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# Purification and Functional Characterization of $\phi$X174 Lysis Protein E[†]

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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-amino acid membrane protein encoded by the prototype microvirus, $\phi$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 overexpression because of its lethality to *Escherichia coli*. Moreover, using a fluorescently labeled analogue of the sugar-nucleotide substrate, we demonstrate that E acts as a noncompetitive 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 heteromultimeric 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 the fact that the result of this binding is a conformational change that inactivates the enzyme.

Small, single-stranded nucleic acid bacteriophages effect host lysis by expression of a single gene 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 gene *E* of the classic coliphage and prototype of the ubiquitous *Microviridae*, $\phi$X174. *E*, entirely embedded in the +1 reading frame of the unrelated essential gene *D*, encodes a membrane protein of 91 residues (Figure 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, 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 35 N-terminal amino acids, including the sole predicted transmembrane domain (TMD),[1] are required for lytic activity (*5*, *9*, *10*), which fits well with the multispanning integral membrane protein character of MraY (Figure 1).

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 *Escherichia coli*, phosphate-N-acetylmuramic acid-L-Ala-$\gamma$-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 complex has been proposed (*11*) (Figure 2).

The mechanism of E-mediated inhibition of MraY function was initially addressed by genetics and physiological experiments. In vivo, lysis by the wild-type (wt) E protein, but not E fusions where the C-terminal domain is replaced with a heterologous domain (*9*), requires SlyD, a cytoplasmic FKBP (FK506 binding protein)-type peptidyl-prolyl cis-trans isomerase (PPIase) (*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 *Epos* (plates on *slyD*), that increase the rate of translation of the *E* mRNA and thus return the E protein to

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[1]Abbreviations: TMD, transmembrane domain; P-MurNAc-pentapeptide, phosphate-N-acetylmuramic acid-L-Ala-$\gamma$-D-Glu-*meso*-diaminopimelic acid-D-Ala-D-Ala; undecaprenol-P, undecaprenol phosphate; wt, wild-type; FKBP, FK506 binding protein; PPIase, peptidyl-prolyl cis-trans isomerase; LB, Luria-Bertani; IPTG, isopropyl $\beta$-D-thiogalactopyranoside; UDP-MurNAc-pentapeptide-DNS, UDP-MurNAc-L-$\gamma$-D-Glu-*meso*-diaminopimelic acid (N$\varepsilon$-dansyl)-D-Ala-D-Ala; phytol-P, phytol phosphate; TLC, thin layer chromatography; DDM, *n*-dodecyl $\beta$-D-maltoside; EBB, Empigen BB; IMAC, immobilized metal affinity chromatography; CMC, critical micelle concentration; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine.

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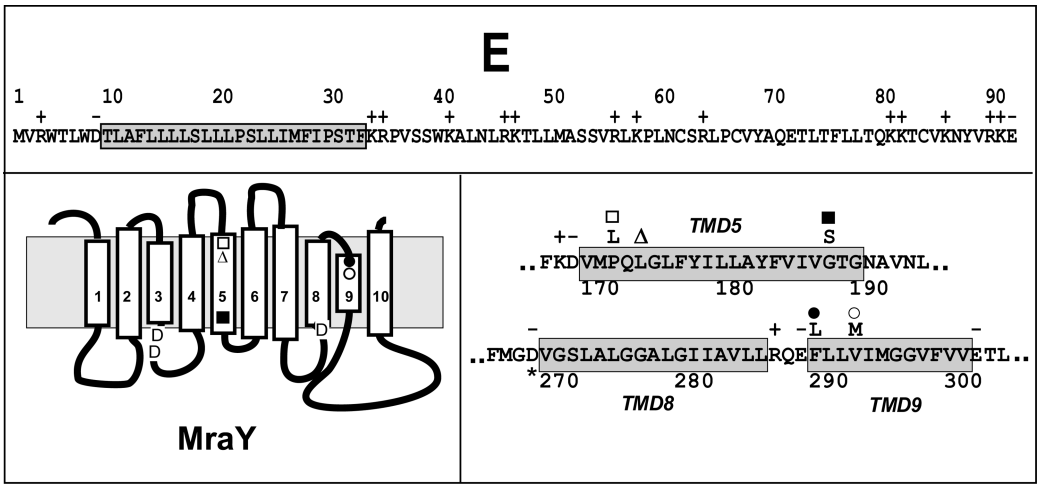


FIGURE 1: Features of E and MraY. The top panel shows the primary structure of E with the putative TMD in the shaded rectangles. The positively and negatively charged residues are indicated. The bottom left panel shows the proposed topology of MraY (*14*). The three conserved Asp residues essential for MraY enzymatic activity are labeled on cytoplasmic loops below TMD 3 and TMD 8 (*44*). The E resistance mutations isolated in *mraY* (*5*, *14*) are denoted as follows: (□) P170L, (△) ΔL172, (■) G186S, (●) F288L, and (○) V291M. The bottom right panel shows 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 with an asterisk. Modified from ref *14*.

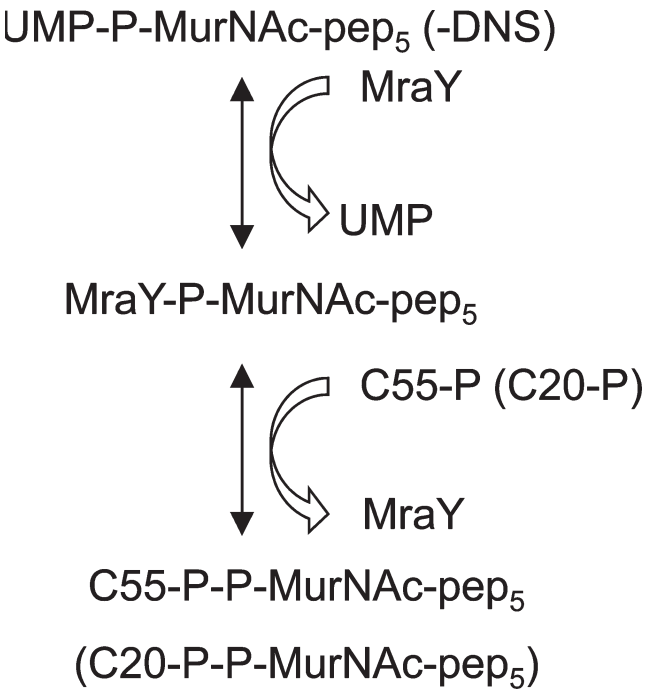


FIGURE 2: Proposed reaction mechanism of MraY. The MraY-catalyzed reaction has been proposed to be a two-step process (*45*). In this model, the first step generates a MurNAc-pentapeptide−phosphoenzyme intermediate (MraY-P-MurNAc-$pep_5$) and free UMP. In the second step, undecaprenol-P (labeled as C55-P) attacks the intermediate, which results in formation of Lipid I and release of the free enzyme. In parentheses, the substrates and products in the in vitro assay used are indicated: $pep_5$, pentapeptide; C20, phytol.

normal levels, despite its continued instability (*13*). Thus, SlyD has no role in inhibition of MraY, despite the strict *slyD*-dependent lysis phenotype of $\phi$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 $\phi$X174, have been isolated. These mutations map to TMD 5 and 9 of the proposed topological map of MraY (Figure 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 *Bacillus 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 have been hampered by the fact that E is lethal to *E. coli*. Experiments with quantifiable *EΦlacZ* gene fusions indicated that lysis was 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 37 N-terminal 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 overexpressed 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 heteromultimeric 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.

## 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 ampicillin (100 $\mu$g/mL), chloramphenicol (10 $\mu$g/mL), and kanamycin (40 $\mu$g/mL) or the inducers isopropyl $\beta$-D-thiogalactopyranoside (IPTG) and arabinose (*16*). The hosts for overexpression of *E* and *mraY* were BL21 (DE3) and BL21(DE3)plysS, respectively (Novagen). Tunicamycin (mixture of isomers A−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. Plasmids pETE6his and pETMY contain the ϕX174 *E* gene extended with six histidine codons (*13*) and the wt *E. coli mraY* gene (*5*), respectively, inserted between the NdeI and BamHI sites of the pET11a vector (Novagen). The pBsMraYKan plasmid 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.

*Overproduction and Purification of E*. To determine optimum conditions for overproduction of $E_{6his}$, BL21(DE3) cells harboring either the pETE6his plasmid alone or both the pBsMraYKan and pETE6his plasmids were induced at an $A_{550}$ of ~0.6 with either 1 mM IPTG or 0.2% arabinose and 1 mM IPTG (added 2 min 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 an $A_{550}$ of 0.6 with 0.2% arabinose and 1 mM IPTG (added 2 min 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, and 1 mM DTT] and disrupted with a 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 130000*g* for 1 h in the cold, using a type 50.2 Ti rotor (Beckman). Membrane pellets were resuspended in buffer containing 50 mM Tris (pH 8.0), 170 mM KCl, and 10 mM $MgCl_2$ (buffer R) with various detergents, as indicated, and shaken gently overnight at 4 °C. Insoluble material was removed by centrifugation at 130000*g* for 1 h 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 of Talon Metal Affinity resins (Clontech) in a Poly-Prep column (Bio-Rad) using gravity elution. The elution buffer was buffer R supplemented with 100 mM imidazole and 0.06% EBB. Fractions containing $E_{6his}$ protein were identified by Western blot analysis, as described previously (*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: $A_{280}$, using a molar absorption coefficient of E calculated as defined by Pace et al. (*18*); or 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, and ampicillin at 37 °C, induced with IPTG and arabinose at an $A_{550}$ of 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 with a 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 100000*g* for 1 h in the cold, using a TLA-100.3 rotor (Beckman). Membranes were resuspended in 100 μL of 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 previously (*6*). The dansylated UDP-MurNAc-pentapeptide, UDP-MurNAc-L-γ-D-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 previously (*19*). Phytol phosphate (phytol-P), a C-20 analogue of undecaprenol-P, was used as the lipid substrate and was prepared by the chemical phosphorylation of phytol following the method of Danilov et al. (*20*). The phospholipid product is detected by thin layer chromatography (TLC) using a chloroform/methanol/water mixture (60:25:4) as the mobile phase and confirmed as a single species using both $I_2$ and phospray staining (Silica Gel TLC plates, Whatman). The concentration of phytol-P was determined by measuring the amount of free phosphate after hydrolysis with 4 N HCl at 90 °C, as described previously (*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 with E protein or mock E extract (see below), as indicated. Phytol-P was provided in buffer R with 4% DDM; the final concentration of DDM in the total reaction mixture 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 amounts of time. For the 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 6−7 h using an isobutyric acid/$NH_4OH$/water mixture (66:1:33) as the mobile phase. Quantification of the product was done by scraping the spot, extracting with methanol, and measuring the fluorescence intensity. Fluorescence measurements were conducted in a Koala spectrofluorometer (ISS) (excitation at 340 nm, emission at 535 nm), 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 an $A_{550}$ of 0.6 with 1 mM IPTG for 1 h and then harvested by centrifugation (JA-10 rotor, Beckman) at 8K rpm (7000*g*) for 15 min in the cold. The cells were resuspended in 1/100 volume of cold French press buffer and disrupted with a French press. The lysate was cleared of whole cells by centrifugation in a JA-20 rotor (Beckman) at 5000*g* for 10 min in the cold. Membranes were collected by

centrifugation at 38K rpm (130000*g*) for 1 h in the cold, using a type 50.2 Ti rotor (Beckman). To extract MraY activity, membrane pellets were resuspended in buffer R with 1% DDM and stirred for 1 h in the cold. Insoluble material was removed by centrifugation at 50K (100000*g*) for 1 h in a TLA-100.3 rotor (Beckman). The supernatant from this clearing step was used without further treatment for MraY reactions.

## RESULTS

*Overproduction 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 min (Figure 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 (Figure 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 (Figure 3), and $E_{6his}$ protein accumulated to a much higher level (Figure 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 $\mu$M $E_{6his}$ protein (Figure 4C) that was 84% pure. This corresponds to a yield of 27 $\mu$g per liter of induced culture. A mock purification using the empty vector yielded the same background species (compare lanes 3−8 with lanes 9−15 in Figure 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 to quantify 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 $\beta$-galactosidase activity of E−$\beta$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 via SDS−PAGE (Figure 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

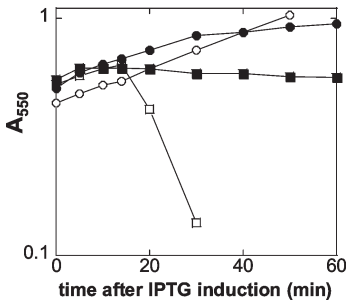


FIGURE 3: $^{Bs}$MraY prevents lysis from overexpression of *$E_{6his}$*. Cultures of BL21(DE3) harboring the indicated plasmids were induced at an $A_{550}$ of 0.5 via addition of 1 mM IPTG and monitored for culture turbidity as described in Experimental Procedures. The pBsMraY-Kan plasmid was induced by addition of 0.2% arabinose 2 min before IPTG was added: (○) pET11a, (□) pETE6his, (●) pBsMraYKan and pET11a, and (■) pBsMraYKan and pETE6his.

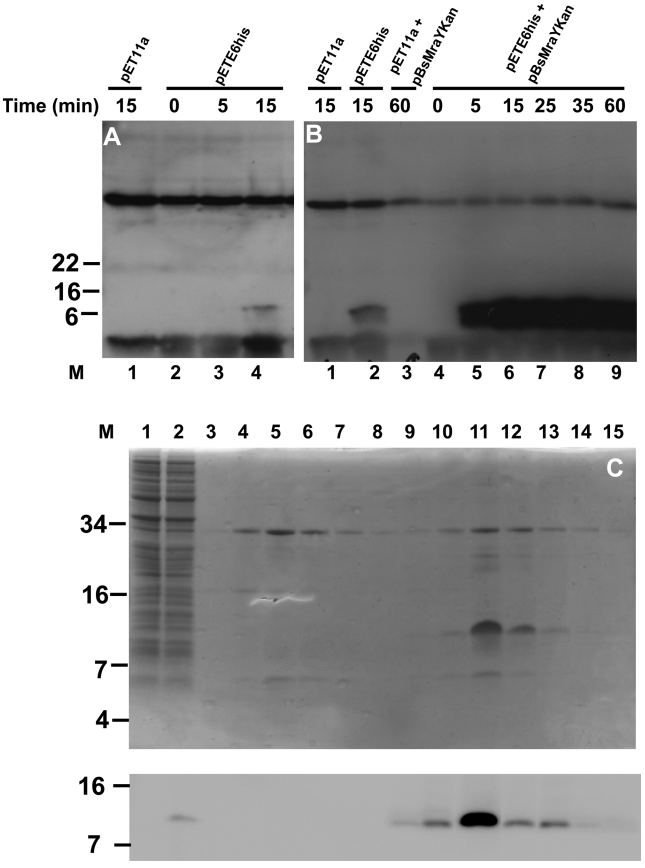


FIGURE 4: Overproduction and purification of $E_{6his}$. (A and B) Accumulation of $E_{6his}$ after induction. Cultures of BL21(DE3) cells harboring either the pETE6his plasmid or the pET11a vector, as indicated, or the pBSMraYKan plasmid, were induced at time zero with IPTG. The cultures were sampled at various times after induction and analyzed by immunoblot, as described in Experimental Procedures. Molecular mass (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, respectively. (B) Lane 1: pET11a, $t$ = 15 min. Lane 2: pETE6his, $t$ = 15 min. Lane 3: pET11a and pBsMraYKan, $t$ = 60 min. Lanes 4−9: pETE6his and pBsMraYKan, $t$ = 0, 5, 15, 25, 35, and 60 min, respectively. (C) Purification of E. Coomassie blue-stained SDS−PAGE (top) and immunoblot (bottom) analysis of steps in $E_{6his}$ purification or mock E purification (see Experimental Procedures), as indicated. M: molecular mass standards. Lanes 1 and 2: whole cell samples, pET11a vector and pETE6his. Lanes 3 and 9: 10 mM imidazole wash fraction, pET11a vector and pETE6his. Lanes 4−8: elution fractions 1−5, respectively, pET11a vector. Lanes 10−15: elution fractions 1−6, respectively, pETE6his.

Table 1: Extraction of $E_{6his}$ from *E. coli* Membranes[a]

| detergent | extraction efficiency (%) | detergent | extraction efficiency (%) |
|---|---|---|---|
| Triton X-100 | 13 | cholic acid | < 5 |
| Tween 20 | < 5 | DHPC | < 5 |
| Nonidet P40 | 18 | EBB | 49 |
| SDS | 70 | saponin | < 5 |

[a] Membranes containing overproduced $E_{6his}$ 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. The concentration of detergents used was 1% for each detergent, except DHPC, the concentration of which was 14 mM (~0.7%).

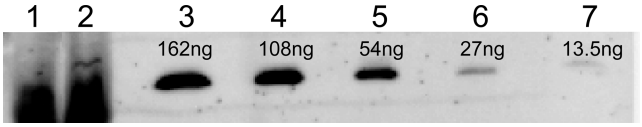


FIGURE 5: Quantification of E in vivo. Immunoblots of membranes from cells lysed by induction of $E_{6his}$, using antibodies against the oligohistidine tag: lane 1, pRW* (vector); lane 2, pRW*$E_{6his}$; lanes 3−7, different amounts of purified $E_{6his}$ as indicated above the E bands.

Table 2: Amounts of E at Lysis in Vivo[a]

| | E (ng) | E (no. of molecules/cell) |
|---|---|---|
| 1 | 17 | 340 |
| 2 | 22 | 440 |
| 3 | 26 | 520 |
| 4 | 28 | 560 |
| average | 23 ± 5 | 465 ± 97 |

[a] The same experiments shown in Figure 5 were repeated four times, and the average number of E molecules per cell at lysis in vivo is shown.

a level of 500 molecules/cell, each functional MraY molecule would have to have a $k_{cat}$ of ~200 $min^{-1}$ to account for the level of murein synthesis in *E. coli* (*26*). This is in reasonable agreement with the $k_{cat}$ of ~320 $min^{-1}$ measured by Al-Dabbagh et al. (*27*) for purified *B. subtilis* 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 overproduction 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 20-carbon analogue of undecaprenol-P (Figure 2). The fluorescently labeled product, phytol-P-P-MurNAc-pentapeptide-DNS, is separated from the reaction mixture by TLC (Figure 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 ± 0.09 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 (Figure 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 (Figure 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 (Figure 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 solubilized MraY was determined with respect to both the lipid and sugar-nucleotide substrates (Figure 9) by measuring MraY activity in the presence of varying concentrations of $E_{6his}$. Experiments with a goal of obtaining 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 noncompetitive inhibitor of MraY with respect to both lipid and sugar-nucleotide substrates, with an average $K_i$ of 0.53 ± 0.12 μM (Figure 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 three classes according to the degree of inhibition at 2.7 μM $E_{6his}$. $MraY_{G186S}$ and $MraY_{V291M}$ are almost as sensitive to E as the wt protein; $MraY_{F288L}$ is, like $^{Bs}$MraY, not detectably inhibited, and $MraY_{P170L}$ and $MraY_{\Delta L172}$ are inhibited to a degree intermediate between the first two classes (Figure 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 superfamily, 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 overproduction 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

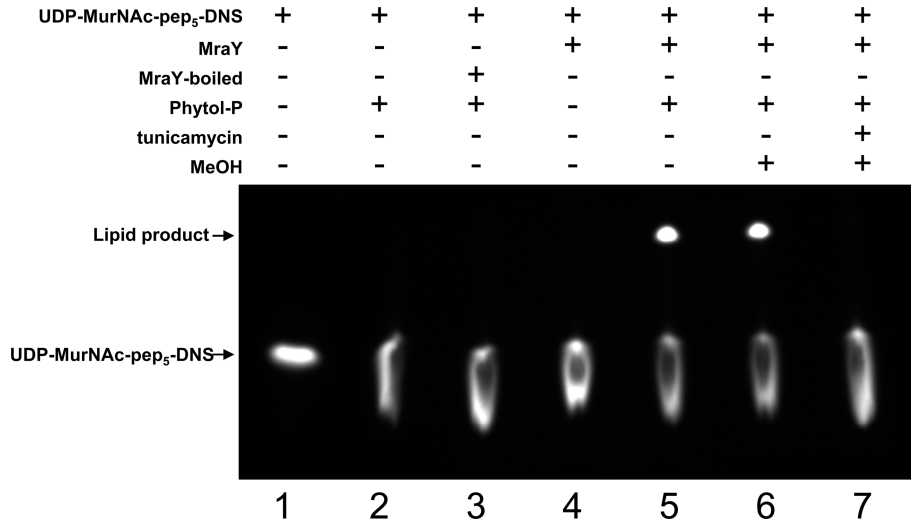


FIGURE 6: Fluorescence-based assay for MraY. Reaction mixtures containing the fluorescent substrate UDP-MurNAc-pentapeptide-DNS (UDP-MurNAc-pep$_5$-DNS) in buffer R (lanes 2−7), supplemented with DDM-solubilized MraY (lanes 4−7) or heat-inactivated MraY (lane 3), phytol-P (lanes 2, 3, and 5−7), tunicamycin in methanol (lane 7), or methanol (lane 6), were incubated at 37 °C for 30 min, quenched by being heated at 100 °C for 2 min, and then analyzed by TLC, as described in Experimental Procedures. Lane 1 contained the UDP-MurNAc-pentapeptide-DNS alone. Reaction components are also listed above the panel, with plus and minus signs indicating inclusion and exclusion, respectively. Positions of the fluorescent substrate and product are indicated.

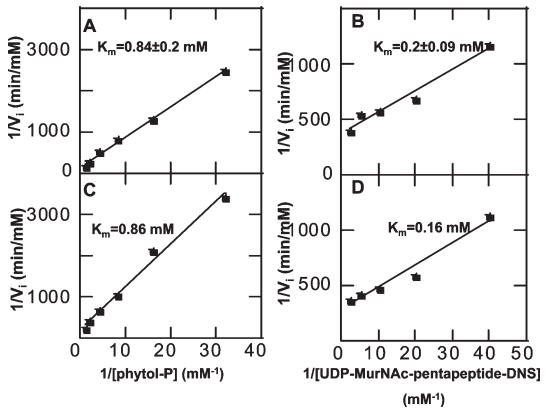


FIGURE 7: Determination of $K_m$ values. (A and B) $K_m$ values of wt MraY for phytol-P and UDP-MurNAc-pentapeptide-DNS. (C and D) $K_m$ values of the MraY$_{F288L}$ mutant for phytol-P and UDP-MurNAc-pentapeptide-DNS. Initial rates were measured as described in Experimental Procedures, first varying the phytol-P concentration with UDP-MurNAc-pentapeptide present at 0.2 mM and then varying the 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 (*30*).

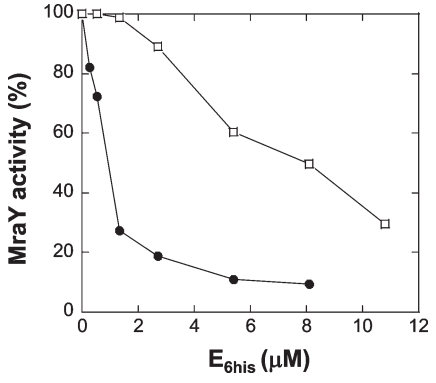


FIGURE 8: Inhibition of both particulate and detergent-solubilized MraY by $E_{6his}$. Activity was measured by the in vitro assay as described in Experimental Procedures. Data are expressed as percentage activity. Elution fraction 2 of a parallel purification of material from cells carrying the vector, pET11a (see Figure 4C, lane 5), served as the null E control (0 $\mu$M $E_{6his}$): (●) detergent-solubilized MraY and (□) membranes containing MraY.

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 noncompetitive 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 α-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

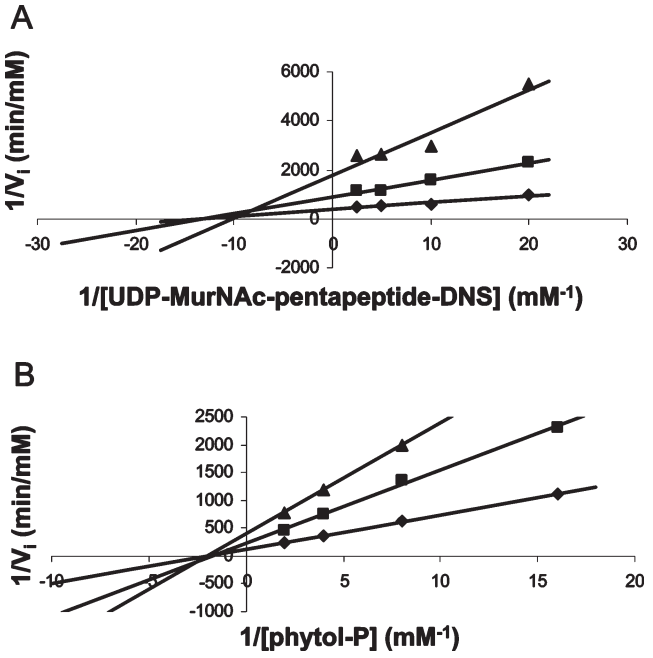


FIGURE 9: $E_{6his}$ is a noncompetitive inhibitor of MraY with respect to both soluble and lipid substrates. Assays were conducted as described in Experimental Procedures at fixed $E_{6his}$ concentrations: (◆) 0 $\mu$M [mock E extract (Figure 4C, lane 5)], (■) 0.7 $\mu$M, and (▲) 1.4 $\mu$M. (A) Varying UDP-MurNAc-pentapeptide-DNS concentration in the presence of a fixed concentration (0.25 mM) of phytol-P. (B) Varying phytol-P concentration in the presence of a fixed concentration (0.2 mM) of UDP-MurNAc-pentapeptide-DNS.

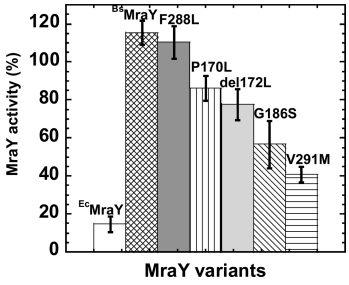


FIGURE 10: Inhibition of MraY mutants by $E_{6his}$. Enzymatic activities of MraY proteins produced from five *mraY* mutant alleles and $^{Bs}$MraY were determined at a fixed $E_{6his}$ concentration (2.7 $\mu$M). Data are expressed as percentage activity.

addition, we have found that E acts in a noncompetitive 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 heteromultimeric 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 *Staphylococcus aureus*, were found to complement *E. coli mraY* defects in vivo (*14*, *41*). The ability of these diverged proteins (43% similarity between $^{Ec}$MraY and $^{Bs}$MraY; 40% between $^{Ec}$MraY and $^{Sa}$MraY; 54% between $^{Bs}$MraY and $^{Sa}$MraY) 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 TMD of E and TMD 5 and 9 of MraY, and this binding results in noncompetitive inhibition of the enzyme by causing a conformational change. The catalytically important aspartate residues of MraY are associated with cytoplasmic loops terminating in TMD 4 (Asp115 and Asp116) and TMD 8 (Asp267) (Figure 1). TMDs almost invariably interact with their adjacent neighbors in primary structure (*42*, *43*), which in this case would include TMD 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 TMD 9 of MraY may localize these residues near the catalytic Asp267, predicted to be at the cytoplasmic interface of TMD 8.

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 noncompetitive 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 TMD 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.

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