Published in final edited form as:

Biochemistry. 2009 June 9; 48(22): 4999–5006. doi:10.1021/bi900469g.

Purification and Functional Characterization of the ϕ X174 Lysis Protein E[†]

Yi Zheng, Douglas K. Struck, and Ry Young*

Department of Biochemistry and Biophysics, Texas A&M University, 2128 TAMU, College Station TX 77843-2128

Abstract

Two classes of bacteriophages, the single-stranded DNA *Microviridae*, and the single-stranded RNA *Alloleviviridae*, accomplish lysis by expressing “protein antibiotics”, or polypeptides that inhibit cell wall biosynthesis. Previously, we have provided genetic and physiological evidence that E, a 91 aa membrane protein encoded by the prototype microvirus, ϕ X174, is a specific inhibitor of the translocase *MraY*, an essential membrane-embedded enzyme that catalyzes the formation of the murein precursor, Lipid I, from UDP-N-acetylmuramic acid-pentapeptide and the lipid carrier, undecaprenol phosphate. Here we report the first purification of E, which has been refractory to over-expression because of its lethality to *E. coli*. Moreover, using a fluorescently-labeled analog of the sugar-nucleotide substrate, we demonstrate that E acts as a non-competitive inhibitor of detergent-solubilized *MraY*, with respect to both soluble and lipid substrates. In addition, we show that the E-sensitivity of five *MraY* mutant proteins, produced from alleles selected for resistance to E, can be correlated to the apparent affinities determined by *in vivo* multicopy suppression experiments. These results are inconsistent with previous reports that E inhibited membrane-embedded *MraY* but not the detergent-solubilized enzyme, which led to a model in which E functions by binding *MraY* and blocking the formation of an essential hetero-multimeric complex involving *MraY* and other murein biosynthesis enzymes. We discuss a new model in which E binds to *MraY* at a site composed of the two transmembrane domains within which the E-resistance mutations map and that the result of this binding is a conformational change that inactivates the enzyme.

Small, single-strand nucleic acid bacteriophages effect host lysis by expression of a single gene and without the elaboration of a muralytic enzyme, in contrast to the larger, double-stranded DNA phages, which invariably employ a holin and an endolysin for lysis (1). The best-studied example is the gene *E* of the classic coliphage and prototype of the ubiquitous *Microviridae*, ϕ X174. *E*, entirely embedded in the +1 reading frame of the unrelated essential gene *D*, encodes a membrane protein of 91 residues (Fig. 1) (2–4). Previously, we have shown that protein E causes lysis by inhibiting *MraY*, the enzyme which catalyzes the synthesis of the murein precursor, Lipid I, and thus blocking cell wall synthesis (5, 6). E has been shown to function in a number of Gram-negative hosts (7, 8). Gene fusion experiments have shown that only its N-terminal 35 amino acids, including the sole predicted transmembrane domain (TMD¹), are required for lytic activity (5, 9, 10), which fits well with the multi-spanning integral membrane protein character of *MraY* (Fig. 1).

[†]This work was supported by Public Health Service grant GM27099 to R.Y., the Robert A. Welch Foundation, and the Program for Membrane Structure and Function, a Program of Excellence grant from the Office of the Vice President for Research at Texas A&M University.

*Corresponding author. phone: 979-845-2087, fax: 979-862-4718, ryland@tamu.edu.

Because there are no useful antibiotics that target MraY, the mechanism by which E functions as an inhibitor has been of interest. *In vivo*, MraY catalyzes the formation of Lipid I by transferring phosphate-N-acetylmuramic acid-pentapeptide (in *E. coli*, phosphate-N-acetylmuramic acid-L-Ala- γ -D-Glu-meso-diaminopimelic acid (DAP)-D-Ala-D-Ala) (P-MurNac-pentapeptide) from UDP-MurNac-pentapeptide to undecaprenol-phosphate (undecaprenol-P). A two-step reaction pathway involving a covalent MraY-P-MurNac-pentapeptide has been proposed (11) (Fig. 2).

The mechanism of E-mediated inhibition of MraY function was initially addressed by genetics and physiological experiments. *In vivo*, lysis by the wt E protein, but not E fusions where the C-terminal domain is replaced by a heterologous domain (9), requires SlyD, a cytoplasmic FKBP (FK506 binding protein)-type peptidyl-prolyl cis-trans isomerase (12). However, this requirement has been shown to be purely a matter of the stability of E and is unrelated to E function (13). Lysis is completely restored in a *slyD* knockout background by mutations, designated as *Epos* (plates on *slyD*), that increase the translation rate of the *E* mRNA and thus return the E protein to normal levels, despite its continued instability (13). Thus SlyD has no role in inhibition of MraY, despite the strict *slyD*-dependent lysis phenotype of ϕ X174.

The original studies identifying MraY as the target of E were based on the isolation of dominant mutants in *mraY* resistant to the expression of a cloned *E* gene (5). To date, five missense mutants of *mraY* providing resistance to E-mediated lysis, and impaired in plaque-formation by ϕ X174, have been isolated. These mutations map to TMDs 5 and 9 of the proposed topological map of MraY (Fig. 1) (14). Experiments in which inductions of plasmid-borne *mraY* genes have been used to protect the host from E lysis have indicated that E binds to MraY *in vivo* and that the five E-resistant *mraY* alleles can be separated into three classes based on apparent affinities for E (14). Moreover, E was found to be incapable of causing lysis when the active form of *B. subtilis* MraY (^{Bs}MraY) was produced in *E. coli*, indicating that the great disparity between the sequences of ^{Bs}MraY and ^{Ec}MraY prevents E binding.

In vitro studies of E inhibition has been hampered by the fact that E is lethal to *E. coli*. Experiments with quantifiable *E* Φ lacZ gene fusions indicated that lysis is brought about when only a few hundred chimeric molecules were present, suggesting that the *in vivo* levels of MraY must also be very low (9). Purification of E has not been reported. However, Mendel *et al.* (15) prepared a synthetic polypeptide, E_{pep}, corresponding to the N-terminal 37 residues of the predicted E sequence. Consistent with the earlier finding that only the N-terminal portion of E is required for lysis, inhibition was observed when SDS-solubilized E_{pep} was added to membranes containing over-expressed MraY. Unexpectedly, it was found that E_{pep} was not able to inhibit the MraY activity in detergent-solubilized membrane extracts. This led to a model in which E effects lysis by preventing the assembly of MraY into an essential hetero-multimeric integral membrane complex with high MraY activity, a complex which would not be formed in detergent. Here we report the first purification of full-length E protein and characterize its ability to inhibit MraY; the results are discussed in terms of a different model for E action.

¹The abbreviations used are: TMD, transmembrane domain; P-MurNac-pentapeptide, phosphate-N-acetylmuramic acid-L-Ala- γ -D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala; undecaprenol-P, undecaprenol-phosphate; FKBP, FK506 binding protein; LB, Luria-Bertani; IPTG, isopropyl- β -D-thiogalactopyranoside; UDP-MurNac-pentapeptide-DNS, UDP-MurNac-L- γ -D-Glu-meso-diaminopimelic acid (N ϵ -dansyl)-D-Ala-D-Ala; phytol-P, phytol-phosphate; TLC, thin layer chromatography; DDM, n-dodecyl- β -D-maltoside; EBB, Empigen BB; IMAC, immobilized metal affinity chromatography; CMC, critical micelle concentration; DHPC, 1,2-diheptanoyl-sn-glycero-3-phosphocholine

EXPERIMENTAL PROCEDURES

Media, chemicals, strains and culture methods

Growth and induction conditions for bacterial cultures have been described, including the use of Luria-Bertani (LB) broth, supplemented as appropriate with these antibiotics: ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), and kanamycin (40 µg/ml) or the inducers isopropyl-β-D-thiogalactopyranoside (IPTG) and arabinose (16). The hosts for over-expression of *E* and *mraY* were, respectively, BL21(DE3) and BL21(DE3)plysS (Novagen). Tunicamycin (mixture of isomers A, B, C, and D), phytol and phospray, a reagent for detection of phospholipid, were purchased from Sigma. The sources of detergents were as follows: Tween 20, SDS, and Empigen BB (EBB) from Sigma; cholic acid, saponin, and Triton X-100 from EMD; Nonidet P40 from Bethesda Research Laboratories; 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC) from Avanti Polar Lipids; *n*-dodecyl-β-D-maltoside (DDM) from Anatrace.

Plasmids

Plasmids were constructed using standard PCR, digestion and ligation methods, as previously described; all final constructs were verified by sequencing at the Laboratory for Plant Genome Technology at the Texas Agricultural Experiment Station. The plasmids pETE6his and pETMY contain, respectively, the ϕ X174 *E* gene extended with 6 histidine codons (13) or the wild type *E. coli mraY* gene (5), inserted between the NdeI and BamHI sites of pET11a vector (Novagen). The plasmid pBsMraYKan contains *Bs mraY* under the control of the P_{BAD} promoter and was derived from pBAD30-BsMraY (14) by deleting the *bla* gene and inserting a kanamycin-resistance cassette from pZS*24-MCS-1 (17) into the unique ClaI site.

Over-production and purification of E

To determine optimum conditions for over-production of E_{6his}, BL21(DE3) cells harboring either the pETE6his plasmid alone or both the pBsMraYKan and pETE6his plasmids were induced at A₅₅₀ ~ 0.6 with either 1mM IPTG or 0.2% arabinose and 1mM IPTG (added 2 minutes after arabinose), respectively. At various times, the cells from 1 ml aliquots were chilled, collected by centrifugation in the cold, resuspended in SDS-PAGE buffer in volumes chosen to normalize for constant cell mass, and analyzed by SDS-PAGE and immunoblotting, as described previously (16).

A total of 5 L of culture of BL21(DE3) cells harboring the pBSMraYKan and pETE6his plasmids was induced at A₅₅₀ = 0.6 with 0.2% arabinose and 1mM IPTG (added 2 minutes after arabinose) for 30 min and then harvested by centrifugation (rotor JA-10, Beckman) at 8K rpm (7000 × g) for 15 min in the cold. The cells were resuspended in 1/200 volume of cold French press buffer (50 mM Tris pH 8.0, 170 mM KCl, 5 mM EDTA, 1 mM PMSF, 1 mM DTT) and disrupted by French press. Whole cells and debris were removed by centrifugation at 5000 × g in a JA-20 rotor (Beckman), and membranes were collected from the supernatant by centrifugation at 130,000 × g for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman). Membrane pellets were resuspended in buffer containing 50 mM Tris pH8.0, 170 mM KCl, 10 mM MgCl₂ (Buffer R) plus various detergents, as indicated, and shaken gently overnight at 4°C. Insoluble material was removed by centrifugation at 130,000xg for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman).

The E_{6his} polypeptide, extracted in 2% EBB, was purified by immobilized metal affinity chromatography (IMAC) with 250 µl Talon Metal Affinity resins (Clontech) in a Poly-Prep column (Bio-Rad) using gravity elution. The elution buffer was the Buffer R supplemented with 100 mM imidazole and 0.06% EBB. Fractions containing E_{6his} protein were identified

by western blot, as described (16). A mock E extract was prepared in the same way using a strain carrying the pET11a vector. The concentration of purified E_{6his} was determined by two independent methods: A₂₈₀, using a molar absorption coefficient of E calculated as defined by Pace *et al.* (18); or by densitometry using Coomassie-blue stained SDS-PAGE gels, with egg white lysozyme (Sigma) as a standard. The two methods gave the same results.

Quantification of E in vivo

As described previously (16) MDS12 *lacI^Q tonA* : : Tn10 carrying plasmids pQ and indicating pRW derivatives was grown in LB-kanamycin-ampicillin at 37 °C, induced with IPTG and arabinose at A₅₅₀ = 0.5, and aerated for 25 min before 20 ml samples were harvested by centrifugation in the cold. The cells were resuspended in 2 ml of cold French press buffer and lysed by French press. Whole cells and debris were removed by centrifugation at 5000 × g in a JA-20 rotor (Beckman). Membranes were collected from 1.5 ml of the supernatant by centrifugation at 100,000 × g for 1 hour in the cold, using a TLA-100.3 rotor (Beckman). Membranes were resuspended in 100 μl sample loading buffer and processed for immunoblotting as described previously (16).

Substrates for the in vitro reaction

UDP-MurNAc-pentapeptide was isolated from *B. subtilis* W23 as described (6). The dansylated UDP-MurNAc-pentapeptide, UDP-MurNAc-L-γD-Glu-*m*-DAP(Nε-dansyl)-D-Ala-D-Ala (UDP-MurNAc-pentapeptide-DNS), was prepared by the reaction of UDP-MurNAc-pentapeptide with dansyl chloride as described (19). Phytol-phosphate (phytol-P), a C-20 analog of undecaprenol-P was used as the lipid substrate and was prepared by the chemical phosphorylation of phytol following the method by Danilov *et al.* (20). The phospholipid product is detected by thin layer chromatography (TLC) using chloroform: methanol: water (60:25:4) as the mobile phase, and confirmed as a single species using both I₂ and phospray staining (Silica Gel TLC plates, Whatman). The concentration of phytol-P was determined by measuring free phosphate after hydrolysis with 4N HCl at 90°C, as described (21). Using this lipid substrate, tunicamycin, a competitive inhibitor of the sugar-nucleotide substrate for this class of enzymes (22–24), exhibited efficient competitive inhibition of UDP-MurNAc-pentapeptide-DNS (data not shown).

In vitro reaction catalyzed by MraY

MraY activity was measured using a 10 μl reaction mixture containing Buffer R, 2.5 μl of MraY extract or membranes, and the substrates or substrates plus E protein or mock E extract (see below), as indicated. Phytol-P was provided in Buffer R plus 4% DDM; the final concentration of DDM in the total reaction was 1.25%. The reaction was started by addition of the UDP-MurNAc-pentapeptide-DNS substrate. The reaction mixture was incubated at 37°C for various times. For determination of initial rates, reactions were terminated at 5 min, which was determined to be within the linear phase of the reaction. After termination by boiling for 2 min, the reaction mixture was spotted on a TLC plate (Silica Gel, EMD Chemicals). The plate was developed for 6 to 7 hours using isobutyric acid: NH₄OH: water (66:1:33) as the mobile phase. Quantification of the product was done by scraping the spot, extracting with methanol, and measuring the fluorescent intensity. Fluorescence measurements were done in a Koala spectrofluorometer (ISS) (excitation = 340nm, emission = 535nm), with a volume of 100 μl in a 130-μl fluorescence micro cell (Hellma).

Preparation of an MraY-enriched membrane fraction

A total of 10 L of culture of BL21(DE3)plysS cells harboring the pETMY plasmid were induced at A₅₅₀ = 0.6 with 1 mM IPTG for one hour and then harvested by centrifugation

(rotor JA-10, Beckman) at 8K rpm ($7000 \times g$) for 15 min in the cold. The cells were resuspended in 1/100 volume of cold French press buffer and disrupted by French press. The lysate was cleared of whole cells by centrifugation in a JA-20 rotor (Beckman) at $5000 \times g$ for 10 min in the cold. Membranes were collected by centrifugation at 38K rpm ($130,000 \times g$) for 1 hour in the cold, using a Type 50.2 Ti rotor (Beckman). To extract MraY activity, membrane pellets were resuspended in Buffer R plus 1% DDM and stirred for 1 hour in the cold. Insoluble material was removed by centrifugation at 50K ($100,000 \times g$) for 1 hour in a TLA-100.3 rotor (Beckman). The supernatant from this clearing step was used without further treatment for MraY reactions.

RESULTS

Over-production and purification of E

Purification of E protein is especially challenging since it is lethal to *E. coli*, and many other Gram-negative bacteria, and thus not readily overproduced. Induction of an E allele encoding a C-terminal oligohistidine tag caused lysis within 15 minutes (Fig. 3), making recovery of membrane material from the large culture volume difficult and allowing only a small amount of E to accumulate in the membranes (Fig. 4A, lane 4). To overcome this obstacle, we took advantage of the insensitivity of ^{Bs}MraY to E inhibition. In the presence of the heterologous enzyme, lysis did not occur, making recovery of the membrane material much more efficient (Fig. 3), and E_{6his} protein accumulated to a much higher level (Fig. 4B). E_{6his} could be efficiently extracted from membranes of these induced cells with the zwitterionic detergent EBB or with SDS, but not with other commonly-used detergents (Table 1). The EBB-solubilized material was purified by IMAC, yielding a preparation 54 μ M E_{6his} protein (Fig. 4C) that was 84% pure. This corresponds to a yield of 27 μ g per liter of induced culture. A mock purification using the empty vector yielded the same background species (compare lanes 3 -8 with 9 - 15 in Fig. 4C) and was used as the negative control in all experiments with E.

Quantification of E in vivo

The lysis function of E depends on it having at least a stoichiometric relationship with MraY. Previous attempts at quantifying E were based on indirect *in vivo* approaches, one involving relative incorporation of label into E versus virion proteins (25) and the other based on the β -galactosidase activity of E- β gal chimeras (9), yielding estimates of 100–300 molecules and ~1000 molecules per cell, respectively. Here, using the purified E_{6his} as a standard for quantitative immunoblotting, a direct quantification of the amount of E in membranes could be attempted. Although the large amounts of lysate needed tended to distort visualization of the E species in SDS-PAGE (Fig. 5), nevertheless reproducible results were obtained that allowed an estimate of ~ 500 molecules of E at the time of lysis (Table 2). This confirms the more indirect estimates made previously and puts an upper limit on the number of MraY molecules that can be present *in vivo*. At 500 molecules/cell, each functional MraY molecule would have to have $k_{cat} \sim 200 \text{ min}^{-1}$ to account for the level of murein synthesis in *E. coli* (26). This is in reasonable agreement with $k_{cat} \sim 320 \text{ min}^{-1}$ measured by Al-Dabbagh *et al.* (27) for purified *E. coli* MraY.

MraY preparation and fluorescence assay

Initially we constructed ^{H6}*mraY* an allele of *mraY* encoding an N-terminal oligohistidine-tagged variant, to facilitate enrichment of MraY activity by IMAC, as reported by Bouhss *et al.* (28). However, this allele failed a stringent complementation test with a chromosomal Δ *mraY* (data not shown) (14). This result was not surprising, since the N terminus of MraY is predicted to be periplasmic, and an appended oligopeptide tag could interfere with proper folding in the membrane. Since our goal was biochemical characterization of E-mediated

inhibition, which might have stringent requirements for *MraY* folding, we decided to use the wt *mraY* allele for over-production and enrichment.

To measure *MraY* activity, we developed a fluorescence-based transfer assay for measuring *MraY* enzymatic activity in both crude membrane preparations and detergent-solubilized forms. The substrates used are UDP-MurNAc-pentapeptide-DNS, a fluorescent substrate analogue, and phytol-P, a twenty-carbon analogue of undecaprenol-P (Fig. 2). The fluorescently-labeled product, phytol-P-P-MurNAc-pentapeptide-DNS, is separated from the reaction mixture by TLC (Fig. 6) and quantified by fluorescence spectroscopy. Using this assay, Michaelis-Menten kinetics was observed for both substrates. Using Ping Pong Bi Bi formalism (29, 30), the K_m for UDP-MurNAc-pentapeptide-DNS was found to be $0.2 \pm .09$ mM, consistent with the estimated *in vivo* concentration of this substrate (31), and the apparent K_m for phytol-P was 0.84 ± 0.2 mM (Fig. 7). We also determined the K_m parameters for the *MraY*_{F288L} mutant protein, which has been shown to be the most extreme mutant, in terms of E-resistance, among the five (14), and obtained the same values (Fig. 7). This indicates that E-resistance is not due to an altered substrate affinity.

E-mediated inhibition of *MraY*

Next, the purified detergent-solubilized *E*_{6his} protein was examined for its ability to inhibit *MraY* *in vitro*. In contrast to the findings of Mendel *et al.* (15) with a synthetic peptide corresponding to the first 37 residues of *E*, we found the purified *E*_{6his} protein inhibited solubilized *MraY* efficiently (Fig. 8). We also observed inhibition by *E*_{6his} with membranes containing *MraY*, consistent with both the findings of Mendel *et al.* (15) and our original demonstration that *E* inhibits *MraY* specifically when both are present in the same membranes (6). For a given concentration of *E*, the extent of inhibition is lower than for the solubilized enzyme, which is not surprising considering that, in the assays with particulate *MraY*, the *E* protein must somehow enter the membrane from its detergent-solubilized state. Next, the mode by which *E* inhibited the solubilized *MraY* was determined with respect to both the lipid and sugar-nucleotide substrates (Fig. 9) by measuring *MraY* activity in the presence of varying concentrations of *E*_{6his}. Experiments to obtain the kinetic data were performed with crude *MraY* sample containing membrane detergent extracts. Kinetic analysis revealed that the K_m parameters for both UDP-MurNAc-pentapeptide-DNS and phytol-P were unchanged in the presence of *E*_{6his}, whereas in both cases V_{max} was decreased. Thus, *E* is a non-competitive inhibitor of *MraY* with respect to both lipid and sugar-nucleotide substrates, with an average $K_i = 0.53 \pm 0.12 \mu\text{M}$ (Fig. 9).

Sensitivity of *MraY* mutant alleles

We then investigated the ability of *E* to inhibit the *MraY* proteins from the five mutant alleles isolated by selecting for resistance to the lysis protein. The five mutants fall into 3 classes according to the degree of inhibition at $[E_{6his}] = 2.7 \mu\text{M}$: *MraY*_{G186S} and *MraY*_{V291} are almost as sensitive to *E* as the wt protein; *MraY*_{F288L} is, like ^{Bs}*MraY*, not detectably inhibited; and *MraY*_{P170L} and *MraY*_{ΔL172} are inhibited to a degree intermediate between the first two classes (Fig. 10). These results match the classes of apparent affinities determined *in vivo* by comparing the ability of multicopy plasmids carrying these alleles to protect the wt protein from *E* inhibition (14).

DISCUSSION

In the more than 40 years since *E* was defined as the lysis gene of the paradigm *Microvirus* φX174 (32), many hypotheses have been advanced for the molecular basis of its ability to effect lysis in the absence of detectable muralytic activity (32–37). Recently, genetic and physiological studies from our laboratory provided evidence that *E* acts as a specific

inhibitor of MraY, the first membrane-embedded enzyme in the pathway of murein precursor biosynthesis (5, 6). Specifically, mutations that conferred resistance to E-mediated lysis mapped to two TMDs of MraY, and MraY activity, but not the activity of an unrelated member of the translocase super-family, was inhibited in membranes containing E (6). *In vitro* studies needed to extend these studies have been stymied by the inability to obtain purified E protein.

Here we report over-production of E achieved by providing an E-insensitive heterologous MraY protein in trans. This is the first purification of any of the single-gene phage lysis proteins and opens the way for the structural and biophysical characterization of E. Moreover, the availability of E protein makes it possible now to study the E-SlyD interaction with purified components. PPIases are ubiquitous, found in all cells and in all major cellular compartments, but their biological roles, at least in the absence of drugs, have been elusive. Although SlyD is irrelevant to E function, its interaction with E remains the most genetically tractable PPIase-protein substrate phenotype known in biology. SlyD is easily purified and in fact has been inadvertently purified by many laboratories, since it possesses a histidine-rich C-terminal tail and thus contaminates IMAC purifications of soluble (but not membrane) proteins (38–40).

In the work reported here, the purified E protein was used to investigate its mode of inhibition of MraY. Using solubilized extracts of membranes enriched in MraY, we have demonstrated that E protein acts as a non-competitive inhibitor with respect to both the lipid and sugar-nucleotide substrates of MraY. Our results are significantly different from those of Mendel *et al.* (15), who reported that E_{pep}, a synthetic peptide corresponding to the first 37 residues of E, inhibited membrane-embedded MraY but not detergent-solubilized MraY. The discrepancy may arise from differences in the assays used. Mendel *et al.* used SDS-solubilized E_{pep} for their inhibition studies, resulting in SDS concentrations at or above its CMC (critical micelle concentration) in the final reaction mixes. Although the authors report that the detergent alone at this concentration had no effect on MraY activity, it is possible that it affects E-MraY interactions. In our hands, 1% SDS completely destroys MraY activity (data not shown). Although the authors reported CD measurements indicating alpha-helical character, it is also possible that the synthetic 37 residue polypeptide used was not correctly folded, perhaps due to the lack of a C-terminal domain. The C-terminal hydrophilic domain of E can be replaced by some, but not all, heterologous protein folding domains (9, 10), and there have been no reports that simple C-terminal truncations of E are lytic *in vivo*. Most importantly, here we have shown that the E-sensitivity of solubilized MraY proteins *in vitro* correlates with the allelic state of *mraY* and with our previous assessments of apparent E-MraY binding *in vivo* (5, 14). In the absence of commensurate genetic validation, results obtained with the synthetic polypeptide must be interpreted with caution.

We have now demonstrated that E inhibits MraY specifically *in vivo* (5), in membranes (6), and in solubilized extracts. In addition, we have found that E acts in a non-competitive fashion with respect to both its lipidic and soluble substrates. Taken together, these results obviate the need to invoke the existence of a detergent-sensitive, E-sensitive hetero-multimeric complex of membrane proteins required for the biological activity of MraY (15). The existence of such a complex was difficult to reconcile with the fact that the MraY enzymes from two Gram-positive bacteria, *B. subtilis* and *S. aureus*, were found to complement *E. coli mraY* defects *in vivo* (14, 41). The ability of these diverged proteins (43% similarity between E^cMraY and B^sMraY; 40% between E^cMraY and S^aMraY; 54% between B^sMraY and S^aMraY) to complement would not seem compatible with a model requiring intimate interactions with other *E. coli cell* division and murein synthesis proteins. The data presented here, taken with the genetic studies and the *in vivo* protection experiments reported previously (5, 14), suggest a simpler model in which E binds to MraY

by interactions between the single transmembrane domain (TMD) of E and TMDs 5 and 9 of MraY, and this binding results in non-competitive inhibition of the enzyme by causing a conformational change. The catalytically important aspartate residues of MraY are associated with cytoplasmic loops terminating in TMDs 4 (Asp115, 116) and 8 (Asp267) (Fig. 1). TMDs almost invariably interact with their adjacent neighbors in primary structure (42, 43), which in this case would include the TMDs 5 and 9 that appear to define the E binding site, so an E-dependent conformational change based on transmembrane-helix interactions would not be difficult to conceive. Alternatively, it is worth noting that E has two basic residues, Lys33 and Arg34, predicted to be at the cytoplasmic interface of the membrane; binding of E to TMD9 of MraY may localize these residues near to the catalytic Asp267, predicted to be at the cytoplasmic interface of TMD8.

Protein inhibitors of biosynthetic enzymes are rare, so in this respect alone the ability of E to inhibit MraY is of interest. Moreover, MraY is universally conserved in bacteria, so understanding how E mediates non-competitive inhibition may be useful in the development of new antibacterial agents. E is particularly attractive as a probe for the mechanistic investigation of MraY because, unlike small molecule inhibitors, such as mureidomycin and tunicamycin, E is a genetic system on its own. This offers many advantages, especially since E can be tagged with any number of C-terminal protein moieties without affecting its inhibitory function. Also, the existence of the three classes of E-resistant mutants of MraY indicates that the affinity of E could be tuned by manipulation of the E sequence too. Indeed, a suppressor analysis looking for E missense changes that overcome the E-resistance mutations in TMDs 5 and 9 may allow a point-to-point interaction map to be generated, if allele-specificity can be demonstrated. Recently, the MraY of *B. subtilis* has been purified substantially on a small scale (28). Similar progress with the *E. coli* enzyme might allow the use of the genetically tractable *E* lysis protein system to probe MraY at the structural and mechanistic level.

Acknowledgments

We thank the members of the Young laboratory, past and present, for their helpful criticisms and suggestions.

References

1. Young, R.; Wang, IN. Phage Lysis. In: Calendar, R., editor. The Bacteriophages. 2. Oxford University Press; Oxford: 2006. p. 104-126.
2. Sanger F, Air GM, Barrell BG, Brown NL, Coulson AR, Fiddes JC, Hutchison CA III, Slocombe PM, Smith M. Nucleotide sequence of bacteriophage ϕ X174 DNA. *Nature*. 1977; 265:687-695. [PubMed: 870828]
3. Altman E, Young K, Garrett J, Altman R, Young R. Subcellular localization of lethal lysis proteins of bacteriophages λ and ϕ X174. *J Virol*. 1985; 53:1008-1011. [PubMed: 3156254]
4. Bläsi, U.; Geisen, R.; Lubitz, W.; Henrich, B.; Plapp, R. Localisation of the bacteriophage ϕ X174 lysis gene product in the cell envelope of *Escherichia coli*. In: Hakenbeck, R.; Holtje, JV.; Labischinski, H., editors. The Target of Penicillin. Walter de Gruyter; Berlin: 1983. p. 205-210.
5. Bernhardt TG, Roof WD, Young R. Genetic evidence that the bacteriophage ϕ X174 lysis protein inhibits cell wall synthesis. *Proc Natl Acad Sci USA*. 2000; 97:4297-4302. [PubMed: 10760296]
6. Bernhardt TG, Struck DK, Young R. The lysis protein E of ϕ X174 is a specific inhibitor of the MraY-catalyzed step in peptidoglycan synthesis. *J Biol Chem*. 2001; 276:6093-6097. [PubMed: 11078734]
7. Eko FO, Witte A, Huter V, Kuen B, Furst-Ladani S, Haslberger A, Katinger A, Hensel A, Szostak MP, Resch S, Mader H, Raza P, Brand E, Marchart J, Jechlinger W, Haidinger W, Lubitz W. New strategies for combination vaccines based on the extended recombinant bacterial ghost system. *Vaccine*. 1999; 17:1643-1649. [PubMed: 10194817]

8. Jalava K, Henselb A, Szostaka M, Resch S, Lubitz W. Bacterial ghosts as vaccine candidates for veterinary applications. *J Controlled Release*. 2002; 85:17–25.
9. Maratea D, Young K, Young R. Deletion and fusion analysis of the ϕ X174 lysis gene *E*. *Gene*. 1985; 40:39–46. [PubMed: 2936651]
10. Buckley KJ, Hayashi M. Lytic activity localized to membrane-spanning region of ϕ X174 E protein. *Mol Gen Genet*. 1986; 204:120–125. [PubMed: 3018438]
11. Hammes WP, Neuhaus FC. On the specificity of phospho-N-acetylmuramyl-pentapeptide translocase. The peptide subunit of uridine diphosphate-N-actylmuramyl-pentapeptide. *J Biol Chem*. 1974; 249:3140–3150. [PubMed: 4208473]
12. Roof WD, Young R. ϕ X174 lysis requires *slyD*, a host gene which is related to the FKBP family of peptidyl-prolyl cis-trans isomerases. *FEMS Microbiol Rev*. 1995; 17:213–216. [PubMed: 7669348]
13. Bernhardt TG, Roof WD, Young R. The *Escherichia coli* FKBP-type PPIase SlyD is required for the stabilization of the E lysis protein of bacteriophage ϕ X174. *Mol. Microbiol*. 2002; 45:99–108.
14. Zheng Y, Struck DK, Bernhardt TG, Young R. Genetic analysis of *MraY* inhibition by the ϕ X174 protein E. *Genetics*. 2008; 180:1459–1466. [PubMed: 18791230]
15. Mendel S, Holbourn JM, Schouten JA, Bugg TD. Interaction of the transmembrane domain of lysis protein E from bacteriophage ϕ X174 with bacterial translocase *MraY* and peptidyl-prolyl isomerase *SlyD*. *Microbiology*. 2006; 152:2959–2967. [PubMed: 17005977]
16. Zheng Y, Struck DK, Dankenbring CA, Young R. Evolutionary dominance of holin lysis systems derives from superior genetic malleability. *Microbiology*. 2008; 154:1710–1718. [PubMed: 18524925]
17. Lutz R, Bujard H. Independent and tight regulation of transcriptional units in *Escherichia coli* via the *LacR/O*, the *TetR/O* and *AraC/I1-I2* regulatory elements. *Nucleic Acids Res*. 1997; 25:1203–1210. [PubMed: 9092630]
18. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci*. 1995; 4:2411–2423. [PubMed: 8563639]
19. Weppner WA, Neuhaus FC. Fluorescent substrate for nascent peptidoglycan synthesis. Uridine diphosphate-N-acetylmuramyl-(N-epsilon-5-dimethylaminonaphthalene-1- sulfonyl)pentapeptide. *J Biol Chem*. 1977; 252:2296–2303. [PubMed: 849930]
20. Danilov LL, Druzhinina TN, Kalinchuk NA, Maltsev SD, Shibaev VN. Polyprenyl phosphates: synthesis and structure-activity relationship for a biosynthetic system of *Salmonella anatum* O-specific polysaccharide. *Chem Phys Lipids*. 1989; 51:191–203. [PubMed: 2611960]
21. Hess H, Derr J. Assay of inorganic and organic phosphorus in the 0.5–1 nanomole range. *Anal Biochem*. 1975; 63:607–613. [PubMed: 1122033]
22. Struck DK, Lennarz WJ. Evidence for the participation of saccharide-lipids in the synthesis of the oligosaccharide chain of ovalbumin. *J Biol Chem*. 1977; 252:1007–1013. [PubMed: 838693]
23. Brandish P, Kimura K, Inukai M, Southgate R, Lonsdale J, Bugg T. Modes of action of tunicamycin, liposidomycin B, and mureidomycin A: Inhibition of phospho-N-acetylmuramyl-pentapeptide translocase from *Escherichia coli*. *Antimicrob Agents Chemother*. 1996; 40:1640–1644. [PubMed: 8807054]
24. Meier-Dieter U, Barr K, Starman R, Hatch L, Rick PD. Nucleotide sequence of the *Escherichia coli rfe* gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the *rfe-rff* gene cluster. *J Biol Chem*. 1992; 267:746–753. [PubMed: 1730666]
25. Pollock TJ, Tessman ES, Tessman I. Identification of lysis protein E of bacteriophage ϕ X174. *J Virol*. 1978; 28:408–410. [PubMed: 702655]
26. Wientjes FB, Woldringh CL, Nanninga N. Amount of peptidoglycan in Cell Walls of Gram-negative bacteria. *J Bacteriol*. 1991; 173:7684–7691. [PubMed: 1938964]
27. Al-Dabbagh B, Henry X, El Ghachi M, Auger G, Blanot D, Parquet C, Mengin-Lecreulx D, Bouhss A. Active site mapping of *MraY*, a member of the polyprenyl-phosphate N-acetylhexosamine 1-phosphate transferase superfamily, catalyzing the first membrane step of peptidoglycan biosynthesis. *Biochemistry*. 2008; 47:8919–8928. [PubMed: 18672909]

28. Bouhss A, Crouvoisier M, Blanot D, Mengin-Lecreulx D. Purification and characterization of the bacterial MraY translocase catalyzing the first membrane step of peptidoglycan biosynthesis. *J Biol Chem.* 2004; 279:29974–29980. [PubMed: 15131133]
29. Cook, P.; Cleland, W. *Enzyme kinetics and mechanism.* Garland Science; London and New York: 2007.
30. Brandish PE, Burnham MK, Lonsdale JT, Southgate R, Inukai M, Bugg TD. Slow binding inhibition of phospho-N-acetylmuramyl-pentapeptide-translocase (*Escherichia coli*) by mureidomycin A. *J Biol Chem.* 1996; 271:7609–7614. [PubMed: 8631795]
31. van Heijenoort, J.; Neidhardt, FC. Murein synthesis. In: Neidhardt, FC., editor. *Escherichia coli and Salmonella Cellular and Molecular Biology.* ASM Press; Washington, D.C.: 1996. p. 1025-1034.
32. Hutchison CA III, Sinsheimer RL. The process of infection with bacteriophage ϕ X174. X. Mutations in a ϕ X lysis gene. *J Mol Biol.* 1966; 18:429–447. [PubMed: 5968177]
33. Hutchison CA III, Phillips S, Edgell MH, Gillam S, Jahnke P, Smith M. Mutagenesis at a specific position in a DNA sequence. *J Biol Chem.* 1978; 253:6551–6560. [PubMed: 681366]
34. Bläsi, U.; Halfmann, G.; Lubitz, W. Induction of autolysis of *Escherichia coli* by ϕ X174 gene E product. In: Nombela, C., editor. *Microbial Cell Wall Synthesis and Autolysis.* Elsevier Science Publishers; New York: 1984. p. 213-218.
35. Witte A, Bläsi U, Halfmann G, Szostak M, Wanner G, Lubitz W. ϕ X174 protein E-mediated lysis of *Escherichia coli*. *Biochimie.* 1990; 72:191–200. [PubMed: 2143087]
36. Witte A, Wanner G, Bläsi U, Halfmann G, Szostak M, Lubitz W. Endogenous transmembrane tunnel formation mediated by ϕ X174 lysis protein E. *J Bacteriol.* 1990; 172:4109–4114. [PubMed: 2141836]
37. Witte A, Lubitz W. Biochemical characterization of ϕ X174-protein-E-mediated lysis of *Escherichia coli*. *Eur J Biochem.* 1989; 180:393–398. [PubMed: 2522390]
38. Bolanos-Garcia VM, Davies OR. Structural analysis and classification of native proteins from *E. coli* commonly co-purified by immobilised metal affinity chromatography. *Biochim Biophys Acta.* 2006; 1760:1304–1313. [PubMed: 16814929]
39. McMurry JL, Macnab RM. BD TALON™ resin does not bind *E. coli* SlyD, a common contaminant in Ni-NTA IMAC. *Clontechiques XIX.* 2004;2–4.
40. Parsy CB, Chapman CJ, Barnes AC, Robertson JF, Murray A. Two-step method to isolate target recombinant protein from co-purified bacterial contaminant SlyD after immobilised metal affinity chromatography. *J Chromatogr B.* 2007; 853:314–319.
41. Bouhss A, Mengin-Lecreulx D, Le Beller D, van Heijenoort J. Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. *Mol Microbiol.* 1999; 34:576–585. [PubMed: 10564498]
42. Bowie J. Helix packing in membrane Proteins. *J Mol Biol.* 1997; 272:780–789. [PubMed: 9368657]
43. Chamberlain AK, Faham S, Yohannan S, Bowie J. Construction of helix-bundle membrane proteins. *Adv Protein Chem.* 2003; 63:19–46. [PubMed: 12629965]
44. Lloyd AJ, Brandish PE, Gilbey AM, Bugg TD. Phospho-N-acetyl-muramyl-pentapeptide translocase from *Escherichia coli*: catalytic role of conserved aspartic acid residues. *J Bacteriol.* 2004; 186:1747–1757. [PubMed: 14996806]
45. Heydanek MG Jr, Struve WG, Neuhaus FC. On the initial stage in peptidoglycan synthesis. 3 Kinetics and uncoupling of phospho-N-acetylmuramyl-pentapeptide translocase (uridine 5'-phosphate). *Biochemistry.* 1969; 8:1214–1221. [PubMed: 5781013]

Figure 1. Features of E and MraY

Top panel primary structure of E with the putative TMD in the shaded rectangles. The positively and negatively charged residues are indicated. **Bottom panel, left:** proposed topology of MraY (14). The three conserved Asp residues essential for MraY enzymatic activity are labeled on cytoplasmic loops below TMD3 and TMD8 (44). The E-resistance mutations isolated in *mraY* (5, 14) are indicated by the following symbols: □, P170L; Δ, ΔL172; ■, G186S; ●, F288L; ○, V291M. **Bottom panel, right:** The sequences of three TMDs (TMD 5, 8 and 9) in MraY. In TMD 5 and 9, the E-resistance mutations are shown. The sequences of the TMDs proposed (14) are shown in the shaded rectangles. The proposed catalytic Asp267 residue is indicated by an asterisk. Modified from Zheng *et al.* (14), with permission.

Figure 2. Proposed reaction mechanism of MraY

The MraY-catalyzed reaction has been proposed to be a two-step process (45). In this model, the first step generates a MurNAc-pentapeptide-phosphoenzyme intermediate and free UMP (MraY-P-MurNAc-pep₅). In the second step, undecaprenol-P (labeled as C55-P) attacks the intermediate, and results in formation of lipid I and release of the free enzyme. In parenthesis, the substrates and products in the *in vitro* assay used are indicated; pep₅, pentapeptide; C20 = phytol.

Figure 3. *BsMraY* prevents lysis from over-expression of *E_{6his}*

Cultures of BL21(DE3) harboring the indicated plasmids were induced at $A_{550} = 0.5$ by addition of 1mM IPTG and monitored for culture turbidity as described in Experimental Procedures. The pBsMraYKan plasmid was induced by addition of 0.2% arabinose 2 minutes before IPTG was added. Symbols: ○, pET11a; □, pETE6his; ●, pBsMraYKan and pET11a; ■, pBsMraYKan and pETE6his.

Figure 4. Over-production and purification of E₆his

Panels **A** and **B**: **Accumulation of E₆his after induction.** Cultures of BL21(DE3) cells harboring either the pETE6his plasmid or the pET11a vector, as indicated, or the pBSMrayKan plasmid, were induced at $t = 0$ with IPTG. The cultures were sampled at various times after induction and analyzed by immunoblot, as described in Experimental Procedures. Molecular weight (M) standards are indicated to the left of the blots.

A. Lane 1, pET11a, $t = 15$ min. Lanes 2 – 4: pETE6his, $t = 0, 5$, and 15 min.

B. Lane 1, pET11a, $t = 15$ min. Lane 2, pETE6his, $t = 15$ min. Line3, pET11a + pBsMrayKan, $t = 60$ min. Lanes 4 – 9: pETE6his + pBsMrayKan, $t = 0, 5, 15, 25, 35$ and 60 min.

Panel **C**. **Purification of E.** Coomassie blue-stained SDS PAGE (top panel) and immunoblot (bottom panel) analysis of steps in E₆his purification or mock E purification (see Experimental Procedures), as indicated. M, molecular weight standards. Lanes 1 and 2: whole cell samples, pET11a vector and pETE6his. Lanes 3 and 9: 10mM imidazole wash fraction, pET11a vector and pETE6his. Lanes 4–8: elution fractions 1–5, pET11a vector. Lanes 10–15: elution fractions 1–6, pETE6his.

Figure 5. Quantification of E *in vivo*

Immunoblots of membranes from cells lysed by induction of *E*_{6his}, using antibodies against the oligohistidine tag are shown. Lane 1, pRW* (vector); Lane 2, pRW*E_{6his}; Lane 3–7, different amounts of purified E_{6his} as indicated above the E bands.

Figure 6. Fluorescence-based assay for MraY

Reaction mixtures containing the fluorescent substrate UDP-MurNAc-pentapeptide-DNS (UDP-MurNAc-pep₅-DNS) in Buffer R (lanes 2 – 7), supplemented with DDM-solubilized MraY (lanes 4 – 7) or heat-inactivated MraY (lane 3), phytol-P (lanes 2–3, 5–7), tunicamycin in methanol (lane 7) or methanol (lane 6) were incubated at 37°C for 30 min, quenched by heating at 100°C for 2 min, and then analyzed by TLC, as described in Experimental Procedures. Lane 1 contains the UDP-MurNAc-pentapeptide-DNS alone. Reaction components are also listed above the panel, with +/- signs indicating inclusion or exclusion, respectively. Positions of the fluorescent substrate and product are indicated.

Figure 7. Determination of K_m values

Panels A and B K_m of wt MraY for phytol-P and UDP-MurNAc-pentapeptide-DNS. **Panel C and D**, K_m of MraY F288L mutant for phytol-P and UDP-MurNAc-pentapeptide-DNS. Initial rates were measured as described in Experimental Procedures, first varying phytol-P concentration with UDP-MurNAc-pentapeptide present at 0.2 mM, then varying UDP-MurNAc-pentapeptide concentration with phytol-P present at 0.25 mM. K_m values were determined by linear regression using equations for a Ping Pong Bi Bi system (29), as previously described[add Brandish ref].

Figure 8. Inhibition of both particulate and detergent-solubilized MraY by E_{6his}

Activity was measured by the *in vitro* assay as described in experimental procedures. Data are expressed as percentage activity. Elution fraction #2 of a parallel purification of material from cells carrying the vector, pET11a (see Fig. 6C, lane 5) served as the null E control (0 μM E_{6his}). Symbols: ●, detergent-solubilized MraY; □, membranes containing MraY.

Figure 9. E_{6his} is a non-competitive inhibitor of MraY with respect to both soluble and lipid substrates

Assays were carried out as described in Experimental Procedures at fixed E_{6his} concentrations: ◆, 0 μM (mock E extract, figure 6C, lane5); ■, 0.7 μM; ▲, 1.4 μM. **(A)** Varying UDP-MurNAc-pentapeptide-DNS concentration in the presence of fixed concentration (0.25 mM) of phytol-P. **(B)** Varying phytol-P concentration in the presence of fixed concentration (0.2 mM) of UDP-MurNAc-pentapeptide-DNS.

Figure 10. Inhibition of MraY mutants by E_{6his}

Enzymatic activities of MraY proteins produced from five *mraY* mutant alleles and B^sMraY were determined at a fixed E_{6his} concentration (2.7 μM). Data are expressed as percentage activity.

Table 1Extraction of E_{6his} from *E. coli* membranes^a

Detergent	Extraction efficiency (%)
Triton X-100	13
Tween 20	<5
Nonidet P40	18
SDS	70
Cholic acid	<5
DHPC	<5
EBB	49
Saponin	<5

^aMembranes containing over-produced E_{6his} were extracted with the indicated detergents, as described in Experimental Procedures. The distribution of the E_{6his} protein between soluble and insoluble fractions was estimated by immunoblot and densitometry. Concentration of detergents used was 1% for each detergent, except DHPC, which was 14 mM (~0.7%).

Table 2E amount at lysis *in vivo*^b

	E (ng)	E (molecule/cell)
1	17	340
2	22	440
3	26	520
4	28	560
average	23±5	465±97

^bThe same experiments as shown in Fig. 5 were repeated four times, and the average amount of E molecules per cell at lysis *in vivo* is shown.