivo and in vitro. We find that expression of Emyc, encoding an epitope-tagged E protein, from a multicopy plasmid inhibits the incorporation of [3H]diaminopimelic acid into cell wall and leads to a profile of labeled precursors consistent with MraY inhibition. Moreover, we find that membranes isolated after Emyc expression are drastically reduced in MraY activity, whereas the activity of Rfe, an enzyme in the same superfamily, was unaffected. We therefore conclude that E is indeed a cell wall synthesis inhibitor and that this inhibition results from a specific block at the MraY-catalyzed step in the pathway.

There are at least two distinct mechanisms by which phage promote destruction of the bacterial cell wall and subsequent cell lysis, the choice of which appears to be determined by the phage genome size. Bacteriophages with large genomes encode a holin-endolysin system. In the prototypic λ model, the S holin protein accumulates in the cell membrane and the R endolysin accumulates in the cytoplasm. At a genetically programmed time, S forms a membrane lesion to release the R endolysin into the periplasm where it can degrade the cell wall and cause lysis (1). In contrast, bacteriophages with small genomes can only afford to encode a single lysis protein. Three unrelated single protein lysis systems are known: the E protein from φX174 (ssDNA, Microviridae), and the L and A3 proteins from MS2 (ssRNA, group I) and Qβ (ssRNA, group III) respectively (1–5). The molecular mechanism of lysis caused by any of these phages, indicating that their lytic activity, whereas the activity of Rfe, an enzyme in the same superfamily, was unaffected. We therefore conclude that E is indeed a cell wall synthesis inhibitor and that this inhibition results from a specific block at the MraY-catalyzed step in the pathway.

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The abbreviations used are: MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine; Kan, kanamycin; DAP, diaminopimelic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; undecaprenol-P, undecaprenol-phosphate; GPT enzyme family, UDP-GlcNAc/MurNAc polyisoprenyl-P: GlcNAc/MurNAc 1-P transferase family of enzymes, which we will refer to as the GPT enzyme family (8). Inhibition of MraY by the antibiotic mureidomycin results in lysis of Pseudomonas aeruginosa, and depletion of MraY activity from E. coli also results in concomitant cell lysis (9, 10). These findings, coupled with the isolation of E-resistant mraY mutants, suggest a model in which E inhibits MraY to elicit lysis by a mechanism similar to that of antibiotics like mureidomycin and penicillin that inhibit cell wall synthesis (see Fig. 1A) (11, 12).

The bacterial cell wall is composed of polysaccharide chains of the repeating unit N-acetylglucosamine-β-1,4-N-acetyl-muramic acid (GlcNAcβ1,4MurNAc). Attached to the MurNAc sugar is a pentapeptide side chain (in E. coli, γ-D-Ala-γ-D-Glu-DAP-D-Ala-D-Ala). Adjacent polysaccharide strands are cross-linked by peptide bonds between the free amino group of meso-diaminopimelic acid (DAP) from one peptide chain and the carboxyl group of the penultimate D-Ala of an adjacent peptide. The pathway for cell wall synthesis can be divided into three phases: (i) cytoplasmic reactions, (ii) membrane reactions, and (iii) periplasmic reactions (see Fig. 2) (13). In the cytoplasm, UDP-GlcNAc is converted to UDP-MurNAc in two steps. In the first and committed step in cell wall biosynthesis, an enolpyruvyl moiety from phosphoenolpyruvate is added to C-3 by MurA. MurB catalyzes the reduction of the enolpyruvyl moiety to a lactyl group using reducing equivalents from NAPDH. The pentapeptide is then extended from the lactyl group of UDP-MurNAc, one or two amino acid residues at a time, to generate UDP-MurNAc-pentapeptide. On the cytoplasmic face of the inner membrane, MraY catalyzes the transfer of P-MurNAc-pentapeptide from UMP to the lipid carrier undecaprenol-P, generating undecaprenol-P-MurNAc-pentapeptide or lipid I. GlcNAc is then added to lipid I from UDP-GlcNAc by MurG, generating lipid II. Lipid II is flapped, by an as yet unidentified flipase, to expose the disaccharide-pentapeptide monomer unit on the periplasmic face of the membrane. Once exposed to the periplasm, the disaccharide-pentapeptide is polymerized and cross-linked by multienzyme

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complexes, including the penicillin-binding proteins, to form the mature cell wall (14).

The purpose of this study is to test our model for the mechanism of E-induced lysis (see Fig. 1A) biochemically by monitoring its effect on cell wall synthesis in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Media, Chemicals, and Solvent Systems—Bacterial cultures were grown in Luria-Bertiati (LB) broth (15) or minimal M6 media (16) supplemented with 0.4% glucose and l-amino acids (20 μg/ml His and Tyr; 30 μg/ml Gile, Ile, Phe, Ser, and Val; 40 μg/ml Trp; 50 μg/ml Arg; 60 μg/ml Ala and Asp; 70 μg/ml Asn and Lys; 90 μg/ml Glu; 90 μg/ml Phe; 100 μg/ml Met and Thr; 110 μg/ml Leu). Met and Thr were present at 100 μg/ml to reduce the size of the intracellular DAP pool (17). All cultures were grown in the presence of 40 μg/ml kanamycin (Kan). When indicated, bacterial cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (Alexis, San Diego, CA) at a concentration of 1 mM. [3H]Racemic-DAP (1 mM/cm; 45 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO), and [3H]UDP-MurNAc-pentapeptide (1.6 × 72 cm) was described previously (21). Fractions from the G-25 column were assayed for the presence of N-acetyl sugars as described previously (22). N-Acetyl sugar-containing fractions were pooled, lyophilized, and dissolved in a small volume of H2O. The partially purified material was subjected to TLC analysis on Whatman Al-SIL-G plates using solvent systems A, B, and C and yielded a single UV-absorbing spot in each case. The UDP-MurNAc-pentapeptide and tripeptide were further purified on a 1-mL Hi-Trap Source Q anion exchange column (Amersham Pharmacia Biotech, Piscataway, NJ) using a 0–0.4 M gradient of NH4HCO3 over 20 column volumes and a flow rate of 1 ml/min. For the UDP-MurNAc-pentapeptide preparation, fractions corresponding to the major UV-absorbing peak at 254 nm were pooled, lyophilized, and subjected to analysis using the Texas A&M Laboratory for Biological Mass Spectrometry and quantitative amino acid analysis at the Texas A&M Protein Chemistry Laboratory confirmed that the preparation contained highly purified UDP-MurNAc-pentapeptide (m/z = 1194 and a Ala:Glu:UDP ratio of 3:1:1).

Accumulation of UDP-MurNAc Peptides during Emyc Expression—ET505 pEmycZK and ET505 pJFlacZK were grown in 2 liters of LB-Kan at 37 °C to an A600 of ~0.7 and induced with IPTG. Just before induction, MgCl2 was added to 0.1 mM to stabilize cells undergoing lysis (23). After 45 min of induction, the cells were harvested and UDP-N-acetyl sugars were purified and characterized as described above for the UDP-MurNAc-pentapeptide. For the preparation from ET505 pEmycZK, fractions corresponding to the major UV-absorbing peak in the anion exchange chromatogram were processed for amino acid analysis as described above. The retention time of the UDP-N-acetyl sugar that accumulated during Emyc expression was compared with those of authentic UDP-MurNAc-tripeptide and -pentapeptide standards using the anion exchange column described above with a 0–0.6 M gradient of NH4HCO3 over 20 column volumes and a flow rate of 1 ml/min.

Membrane Preparation—200 ml cultures of ET505 pEmycZK and ET505 pJFlacZK were grown in LB-Kan to an A600 of 0.6 and induced with IPTG. After 9 min of induction, before any observable lysis, the cells were harvested by centrifugation at 9000 × g at 4 °C for 15 min. The pellets were resuspended in 2 ml of French press buffer (50 mM Tris pH 8.0, 100 mM KCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) and disrupted in a French pressure cell (SLM Instruments Inc.) at 16,000 psi. Membranes were pelleted by centrifugation at 100,000 × g for 60 min at 4 °C. Finally, the membrane pellets were resuspended in 300 μl of French press buffer and stored in small aliquots at −80 °C. Protein concentrations of the membrane preparations were determined on aliquots solubilized in 1% SDS with the Bio-Rad DC detergent-compatible protein assay kit using...
bovine serum albumin as a standard. Membranes isolated from ET505 pEmycZK and ET505 pFlacZK contained 17.4 and 21.2 mg/ml protein, respectively.

**MraY Exchange Assay—**MraY activity was assayed in the membrane preparations using an exchange assay monitoring the exchange of [3H]UMP for the UMP moiety of UDP-MurNAc-pentapeptide (24). Reactions contained 1 μl of 0.5 M Tris-HCl (pH 8.0), 1 μl of 0.2 M MgCl₂, 1 μl of 1 mM UMP, 1 μl of [3H]UMP (100,000 cpn), 1.5 μl of 4.8 mM UDP-MurNAc-pentapeptide, and 5-μl membranes. When indicated, 0.25 μl of 0.5 M penicillinase stock in methanol was added. Methanol alone did not affect the reaction. Reactions were incubated at 37 °C for 20 min and terminated by boiling for 3 min. After boiling, the membrane debris was pelleted by centrifugation at maximum speed for 5 min in a microcentrifuge. The supernatant was removed to a fresh tube, and the pellets were washed with 10 μl of distilled H₂O and centrifuged as before. The wash was pooled with the previous supernatant and 10 μl was spotted on a TLC plate (Whatman AL-SIL-G) and developed with solvent system C. UMP and UDP-MurNAc-pentapeptide (0.02 μmol each) were spotted in each lane as standards. The UV-absorbing spots corresponding to UMP (Rₚ = 0.6) and UDP-MurNAc-pentapeptide (Rₚ = 0.3) were scraped, and the radioactivity in each spot was determined by liquid scintillation counting as described above and normalized to total protein.

**Rf Exchange Assays—**Rf activity was measured using an exchange assay monitoring the exchange of [3H]UMP for the UMP moiety of UDP-GlcNAc. Rf reaction compositions were exactly as those for MraY except that 1.5 μl of 5 mM UDP-GlcNAc was added in place of UDP-MurNAc-pentapeptide, and 1 μl of [3H]UMP (200,000 cpn) was used. The reactions were terminated and processed as described above except that solvent system A was used for TLC. UMP and UDP-GlcNAc (0.02 μmol each) were spotted in each lane as standards. The UV-absorbing spots corresponding to UMP (Rₚ = 0.3) and UDP-GlcNAc (Rₚ = 0.2) were scraped, and the radioactivity in each spot was determined by liquid scintillation counting as described above.

**RESULTS**

**E Is a Cell Wall Synthesis Inhibitor—**The isolation of lysis-resistant mraY mutants suggested that E is a cell wall synthesis inhibitor that blocks the MraY-catalyzed step in the pathway (Fig. 1A). To test this model, we monitored the effect of E expression on cell wall synthesis in vivo. The mature cross-linked cell wall is the only cellular material that remains insoluble when cells are boiled in 4% SDS (17). Therefore, a convenient assay for monitoring cell wall synthesis is to measure the incorporation of [3H]DAP, an amino acid unique to the cell wall, into SDS-insoluble material (17). Using this assay we found that [3H]DAP incorporation into the cross-linked cell wall is inhibited 5 min after induction of E expression (Fig. 3A), well before any detectable effect on growth (Fig. 3).

**Cell Wall Precursor Synthesis during Emyc Expression—**To determine the step in cell wall synthesis that is blocked by Emyc, we monitored the synthesis of cell wall precursors during Emyc expression from pEmycZK. After induction of Emyc, cells were pulse-labeled with [3H]DAP for 8 min (Fig. 4A), and the major cell wall precursor fractions were separated by paper chromatography into cell wall, nucleotide intermediates (UDP-MurNAc-tripeptide and -pentapeptide), and lipid intermediates (lipids I and II) (20). As shown in Fig. 4B, only the nucleotide intermediates are labeled with [3H]DAP after Emyc induction, whereas all three fractions are labeled in the control strain. Because the ratio of the [3H]DAP-labeled UDP-MurNAc-tripeptide and -pentapeptide species in the nucleotide fraction could not be determined in this experiment, the data are consistent with an Emyc-mediated block at either the MurF- or the MraY-catalyzed steps of cell wall synthesis (Fig. 2).

**Accumulation of UDP-MurNAc-pentapeptide during Emyc Expression—**For a more precise determination of which nucleotide intermediates are synthesized following Emyc induction, we purified the UDP-N-acetyl sugars that accumulate during Emyc expression and compared them to those isolated from the control strain (see “Experimental Procedures”). By N-acetyl-sugar assay (22) and A₆₀₂₅ measurements, ~5 times more UDP-N-acetyl sugars were isolated after Emyc expression than in the control (1.6 versus 0.3 μmol). Anion exchange chromatography of this material revealed one major UV absorbing peak eluting between 25 and 30 ml (Fig. 5A), whereas the control preparation was a complex mixture containing multiple peaks of similar absorbance (Fig. 5B). Using a slightly different elution gradient, authentic UDP-MurNAc-tripeptide and -pentapeptide standards are clearly separable, and the intermediate that accumulates during Emyc expression has an identical retention time to UDP-MurNAc-pentapeptide. However, quantitative amino acid analysis found a 2:1:1 Ala:DAP:Glu ratio for the peak fractions shown in Fig. 5B, which is consistent with an equimolar mixture of the tripeptide and pentapeptide species. The reason for this discrepancy is not known but may be due to contaminants found in the preparation from E. coli that are absent in the standard preparations from B. subtilis. Based on the chromatographic analysis, we conclude that UDP-MurNAc-pentapeptide is synthesized and accumulates to high levels after Emyc induction. Therefore, the step of cell wall synthesis inhibited by Emyc must be the one catalyzed by MraY.

**MraY Activity Is Dramatically Reduced in Membranes Isolated after Emyc Expression—**The in vivo experiments described above indicate that it is the MraY step that is blocked upon Emyc expression. To test this directly, we measured MraY activity in vitro using membranes isolated with and without prior expression of Emyc. MraY catalyzes the reaction, C₅₅-P + UDP-MurNAc-pentapeptide

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\text{C}_{55}-\text{P} + \text{UDP-MurNAc-pentapeptide} \rightarrow \text{C}_{55}-\text{P-MurNAc-pentapeptide} + \text{UMP}
\]

**REACTION I**
where C55-P is undecaprenol-P. Because this reaction is readily reversible (24), addition of UDP-MurNAc-pentapeptide and [3H]UMP to inner membrane vesicles, which contain the lipid substrate in addition to MraY, leads to the formation of [3H]UDP-MurNAc-pentapeptide. In this reaction, the UMP released from the enzyme as a result of the forward reaction is exchanged with [3H]UMP from solution, leading to the formation of [3H]UDP-MurNAc-pentapeptide by the reverse reaction.

We took advantage of this exchange reaction and monitored the formation of [3H]UDP-MurNAc-pentapeptide as a measure of MraY activity. As shown in Fig. 6A, MraY activity is dramatically reduced in membranes isolated after Emyc expression relative to the activity in control membranes. This level of inhibition was similar to that caused by the MraY inhibitor tunicamycin (25).

DISCUSSION

In a previous report we described the isolation of dominant mutants in the host gene mraY that resulted in resistance to E-induced lysis (6). In addition, we found that overexpression of mraY from a medium-copy plasmid could also confer resistance (6). Given these results, we proposed a model in which E inhibits cell wall synthesis at the MraY-catalyzed step in the pathway to promote lysis (Fig. 1A). The results presented in

Fig. 4. A, pulse-labeling time course. Shown is the time course of [3H]DAP pulse labeling to monitor cell wall precursor synthesis following plasmid induction with IPTG. Strains and symbols are identical to those in Fig. 3. The arrow with an asterisk indicates the time of [3H]DAP addition, and the arrow indicates the time cells were harvested for analysis. B, [3H]DAP incorporation into cell wall and its precursors. [3H]DAP incorporation for the control, ET505 pJFlacZK, and for ET505 pEmycZK, are given in the black and gray boxes, respectively. The results are the average of three samples normalized to A550 at the end of the time course. Error bars represent the S.D. of the triplicate samples.

Fig. 5. UDP-MurNAc-pentapeptide accumulation during Emyc expression. Shown are anion exchange chromatograms of UDP-N-acetyl sugars that were accumulated in 2-liter cultures of ET505 pJ-FlacZK (A) and ET505 pEmycZK (B). For C, the retention time of the major peak in B (dotted line) was compared with those of authentic UDP-MurNAc-tripeptide (gray line) and UDP-MurNAc-pentapeptide (black line) standards using anion exchange chromatography. A slightly different gradient was used in C resulting in retention times that differ from those in A and B (see “Experimental Procedures”).

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Rfe Activity Is Normal in Membranes Containing Emyc—Besides MraY, the only other known member of the GPT enzyme family in E. coli is Rfe (8, 26). Rfe catalyzes the formation of the first lipid intermediate in enterobacterial common antigen synthesis, undecaprenol-P-P-GlcNAc (26). To determine whether Emyc is a specific inhibitor of MraY like the antibiotic mureidomycin (27) or a general GPT enzyme inhibitor like tunicamycin (25, 26), we measured Rfe activity in the membranes described above using an exchange reaction similar to that catalyzed by MraY. Rfe activity was nearly identical in membranes isolated with and without prior Emyc expression (Fig. 6B). As expected, Rfe activity was completely inhibited by the general inhibitor tunicamycin (Fig. 6B).

Fig. 6. A, MraY exchange activity measurements. The level of [3H]UDP-MurNAc-tripeptide formed in MraY exchange reactions with cell membranes isolated from ET505 pJFlacZK (1) and ET505 pEmycZK (2) are shown. In 3, ET505 pJFlacZK membranes were treated with 100 μg/ml tunicamycin. The results are the average of three independent experiments, except for the tunicamycin-treated membranes, where duplicate experiments were averaged. Error bars represent the S.D. of triplicate reactions. B, Rfe exchange activity measurements. The level of [3H]UDP-GlcNAc formed in Rfe reactions with the cell membranes described above is shown. The results are the average of three independent experiments, and error bars represent the S.D. The labels are identical to those in A.

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this study provide compelling biochemical support for this model. By monitoring cell wall synthesis in vivo, we observed complete inhibition of \(^{3}H\)DAP incorporation into cell wall after only 5 min of Emcy expression. We also found that the cytoplasmic nucleotide precursors are the last intermediates labeled with \(^{3}H\)DAP (Fig. 4) and that UDP-MurNAc-pentapeptide accumulates to high levels during Emcy expression (Fig. 5). Taken together, the in vivo experiments indicate that it is the MraY step in the pathway that is blocked. This blockage was demonstrated unequivocally by the finding that in vitro MraY exchange activity is almost completely inhibited in membranes containing Emcy (Fig. 6A).

There are several antibiotics that block the MraY step, and they can be classified based on their mode of inhibition. They are either direct inhibitors of MraY, like mureidomycin and bacitracin, or indirect inhibitors like amphotycin, bacitracin, and colicin M (29–31), all of which are known to induce bacteriolysis (9, 29, 31–33). The direct inhibitors all resemble the nucleotide substrate with additional hydrophobic moieties attached. They can be further subdivided as specific MraY inhibitors, like mureidomycin (27), or general GPT enzyme inhibitors like tunicamycin (25, 26, 34). The indirect inhibitors limit the availability of the substrate undecaprenol-P by sequestering it or interfering with its recycling (30, 35). In addition to MraY, the general GPT enzyme inhibitors like tunicamycin inhibit the eukaryotic GPT enzyme involved in lipid glycosylation, and the indirect inhibitors will bind its lipid substrate, dolichol-P (35, 36). Therefore, only specific MraY inhibitors are likely to have potential therapeutic applications in treating bacterial infections.

E is a 91-amino acid membrane protein. Protein fusion analysis has revealed that only the amino-terminal 29 amino acids of the molecule, encompassing its putative transmembrane domain (TMD), are required for lytic function (37, 38). With these observations in mind, E likely inhibits MraY in one of two ways, either by direct inhibition through a TMD-TMD interaction or by one of the indirect modes described above. To distinguish between these modes of inhibition, we monitored the effect of Emcy on the activity of the other known E. coli GPT enzyme, Rfe, which catalyzes the formation of the first lipid intermediate in enterobacterial common antigen biosynthesis, undecaprenol-P-GlcNAc (26). We observed no inhibition of Rfe activity in membranes containing Emcy (Fig. 6B). If Emcy were a general or an indirect GPT inhibitor, it should have also inhibited Rfe. We therefore conclude that Emcy is a specific MraY inhibitor. This apparent specificity coupled with the small size of its lethal domain and its selectable lytic phenotype make E an attractive system to probe MraY function and inhibition. It may also help in the development of new specific MraY inhibitors with therapeutic applications. Coprecipitation and inhibition studies using a more purified system are underway to investigate E inhibition of MraY in more detail.

Now that there is both biochemical and genetic evidence that cell wall synthesis inhibition is the mechanism of E-induced lysis, it is a reasonable hypothesis that other single protein lysis systems also act in the same way. We are currently taking similar genetic and biochemical approaches to investigate whether the Q8 Aq and MS2 L lysis proteins are also cell wall synthesis inhibitors, and if so, at which step they act.

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\(^2\) W. D. Roof and R. Young, unpublished results.
The Lysis Protein E of φX174 Is a Specific Inhibitor of the MraY-catalyzed Step in Peptidoglycan Synthesis

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