Genetic evidence that the bacteriophage ϕX174 lysis protein inhibits cell wall synthesis

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Protein E, a 91-residue membrane protein of ϕX174, causes lysis of the host in a growth-dependent manner reminiscent of cell wall antibiotics, suggesting E acts by inhibiting peptidoglycan synthesis. In a search for the cellular target of E, we previously have isolated recessive mutations in the host gene *slyD* (sensitivity to lysis) that block the lytic effects of E. The role of *slyD*, which encodes a FK506 binding protein-type peptidyl-prolyl *cis-trans* isomerase, is not fully understood. However, E mutants referred to as *Epos* (plates on *slyD*) lack a *slyD* requirement, indicating that *slyD* is not crucial for lysis. To identify the gene encoding the cellular target, we selected for survivors of *Epos*. In this study, we describe the isolation of dominant mutations in the essential host gene *mraY* that result in a general lysis-defective phenotype. *mraY* encodes translocase I, which catalyzes the formation of the first lipid-linked intermediate in cell wall biosynthesis. The isolation of these lysis-defective mutants supports a model in which translocase I is the cellular target of E and that inhibition of cell wall synthesis is the mechanism of lysis.

Most bacteriophages achieve release of progeny virions by causing lysis of the host cell. Double-stranded DNA phages generally encode at least two genes to elicit host cell lysis: an endolysin, which is an enzyme that degrades the cell wall, and a holin, which at a genetically defined time allows endolysin to cross the cytoplasmic membrane and gain access to the cell wall (1). Once endolysin degrades the cell wall, the cell lyses because of its internal osmotic pressure. In contrast, the small single-stranded DNA phage ϕX174 has a single lysis gene *E* of 91 codons (2). Expression of *E* from a multicopy plasmid is sufficient to cause lysis of *Escherichia coli* (3, 4). Gene fusion analysis has indicated that only the amino-terminal 35 aa of the E polypeptide, containing a hydrophobic region thought to be a transmembrane domain (TMD), is required for lytic activity (6, 7). The E protein has no detectable cell wall degrading activity, and given its simple primary structure, it is unlikely to have any enzymatic activity at all. Therefore, it must be promoting cell lysis by a mechanism distinct from direct degradation of the cell wall.

The mechanism by which E causes host cell lysis is controversial. Many models have been proposed to explain its lytic function. E-mediated lysis is strikingly similar to that caused by the antibiotic penicillin. Penicillin causes cell lysis by inhibiting cell wall synthesis at the level of peptide cross-link formation between strands of peptidoglycan. Both E and penicillin require cell growth for lysis, and light and electron microscopy have revealed that both result in lesions at the cell septum as intermediates in lysis (8–11). These observations led to a model in which E targets and inhibits a component of the cell wall synthesis pathway to cause cell lysis. However, it also has been reported that E-mediated lysis of hosts deficient in autolytic enzymes is impaired, leading to the hypothesis that E functions by targeting and activating a component of the *E. coli* autolytic system (12). Moreover, in scanning electron micrographs of cells undergoing E-mediated lysis, Lubitz and coworkers (13) have observed discrete 50- to 200-nm holes located at the cell septum and occasionally at the poles. This observation has led them to propose a model in which the E protein oligomerizes to form a "transmembrane tunnel" spanning the entire cell envelope to release the cytoplasmic contents, including progeny virions (13).

The confused picture for the mechanism of E lysis primarily is caused by the lack of molecular or genetic evidence to support any single model. As a consequence, we have taken a genetic approach to identify host genes required in E-mediated lysis. We previously have isolated recessive mutations in the host gene *slyD* that absolutely block the lethal and lytic effects of E (14). *slyD* encodes an FK506 binding protein-type peptidyl-prolyl *cis-trans* isomerase (PPIase) (15). *In vitro*, PPIases have been shown to catalyze the folding of proteins limited by peptidyl-prolyl isomerization in their folding pathway. In addition, PPIases have been implicated in protein folding *in vivo* (16–19). E has five peptidyl-prolyl bonds, including three in the essential hydrophobic domain. The lysis block in the *slyD* mutant is associated with the failure of E to accumulate in the membrane, suggesting that SlyD is involved in folding or membrane insertion of the lysis protein (T.G.B., unpublished data). *E* mutants that bypass the *slyD* lysis defect were isolated by selecting for ϕX174 plaque formation on a *slyD*-null lawn. These *Epos* mutants all were found to contain the missense changes R3H or L19F, or, in the most efficient plaque formers, both changes (W.D.R., unpublished data). Moreover, the Epos protein with both changes accumulates and lyses a *slyD* host, possibly reflecting the ability of the altered protein to undergo spontaneous folding or membrane insertion, or to access a different folding catalyst. Irrespective of the molecular mechanism by which the *pos* mutations bypass the SlyD requirement, it seems clear that SlyD cannot be the target of E lytic function. Here we describe the results of a mutant selection undertaken to identify this target.

Materials and Methods

Media, Chemicals, and General Methods. LB broth was used for all cultures and agar plates (21). When indicated, media was supplemented with ampicillin, chloramphenicol (Cam), kanamycin (Kan), rifampicin, naladixic acid, and tetracycline at final concentrations of 100, 10, 40, 100, 10, and 10 μg/ml, respectively. Isopropyl β-D-thiogalactopyranoside (IPTG) (Alexis, San Diego, CA) was added to a final concentration of 1 mM. Unless otherwise indicated, all chemicals were purchased from Sigma.

DNA Techniques and PCR. All DNA manipulations were performed according to standard and published procedures (22, 23). F' DNA was isolated by using the Nucleobond AX 20 Kit (Macherey & Nagel) according to instructions. All enzymes were purchased from Promega with the exception of T4 DNA ligase

(Boehringer Mannheim) and Pfu polymerase (Stratagene). Ligation reactions were performed by using the Rapid DNA Ligation Kit from Boehringer Mannheim according to the manufacturer's instructions. Boilates were prepared for chromosomal DNA templates by resuspension of 1 ml of an overnight culture in 100 μl of TE buffer (10 mM Tris·HCl, pH 7.6/1 mM EDTA) and boiling for 5 min. The debris was removed by centrifugation before use. PCR compositions unless otherwise indicated are: 10 mM Tris·HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 0.2 mM in each dNTP, 1.5 mM MgCl$_2$, 0.5 μM in each primer, 3 units of Pfu DNA polymerase, and approximately 200 ng of plasmid DNA or 1 μl of a boilate as template. Unless otherwise indicated, cycling parameters were 94°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min for a total of 25 cycles.

Bacterial Strains and Phage. XL1-Blue (*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *supE*44 *relA*1 *lac* [F′::Tn*10 proA*$^+$*B*$^+$ *laqI*^{q} Δ*(lacZ)*M15]) was used for all plasmid constructions. As indicated, CCX1 (*E. coli* C *slyD1*) (24), Eps4 (CCX1 *mraY*4), Eps15 (CCX1 *mraY*15), Eps39 (CCX1 *mraY*39), RY7281 (CCX1 *zac*::Tn*10kan*), RY7283 (Eps4 *zac*::Tn*10kan*), RY7278 (CCX1 *recA1 srl*::Tn*10*), KL723 (contains F′104 with the 99.8–7.4 min region) (25), CAG12095 (MG1655 *zac*::Tn*10*) (26), CAG12131 (MG1655 *zac*::Tn*10kan*) (26), CQ21 (27), RY7425 (CQ21 *zac*::Tn*10kan zhd*::Tn*10*), RY7426 (RY7425 *slyD1*), RY7427 (RY7425 *mraY*4), RY7428 (RY7425 *slyD1 mraY*4), RY7288 (RY7278 *rif*^{R}), LE392 (*hsdR*514 *supE*44 *supF*58 *lacY*1 *galK*2 *galT*22 *metB*1 *trpR*55), RY3270 (LE392 *slyD1 zhd*::Tn*10* pRY104B), RY3272 (RY3270 *mraY*4 *zac*::Tn*10kan*), RY7285 (RY7278 [F′104 *zac*::Tn*10kan mraY*4]), RY1275 (Hfr KL16 *recA*58 *srl*::Tn*10 ilv val thr spc*^{R} [P1*tsCam*]) (25), and DH5α (ϕ80 *lacZ*DM15 *recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *supE*44 *relA*1 *deoR* Δ*lacZ*U169) were used for mutant selection and characterization. ϕX174*Epos4B*, referred to as ϕX174*Epos*, was isolated as a spontaneous plaque former on a *slyD* mutant lawn (W.D.R., unpublished results). The *Epos4B* allele contains both the R3H and L19F missense mutations and henceforth will be referred to as *Epos*.

Plasmids. pKN104B is a Kan^R derivative of pRY104B and contains *Epos* under control of the *tac* promoter. pRY104B is identical to pRY100 (24) except that it contains *Epos* in place of *E*. pRY104B was digested with *Sca*I to disrupt *bla*, and a blunt-ended Kan^R cassette was inserted. The Kan^R cassette was obtained from *Sma*I digestion of pWJC3 and gel purification (28). pEmycZ and pEposmycZ are identical to pRY100 (24) except they contain c-*myc*-tagged *E* and *Epos*, respectively under *tac* promoter control. pEmycZK is a Kan^R derivative of pEmycZ constructed in the same manner as pKN104B. pE$_{35}$GFP is a pQE30 (Qiagen, Chatsworth, CA) derivative containing the *E*$_{35}\phi$*gfp* fusion under the control of a hybrid *lac*-T5 phage promoter (W.D.R., unpublished results). pmraY contains *mraY* under *tac* control. pmraY4 and pmraY39 are identical to pmraY except they contain the mutant *mraY* allele indicated by the number. The pmraY series of plasmids was constructed by amplifying the desired *mraY* allele from boilates of CCX1 and Eps strains with the primers ForMraY (5′-tatcccgggatccccgccatggaagaggtagtacg-3′) and RevMraY (5′-tatcccgggaagcttgccattcatcattcagactgcc-3′). The PCR products were digested with *Bam*HI and *Hin*dIII and ligated into appropriately digested pJF118 (29). The pMY30 and pMY304 plasmids are pBAD30 (30) derivatives containing *mraY* and *mraY*4, respectively under control of the *araBAD* promoter. The same PCR products used for pmraY construction were digested with *Sma*I and *Hin*dIII and ligated into appropriately digested pBAD30 (30). pSD1100 is a pACYC184 (31) derivative containing *slyD* under control of its native promoter. *slyD* and its upstream promoter region were amplified from the chromosome of MC4100 by PCR using the primers FarForSlyD (5′-cgcggatcctcacgttcgcaaagatgagc-3′) and

RevSlyD (5′-gcggatccagggcgagcgcaagcttgaagaaacgccaccgccaca-3′). The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into appropriately digested pACYC184.

Selection and Screen for *Epos*-Resistant Mutants. A culture of CCX1 pKN104B was grown to an A$_{550}$ of 0.18, and *Epos* expression was induced with IPTG. After lysis was complete (approximately 3.5 h), 0.1 ml of the culture was plated on LB-Kan-IPTG to yield approximately 200 colonies per plate. A total of about 2,000 survivors were isolated and screened for ϕX174*Epos* phage resistance by using cross-streaks. For cross-streaks, approximately 10^7 plaque-forming units were spread down the center of a plate and allowed to dry. Survivor colonies were picked directly from the selection plate and streaked across the spread phage. A streak was scored positive if there was significant and reproducible growth across the phage.

General Bacterial and Phage Methods. Standard bacterial matings were performed essentially as described (21). Triparental matings to generate a merodiploid with *eps*$^+$ on the chromosome and *eps4* on F′104 were performed by mixing 0.5 ml of exponential cultures of KL723 (strain 1), RY7283 (strain 2), and RY7278 (strain 3) and allowing them to stand at 37°C for 5 h. The desired exconjugants were selected by plating dilutions on LB-Kan-tetracycline (Fig. 1). To generate homozygous *eps*$^+$ merodiploids RY7281 was used as strain 2. P1 transductions were performed essentially as described (21). ϕX174*Epos* phage plating was performed as described (24).

Transposon Mutagenesis. MiniTn*cam* transposon mutagenesis was performed on strain RY7285 (the exconjugant selected from triparental matings) by using the delivery phage λNK1324 essentially as described (32) except the transposition mixture contained 0.5 ml of a 30× concentrated exponential culture of RY7285 in LB-IPTG-10 mM MgSO$_4$. Transposon insertions in the F′ were isolated by mating the pool of transposon mutants with RY2788 and selecting on LB-rifampicin-Kan-Cam. Insertions that eliminated the phage-resistant phenotype conferred by the *eps4* allele on the F′ were identified by replica plating for ϕX174*Epos* sensitivity. Replica plating was performed by replicating plates containing about 200 colonies on velvet to plates with and without 10^8 plaque-forming units of ϕX174*Epos*.

Inverse PCR. To identify the location of the transposon insertions that eliminated the dominant phenotype associated with the *eps4* allele on F′104, the phage-sensitive exconjugants described above were mated with DH5α and plated on LB-Cam-naladixic acid. The F′104 DNA was purified and digested with *Rsa*I, which cuts twice in the middle of the mini-Tn*cam* transposon and in many locations in the F′. The digested DNA was circularized by ligation. The ligation-dependent and transposon-specific inverse PCR (33) product, containing transposon sequence and sequence immediately outside of the transposon insertion site, was generated by using the primers TnCam1 (5′-gcggatccagggc-gagcgctcaggctcaggctctccccgtggagg-3′) and TnCam2 (5′-actggcctcaggcatttgag-3′). Approximately 10 ng of ligated F′ DNA digests was used for the template and cycling parameters were 94°C for 30 sec, 50°C for 30 sec, and 72°C for 3 min for a total of 25 cycles. The product was sequenced by using the primer RevEBam (5′-gcggatccagggcgagcgc-3′). The first 20 bases of good sequence outside of the transposon were used to search the *E. coli* genome by using the BLASTN program (www.ncbi.nlm.nih.gov).

DNA Sequencing. Sequencing PCRs contained approximately 50 ng of PCR product, 1.4 μM sequencing primer, 1.3 μl of 5× reaction buffer (400 mM Tris/10 mM MgCl$_2$), and 0.7 μl of Applied Biosystems Big-Dye reaction mixture in a total reaction
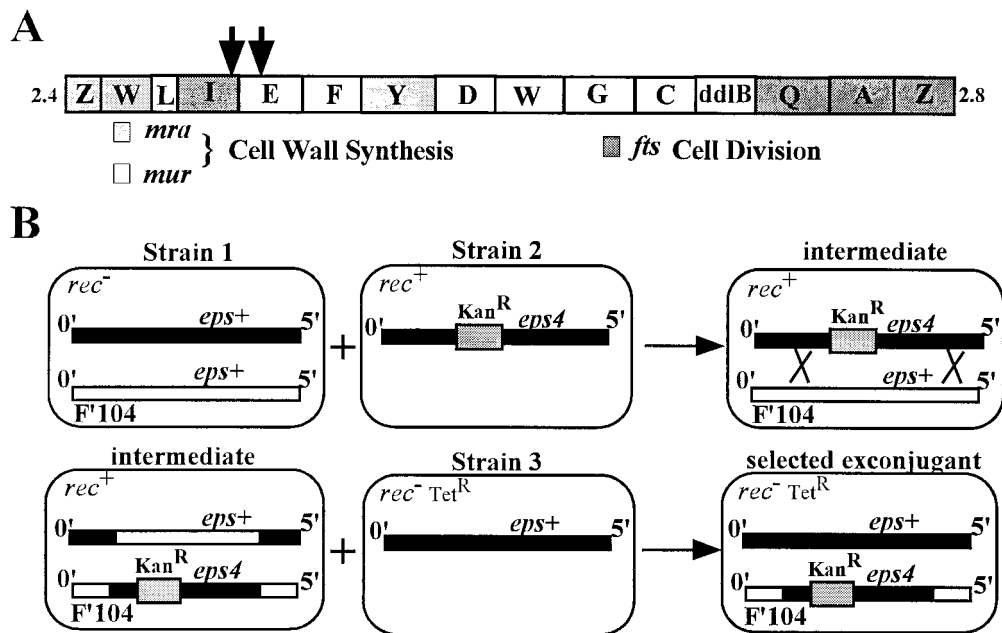
Fig. 1. (*A*) The *mra* locus. Shown is a schematic of the *mra* locus of *E. coli* present at 2 min. The positions of the mini-Tn*cam* insertions that reduce the ability of F′104*eps4* to confer the dominant Eps phenotype are indicated by the arrows. (*B*) Triparental mating strategy to generate merodiploids. Shown is a schematic of the triparental mating strategy used to generate merodiploids with the *eps*⁺ allele on the chromosome and the *eps4* allele on F′104. The thick black line represents the 0- to 5-min region of the chromosome. The open line represents the 0- to 5-min region contained on F′104. Relevant alleles present in the 0- to 5-min regions are indicated above the lines. All other relevant genotypic features are indicated in the top left corner of the cartoon cells. The shaded box represents the transposon Tn*10kan* present at 2 min. The crossed lines represent homologous recombination between the F′ and chromosome. The same strategy was used to generate homozygous *eps*⁺ merodiploids by using a strain 2 with *eps*⁺ linked to the Kan^R transposon marker.

volume of 7 μl. Sequencing was otherwise identical to published methods (23). The entire *mraY* genes of CCX1 and Eps39 were sequenced from PCR products generated from their chromosomal DNA. The PCR products were sequenced by using the primers ForMraY, RevMraY, MYForIN (5′-agttactcttcattt-gtcgccggtggttttgcg-3′), MYRevIN (5′-agttactcttcacacaat-gacgcgcggttccgg-3′), MYForOut (5′-agttactcttcagtgcgtttctggat-tatttcg-3′), and MYRevOut (5′-agttactcttcaaaatacggtcggcata-attgc-3′). This procedure gave good sequence for both strands of the entire gene, and the deletion of codon 172 was the only mutation observed in *mraY39*. For *mraY4* and *mraY15* the sequence of both strands of an approximately 400-bp fragment surrounding the mutation was determined and found to contain the T to C transition in codon 288. This fragment, generated by PCR from the mutant chromosomes using the primers MY-ForIN and MYRevIN, conferred the φX174 *Epos* phage plating phenotype to CCX1 when it replaced the wild-type (wt) fragment of pmraY. This cassette exchange was performed by using the Seamless Cloning Kit (Stratagene) and the primers MYFo-rOut, MYRevOut, MYForIN, and MYRevIN according to the instructions. The fragment was identified because the mutations in *mraY4* and *mraY15* fortuitously destroy an *Eco*RI site contained within it.

Lysis Profiles. Lysis profiles were generated as described (24). Briefly, cultures were grown in LB-ampicillin or LB-Kan-ampicillin at 37°C to an A₅₅₀ of 0.2 to 0.4 and induced with IPTG for expression of the *E* alleles. A₅₅₀ measurements were continued until lysis was essentially complete.

Membrane Topology Prediction. The membrane topology of MraY was predicted by using a hidden Markov model (34) at the TMHMM server (www.cbs.dtu.dk/services/TMHMM-1.0/).

Results and Discussion

Selection for *Epos*-Resistant Mutants. To identify the gene encoding the target of E lysis we selected for spontaneous mutants of

CCX1, *E. coli* C *slyD1*, that gain resistance to *Epos* expression from the plasmid pKN104B. The majority of the selected survivors contained plasmid mutations that eliminated *Epos* expression. To identify host mutants conferring resistance to *Epos* in the high background of plasmid mutants, we screened the survivors by cross-streaking them against the φX174*Epos* phage. Survivors resulting from plasmid mutations are still sensitive to lysis by the phage-encoded *Epos* and are thus phage sensitive. We expected true *Epos*-resistant host mutants to be resistant to *Epos* from the phage as well as the plasmid. Approximately 2,000 survivors were screened, and 17 *eps* (*Epos* sensitivity) mutants scored positive for phage resistance. Two types of *eps* mutants were isolated, 14 with a partial phage-resistance phenotype and three with a tight resistance phenotype. We focused on the three tight mutant strains, Eps4, Eps15, and Eps39, for this investigation. Except for Eps39, the tight mutant strains exhibited no growth or colony morphology phenotypes other than lysis resistance. Eps39 appeared to be somewhat unstable, yielding both small and large colonies upon restreaking, which may indicate the accumulation of suppressor mutations. However, both types of colonies remained resistant to the phage in cross-streaks.

Phenotypic Characterization of the *eps* Mutants. Each of the *eps* mutants were completely resistant to *Epos* expressed from pKN104B (data not shown). To ensure that the pKN104B plasmids from the mutant strains were still functional, their plasmid DNA was isolated and transformed into the parental strain CCX1. All plasmids lysed CCX1 with normal efficiency (data not shown), indicating that the mutants did not survive the selection as a result of a plasmid mutation. The pKN104B plasmid was cured from the mutant strains and the effect of the mutations on the plating efficiency of φX174*Epos* was determined (Table 1). As shown, the plating efficiency of φX174*Epos* was significantly reduced for the *eps* mutants compared with the

Table 1. ϕX174 Epos efficiency of plating (e.o.p.) on mutant strains

Strain	e.o.p.	Plaque size, mm
CCX1	1	3–4
Eps4	0.08	Pin-point
Eps15	0.09	Pin-point
Eps39	0.3	0.5–1
CCX1 pJF118	1	3–4
CCX1 pmraY+	0.8	2–3
CCX1 pmraY4	0.04	0.5–1
CCX1 pmraY39	0.06	0.5–1

parental CCX1 strain. In addition, the average plaque diameter was significantly smaller on *eps* mutant lawns. A one-step growth experiment was performed, and ϕX174*Epos* phage accumulation was observed in the Eps4 mutant strain (data not shown). This indicates that, at least in the case of the *eps4* allele, the mutation affects only lysis and not phage development. Introduction of pSD1100, a medium copy plasmid containing *slyD* under control of its chromosomal promoter, suppressed the ϕX174*Epos* plating defect of the mutants (data not shown; see below).

Genetic Mapping of the *eps* Mutations. Hfr and P1 mapping localized the *eps* mutations to the 2-min region of the *E. coli* chromosome (60% cotransducible with a *Tn*10 marker at 2 min). The 2-min region contains the *mra* locus that is rich in genes for cell wall synthesis and cell division (Fig. 1*A*) (35, 36). To assess the recessive or dominance of the *eps4* allele, a triparental mating of F′104 was used to generate merodiploids of the 0- to 5-min region of the chromosome (Fig. 1*B*). Merodiploids containing two wt copies of the 0- to 5-min region showed normal ϕX174 *Epos* plating efficiency and plaque size. However, merodiploids containing an *eps*⁺ allele on the chromosome and an *eps4* allele on F′104 had the phage-resistant phenotype. Therefore, the *eps4* allele is dominant over wt.

The *eps* Mutations Are Located in *mraY*. To identify the gene containing the dominant *eps4* mutation, a strain carrying F′104*eps4* was mutagenized with mini-Tn*cam* and mated with an *eps*⁺ strain. Exconjugants, carrying transposon insertions in the F′, were screened for ϕX174*Epos* sensitivity by replica plating. The positions of mini-Tn*cam* insertions that eliminated the phage-resistance phenotype associated with the F′ were determined by inverse PCR and sequencing. Two insertions mapping to *ftsI* and *murE* were obtained (Fig. 1*A*), both with partial phage sensitivity, suggesting the insertions were polar on the *eps* locus rather than knockouts. Knowing that the lytic function of E is contained in its hydrophobic membrane domain, we reasoned that its cellular target should be a membrane protein. The first gene downstream of the transposon insertions encoding a membrane protein is *mraY*. We amplified the *mraY* alleles from the parental and mutant strains and inserted them under control of the *tac* promoter in the vector pJF118 (29). As shown in Table 1, basal expression of *mraY* cloned from the mutant strains (pmraY4 and pmraY39) conferred the ϕX174*Epos* plating defect to the parental strain CCX1. On the other hand, basal expression of *mraY* cloned from the parental strain (pmraY+) had only a slight phage-plating defect. Therefore, dominant mutations in *mraY*, a gene encoding a membrane-bound enzyme involved in cell wall synthesis, confer the Eps phenotype. We therefore have renamed the *eps* alleles as *mraY4*, *mraY15*, and *mraY39*. Interestingly, it appears that basal expression of *mraY39* from the plasmid in conjunction with *mraY*⁺ from the chromosome confers a more severe phage-plating defect than the *mraY39* allele alone in the chromosome (compare the efficiency of plating for Eps39 with that of CCX1 pmraY39). We do not currently have an explanation for this observation.

The Lysis Phenotype of the *mraY4* Allele Is General. The dominant *mraY* mutants were isolated by selecting for *Epos* resistance in an *E. coli* C *slyD1* background. We therefore were interested in determining whether the lysis-defective phenotype of the *mraY4* allele is general and whether it depended on the allelic state of E and *slyD*. The *mraY4* allele was transduced into *E. coli* K-12 *slyD*⁺ and *slyD1* strains, and the lysis phenotype was assessed by inducing expression of various plasmid-borne E alleles. The *mraY4* allele severely delayed the lysis of wt E in a *slyD*⁺ background and did not affect the E resistance of the *slyD1* strain (Fig. 2*A*). Interestingly, only the *slyD1 mraY4* double mutant was resistant to *Epos* (Fig. 2*B*). This finding is consistent with the observation that *slyD*, in multicopy, suppresses the ϕX174 *Epos*
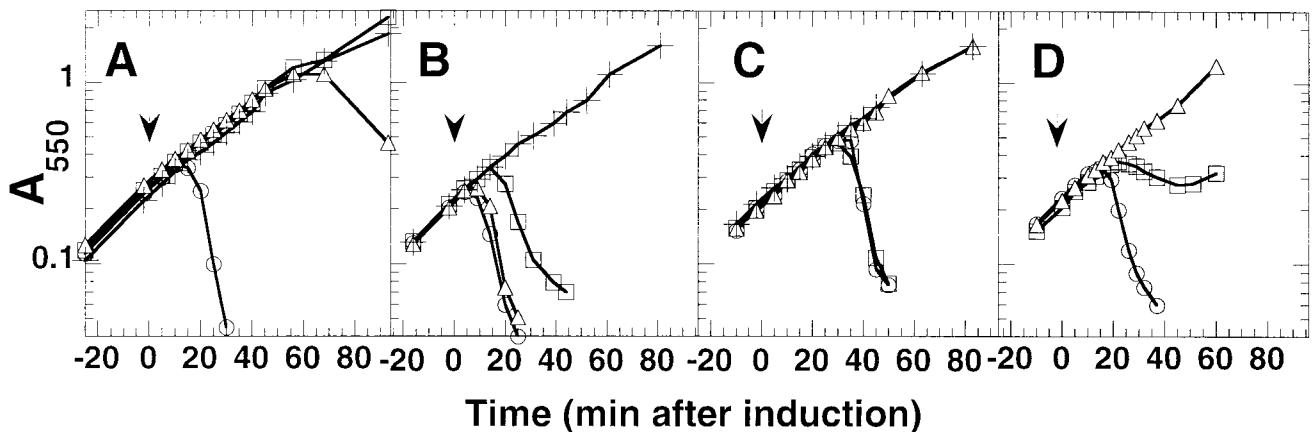

Fig. 2. The *mraY4* allele causes a general lysis defect. (*A*) Lysis profiles from pEmycZ induction in different genetic backgrounds. The allelic state of *slyD* and *mraY* in each strain is as follows: *slyD*⁺ *mraY*⁺ (RY7425) (○), *slyD1 mraY*⁺ (RY7426) (□), *slyD*⁺ *mraY4* (RY7427) (△), and *slyD1* mraY4 (RY7428) (+). The arrow indicates the time of IPTG addition to induce lysis gene expression. (*B*) The strains are the same as indicated in *A* except they contain the plasmid pEposmycZ. (*C*) Same as in *A* except strains contain the plasmid pE₃₅GFP. (*D*) Lysis profiles resulting from increased *mraY* expression. *Emyc* expression was induced from CAG12095 pBAD30 pEmycZK (○), pMY30 pEmycZK (□), and pMY304 pEmycZK (△) at time 0. All cultures were grown in the presence of 0.2% arabinose for constitutive expression of the mraY alleles cloned in the pBAD30 vector.
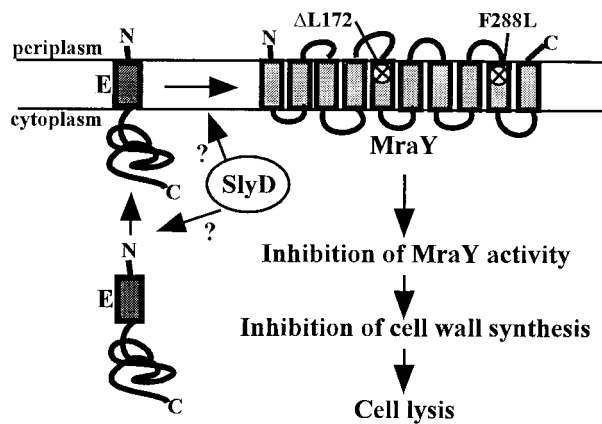
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Fig. 3. Proposed model for the mechanism of E-mediated cell lysis. The E protein is synthesized in the cytoplasm and integrated into the inner membrane. The cytoplasmic SlyD PPIase is required for E protein accumulation, either before or after E reaches the membrane. We propose that as E accumulates in the membrane it interacts with and inhibits MraY. Inhibition of MraY activity would result in the inhibition of cell wall synthesis and cell lysis. The predicted topology of MraY in the membrane is drawn with the positions of the mutations affecting lysis indicated with an x. The topology of the E protein is drawn according to the observation that the C terminus is located in the cytoplasm (R.Y., unpublished data), although it is not known whether the N terminus of E actually traverses the membrane.

plating defect of the original mutants (see above). *Epos* is more lytic than the wt *E* allele in a *slyD*$^+$ strain (compare lysis in Fig. 2 *A* and *B*). Apparently, *Epos* is so efficient in a *slyD*$^+$ strain that the *mraY4* allele has no effect on its ability to cause lysis. However, when the efficiency of *Epos* is reduced because of a *slyD1* null mutation, the presence of the *mraY4* allele completely inhibits lysis (Fig. 2*B*). The *mraY4* allele abolishes lysis by $E_{35}\phi gfp$ irrespective of the allelic state of *slyD* (Fig. 2*C*). Unlike E, lytic chimera, in which the C-terminal two-thirds of the E protein is replaced by a variety of protein domains, are insensitive to the allelic state of *slyD* (W.D.R., unpublished data; ref. 14). This finding has led to criticism that the E fusions are acting by a mechanism distinct from E (37). However, the *mraY4* allele affects the lysis of the $E_{35}\phi gfp$ fusion in addition to E and *Epos*, arguing that all of the *E* alleles, with or without the fusion domains, are acting via the same mechanism and differ only with respect to their *slyD* dependence.

Sequence of the *mraY* Mutations. *mraY* is an essential *E. coli* gene (38). It encodes a 360-aa membrane-bound enzyme, often referred to as translocase I, that catalyzes the formation of the first lipid-linked cell wall precursor, undecaprenol-pyrophospho-*N*-acetylmuramic acid-pentapeptide or lipid I (39). MraY belongs to a ubiquitous superfamily of membrane-bound enzymes that catalyze the transfer of an aminosugar-phosphate from UMP in the cytoplasm to polyisoprenyl-phosphate in the membrane (40). The enzymology of MraY is well established but little is known about its molecular structure (41). MraY has 10 stretches of hydrophobic amino acids predicted to be TMDs and a predicted topology that is supported by recent *bla* fusion analysis (Fig. 3) (42). This topology is also consistent with the topology determined for a hamster enzyme of the same superfamily for which there is also evidence suggesting oligomeric structure (43, 44). Sequencing analysis revealed that both the *mraY4* and *mraY15* alleles have a change creating a F288L substitution near the boundary of predicted TMD 9. The *mraY39* allele was found to have an in-frame deletion of codon 172, located near the boundary of predicted TMD 5 (Fig. 3). Given that the hydrophobic N terminus of the E protein is the lytic

domain, it is not surprising that both *mraY* mutations conferring lysis resistance cause changes in the amino acid sequence of predicted TMDs in MraY. Also, considering that the *mraY* mutants are viable, the mutations must not seriously affect the essential enzymatic function of MraY.

A Model for the Mechanism of E-Mediated Lysis. The isolation of lysis-resistance mutations in *mraY* strongly suggests that E-mediated lysis is caused by an inhibition or derangement of cell wall synthesis. The depletion of MraY from cells causes lysis, as does the inhibition of MraY by the antibiotic mureidomycin (38, 45). Taken together, these data suggest that the target of the E lysis protein is MraY itself (Fig. 3). However, we cannot discount the alternative model that E complexes with undecaprenol-phosphate, making it unavailable as a substrate for MraY. In both cases MraY activity would be depleted from the cell. The simplest idea, that E titrates out the activity of MraY by binding as an inhibitor, leads to the prediction that increasing the cellular MraY concentration would delay or even inhibit lysis. To test this prediction, *mraY* was cloned into a medium copy plasmid under control of the *araBAD* promoter. Expression of *mraY* from this plasmid in conjunction with *E* from the *tac* promoter on a compatible plasmid, severely delayed lysis (Fig. 2*D*). Expression of *mraY4* from the same plasmid completely inhibited lysis (Fig. 2*D*). Thus, as predicted, increasing the level of MraY causes a significant defect in E lysis. The dominance of the *mraY* alleles is also consistent with MraY inhibition. A mutant *mraY* producing a protein that cannot be inhibited by E would be expected to confer dominant lysis resistance over wt *mraY*, producing a protein that is sensitive to E inhibition.

The most likely mode by which E or E fusions could inhibit MraY would be through a direct interaction mediated by contacts between the hydrophobic domain of E and one or more of the TMDs of the oligotopic enzyme. This would explain why the lysis-resistance changes in MraY are localized to putative TMDs. These changes may abolish favorable contacts between E and MraY and alter the interaction of the two proteins.

From the perspective of this model, the failure of E lysis in the *slyD1* background and the failure of the *mraY* mutations to block E or Epos lysis in the *slyD*$^+$ background are related phenotypes pointing to the role of the SlyD PPIase in lysis protein accumulation. In a *slyD* host, E fails to accumulate at all, leaving MraY activity unaffected, whereas in a *slyD*$^+$ host, the presence of active SlyD allows so much E or Epos to accumulate that the reduced sensitivity conferred by the *eps* changes in MraY are overwhelmed and its activity is inhibited (Fig. 3). SlyD could be promoting lysis protein accumulation in a number of ways. It may be directly involved in the folding, stabilization, or membrane insertion of E. Alternatively, E may be unstable unless it is complexed with MraY, and SlyD may promote this interaction, leading to an indirect stabilization of E.

Other Models for E Function. The results reported here are completely inconsistent with the "transmembrane tunnel" model (refs. 13, 37, 46, and 47 and references therein) for E-mediated lysis. In this model, it is proposed that the tunnel, which spans the entire envelope, consists of an oligomer of E protein. Formation of the tunnel is proposed to cause a rapid release of cellular osmotic pressure, resulting in expulsion of the cytosolic contents, including progeny virions (13). A role for SlyD also has been proposed, in which the PPIase activity is involved in catalyzing a dramatic conformational change that leaves the N terminus of E embedded in the inner membrane and externalizes the C-terminal domain from the cytoplasm so it can span the rest of the envelope (46). This makes no sense in view of the isolation of lysis-resistant mutants of *mraY*. The simple primary structure of E and the degeneracy of its C-terminal requirements make it highly unlikely that it could form aqueous pores of such unprec-

edented size. Instead, we believe the large holes reported by Witte *et al.* (13) reflect the consequence of E-mediated inhibition of murein synthesis, which probably manifests itself as weak areas in the envelope. The prevalence of septal locales for these lesions and the established dependence of E-mediated lysis on continued cellular septation probably reflect the fact that the developing septum consists entirely of newly synthesized cell wall (48) and is thus highly susceptible to an interruption of the supply of peptidoglycan precursors. In contrast, during cellular elongation, new murein synthesis occurs at disperse sites in the pre-existing cell wall (5, 48). A lack of cell wall precursors at these sites is thus less likely to be rapidly catastrophic.

Is Cell Wall Synthesis Targeted by All Simple Lytic Phages? The idea that the single lysis gene of the simple lytic phage ϕX174 acts as a "protein antibiotic" not unlike other cell wall synthesis inhibitors suggests that there may be a unifying principle in the two fundamental modes by which bacteriophage achieve host lysis and dispersal of progeny. That is, it is fundamentally necessary to subvert the continuity of the cell wall. This can be done either by elaborating a multigene holin-endolysin system to achieve properly timed degradation of the cell wall (1), or by poisoning cell wall synthesis during cell growth. There are two other known single-gene lysis systems, the *L* gene of group I and II RNA phages and the bifunctional A_2 maturation-lysis gene of the group III and IV RNA phages (20). Neither of these lysis genes are associated with active cell wall degradation. It will be of interest to see whether these single-gene lysis systems also act by attacking the cell wall synthesis machinery, and if so, at which step.

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