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Genetic Analysis of MraY Inhibition by the ϕX174 Protein E

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ABSTRACT

Protein E, the lysis protein of bacteriophage ϕX174, is a specific inhibitor of MraY, the phospho-MurNAc-pentapeptide translocase that catalyzes the synthesis of lipid I in the conserved pathway for peptidoglycan biosynthesis. The original evidence for this inhibition was the isolation of two spontaneous E-resistance *mraY* mutants. Here we report further genetic studies aimed at dissecting the interaction between E and MraY, using a genetic strategy that is facile, rapid, and does not depend on the availability of purified E, purified MraY, or its substrates. This system relies on the ability of *mraY* or its enzymatically inactive D267N allele to protect cells from lysis after induction of a chimeric λ::*E* prophage. Using this approach, the MraY protein from *Bacillus subtilis*, which shares 43% sequence identity with the *Escherichia coli* enzyme, was found to interact weakly, if at all, with E. A potential E binding site defined by transmembrane domains 5 and 9 has been identified by isolating more *mraY* mutants resistant to E inhibition. Genetic analysis indicates that these E-resistant alleles fall into three classes on the basis of the affinity of the encoded proteins for MraY.

IN infections of double-strand DNA phages, host lysis is a strictly regulated, precisely timed, multigenic event, involving up to five proteins, including a holin to permeabilize the cytoplasmic membrane and an endolysin to degrade the cell wall (Young *et al.* 2006). In contrast, host lysis by the much simpler single-strand RNA (ssRNA) and DNA (ssDNA) phages is accomplished by expressing a single gene with no known relationship to any of the lysis genes encoded by more complex phage (Henrich *et al.* 1982; Young and Young 1982; Coleman *et al.* 1983; Karnik and Billeter 1983; Winter and Gold 1983; Bernhardt *et al.* 2002b). There are three unrelated lysis genes encoded by these small phage: *E*, in the prototype microvirus (ssDNA) ϕX174; A_2, in the prototype allolevirus (ssRNA) Qβ; and *L*, in the prototype levivirus (ssRNA) MS2. Although the mechanism of lysis mediated by L remains obscure, it has been established that both E and A_2 operate by inhibiting cytoplasmic steps in cell wall synthesis (Bernhardt *et al.* 2000, 2001a,b).

E has had a prominent role in the history of molecular biology. It was the first gene shown to be completely embedded within another gene in a different reading frame (Sanger *et al.* 1977) (Figure 1) and was the first gene to be subjected to site-directed mutagenesis (Hutchison *et al.* 1978). *E* encodes a 91-amino-acid protein that is encoded by >90% of ϕX174 mRNAs (Hayashi *et al.* 1976) and is localized to the cytoplasmic membrane, presumably by virtue of its putative N-terminal transmembrane domain (TMD) (Altman *et al.* 1983; Bläsi *et al.* 1983). Gene fusion experiments have shown that only the N-terminal 35 amino acids of E, including its putative TMD, are required for its lytic activity (Maratea *et al.* 1985; Buckley and Hayashi 1986). Moreover, EΦβ–galactosidase fusions are lytically active and exhibit β-galactosidase activity, indicating that E has an N-out, C-in topology. We have shown that E causes lysis in growing cells by blocking cell wall synthesis and that this blockage is effected by specific inhibition of MraY, a conserved enzyme in the pathway for murein biosynthesis (Bernhardt *et al.* 2000, 2001a). MraY, also known as translocase I, catalyzes the formation of the precursor lipid I by transferring phospho-MurNAc-pentapeptide from UDP-MurNAc-pentapeptide to undecaprenol-P. MraY has been proposed to have 10 TMDs and to adopt an N-out, C-out topology (Bouhss *et al.* 1999) (Figure 1). Lloyd *et al.* (2004) have shown that aspartate residues at positions 115, 116, and 267 are essential for MraY activity *in vitro*. All three of these residues would reside in cytoplasmic loops of MraY given its predicted topology. D115 and D116 are thought to coordinate the Mg^{2+} ion involved in binding the pyrophosphate moiety of the UDP-MurNAc-pentapeptide substrate, while D267 is predicted to be an active-site nucleophile that attacks its β-phosphate.

In our original study, MraY was identified as the target of E by the isolation of two dominant *mraY* mutations conferring resistance to this lysis protein. One of these

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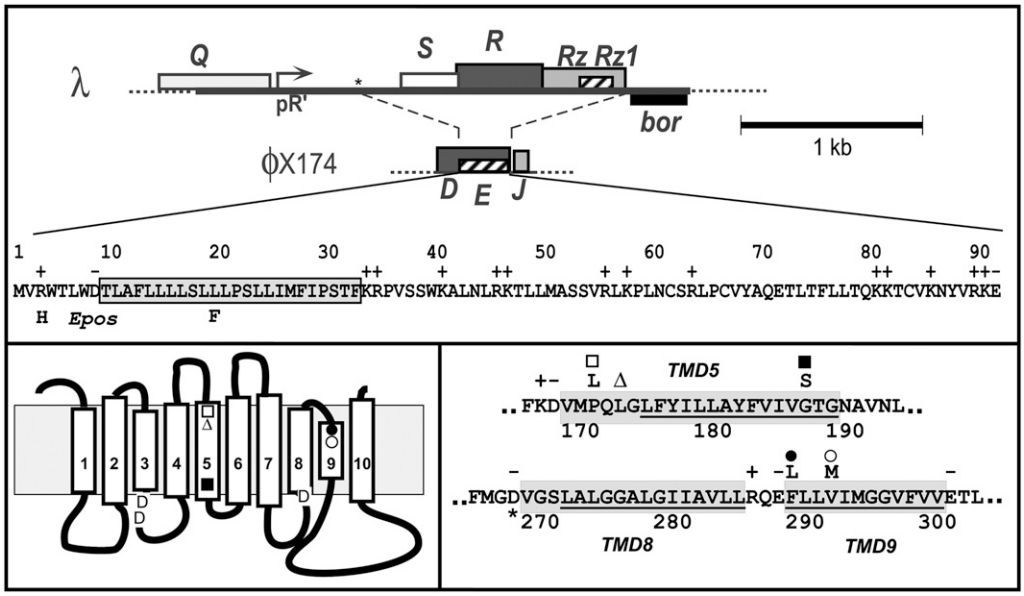

FIGURE 1.—Features of E and MraY. Top: Structure of the lysis gene regions of the phages λ and ϕX174, showing the replacement of *SRRzRz1* with *E* in the chimera λ*E used in this study. The position of a mutation that increases expression of *E* in this chimera is indicated by an asterisk (ZHENG *et al.* 2008) and the two missense changes in Epos are shown at the bottom of the primary structure of E (BERNHARDT *et al.* 2002a). Bottom left: Proposed topology of MraY, based on primary structure analysis using the MemBrain algorithm (SHEN and CHOU 2008), is slightly modified from the topology of BOUHSS *et al.* (1999), mainly in the the placement of TMDs 1, 2, and 5. This topology is still consistent with the results of the β-lactamase fusion study upon which the original topology was based. The beginning and ending residues for each TMD in this model, compared, where different, with those of the previous model, in parentheses, are as follows: TMD1, 25–42 (19–45); TMD2, 70–92 (77–90); TMD3, 97–113; TMD4, 134–153 (134–156); TMD5, 168–188 (174–188); TMD6, 200–220; TMD7, 234–257 (239–251); TMD8, 268–284 (271–284); TMD9, 288–299; and TMD10, 342–358 (343–358). The positions of three conserved Asp residues important for enzyme activity are indicated on cytoplasmic loops at the bottom of TMD3 and TMD8 (LLOYD *et al.* 2004). The positions of *mraY* mutations conferring resistance to E are indicated by: **Δ**, ΔL172, and ●, F288L, reported previously (BERNHARDT *et al.* 2000); and □, P170L; ■, G186S; and ○, V291M. Bottom right: The sequences of two regions of MraY in which E-resistance mutations have been isolated are shown, with the proposed catalytic Asp267 residue indicated by an asterisk. The extent of the TMDs proposed in this study and in the previous work is indicated by shaded rectangles and underlining, respectively.

was a single-codon deletion, ΔL172, in putative TMD5 and the other a missense change, F288L, in putative TMD9 (Figure 1). Here we report studies extending the mutational analysis of the E–MraY interaction and discuss the results in terms of a model for the E-mediated inhibition of MraY.

MATERIALS AND METHODS

Media, chemicals, and general methods: Cultures were grown in standard LB media supplemented with appropriate antibiotics, as described (TRAN *et al.* 2005). Inductions were performed by addition of arabinose to a final concentration of 0.2% and, for lysogenic cultures, beginning 2 min after arabinose induction, by aerating at 42° for 15 min and at 37° thereafter. Lysis profiles were obtained by monitoring A_{550} after induction, as described previously (RAMANCULOV and YOUNG 2001). β-galactosidase activity was assayed according to MILLER (1972b), except that the cells are pelleted and resuspended in assay buffer, as described by M. PRICE-CARTER (unpublished data) (http://rothlab.ucdavis.edu/protocols/beta-galactosidase-3.html). Plasmid DNA isolation, DNA amplification by PCR, DNA transformation, DNA sequencing, and Quikchange (Stratagene) site-directed mutagenesis were performed as previously described (TRAN *et al.* 2005).

Bacterial strains, bacteriophages, and plasmids: The prototroph MDS12 *tonA::*Tn*10* (KOLISNYCHENKO *et al.* 2002; TRAN *et al.* 2005), carrying deletions of all the cryptic prophage sequences of *E. coli*, was used as the host for all lysogenic inductions. The phages λ**E* and λ*Epos* (Figure 1) and the construction of single-copy lysogens have been described (ZHENG *et al.* 2008). The medium-copy plasmid pMY30 has the *E. coli mraY* gene (^{Ec}*mraY*) inserted between the *Sma*I and *Hin*dIII sites of pBAD30 (GUZMAN *et al.* 1995) placing it under the control of the p_{araBAD} promoter (BERNHARDT *et al.* 2000). The plasmid pBAD30-BsMraY was constructed similarly except the *mraY* gene was from *B. subtilis* W23 (nt 1587210–1588202 of the *B. subtilis* genome). A strain with a chromosomal Δ*mraY* was constructed using the protocol of LINK *et al.* (1997). Briefly, the plasmid pKOMY3 was constructed by inserting a DNA fragment spanning nt 95015–98343 of the *E. coli* K-12 genome into the unique *Bam*HI site of pKO3, a vector with a *ts*-replicon. This construct carries *mraY* as well as portions of the upstream and downstream genes *murF* and *murD*. Next the plasmid pKOMY3ΔmraY was constructed, in which the entire *mraY* sequence was deleted (nt 96025–97051 of the *E. coli* genome), leaving 1 and 1.3 kb of homology upstream and downstream of Δ*mraY*, respectively. The strain RY3316 was constructed from MG1655 (*F*$^-$ *ilvG rfb50 rph1*; obtained from the *E. coli* Genetic Stock Center http://cgsc.biology.yale.edu/) by exchanging the deletion from pKOMY3ΔmraY into the chromosome, as described (LINK *et al.* 1997). The strain RY3321, which is RY3316 *recA srl::*Tn*10* pKOMY3, was constructed by P1 transduction and used as the host strain for all complementation experiments. To test the ability of each allele of *mraY* to functionally replace the wild-type (wt) gene in *E. coli*, we first placed it under the control of the *ara* promoter in the vector pBAD30 and transformed the resulting plasmid into RY3321. The ability of the transformants to grow at 42° in the presence, but not the absence, of arabinose was taken as proof that the *mraY* gene on the pBAD30 plasmid was able to complement a chromosomal *mraY* deletion.

Selection of *mraY* mutants resistant to E: Mutants resistant to E-mediated lysis were isolated as previously described

(Bernhardt *et al.* 2000), except that the cells were mutagenized with ethylmethanesulfonate (EMS), essentially as described by Miller (1972a), prior to the selection. The only differences in the protocol used here were that the exposure to EMS was limited to 15–30 min, instead of 2 hr, and that, after the EMS treatment, the cells were washed twice in minimal salts, grown in LB overnight, and stored at −80° after addition of dimethylsulfoxide (85 μl/ml of culture). Individually treated cultures were tested for the frequency of rifampicin resistance as a measure of mutagenesis. Cultures treated for 15 and 30 min exhibited ~60-fold and 100-fold increases in rifampicin resistance, respectively, and were subcultured and used for selection.

Detection of MraY: Bethyl Laboratories (Montgomery, TX) prepared the antibody used for detecting MraY by affinity purification of antisera raised against the peptide RGQRIFRMAPIHHHYEL (residues 314–330 of MraY). For the detection of MraY, logarithmic cultures of MDS12 *tonA::*Tn*10* were induced at A_{550} = ~0.6. After 1 hr, cells were harvested by centrifugation and 1 A_{550} unit was analyzed by SDS–PAGE on a 12% separating gel and immunoblotting, as described (Zheng *et al.* 2008).

RESULTS

Overexpression of active and inactive alleles of *E. coli mraY* protects against E-mediated lysis: Since E is an inhibitor of MraY, it seemed likely that the overexpression of *mraY* would prevent E-mediated lysis. This hypothesis was tested in a "protection assay" using a host with a copy of the *mraY* gene on the chromosome carrying the *E. coli mraY* gene (^{Ec}*mraY*) on an arabinose-inducible plasmid, pBAD30 in *trans* to a thermally inducible λ prophage, λ**E*, in which the lysis gene cassette is replaced by the *E* gene (Zheng *et al.* 2008). When a culture of this strain was sequentially induced with arabinose and a thermal shift in early logarithmic phase (A_{550} = 0.2), the ^{Ec}*mraY* plasmid had no effect on lysis (Figure 2). However, when cultures were induced at a higher culture density, lysis was prevented. Presumably, this reflects increased ^{Ec}*mraY* expression from the plasmid at higher culture densities because of higher cAMP levels and the catabolite-sensitive character of the arabinose promoter of pBAD30 (Guzman *et al.* 1995). This interpretation is supported by the finding that the *lacZ* expression is 2.5-fold higher at A_{550} = 0.5 than at A_{550} = 0.2 (not shown).

Next, we tested a catalytically inactive allele of ^{Ec}*mraY* for its ability to similarly prevent lysis of the induced λ*E lysogen. For this experiment, we used the ^{Ec}*mraY*$_{D267N}$ allele, which encodes an inactive protein thought to be defective because the D267N missense change eliminates a putative active-site nucleophile (Lloyd *et al.* 2004). Once again, arabinose induction at low cell densities had no effect on lysis, but at higher cell densities, lysis was completely blocked (Figure 2). This suggests that, if produced in sufficient quantity, an inactive MraY protein can bind enough of the E protein produced by the induced λ*E lysogen to spare functional MraY, produced from the chromosomal *mraY* gene, from E-mediated inhibition.

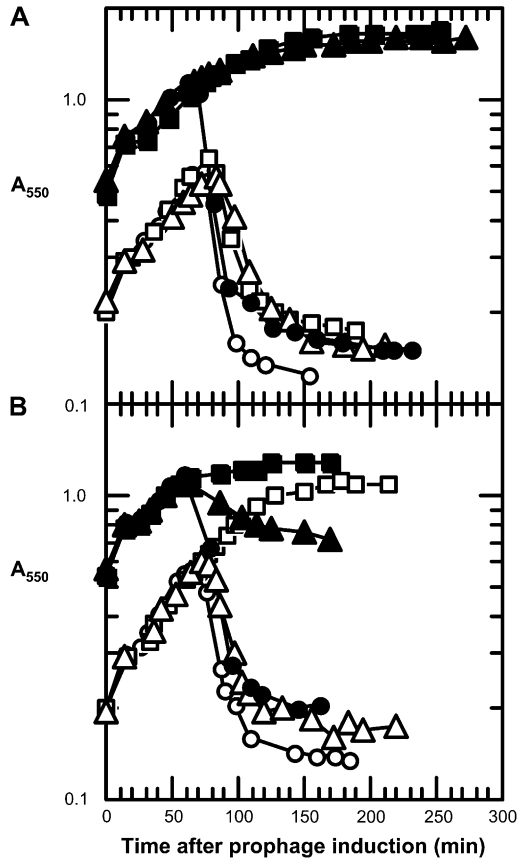

Figure 2.—Induction of plasmid-borned *mraY* alleles can protect against E-mediated lysis. Cultures of MDS12 *tonA::*Tn*10* (λ*E), bearing derivatives of the plasmid pBAD30 carrying the indicated alleles of *mraY* of *E. coli* (A) or *B. subtilis* (B) were induced at either A_{550} = 0.2 (open symbols) or A_{550} = 0.5 (solid symbols) and monitored for culture turbidity, as described in materials and methods. ○ and ●, pBAD30 vector; □ and ■, pBAD30 carrying the wt *mraY* gene; and △ and ▲, pBAD30 carrying the inactivated gene (^{Ec}*mraY*$_{D267N}$ or ^{Bs}*mraY*$_{D231N}$).

A heterologous MraY protein does not interact with E: Since the MraY proteins from gram-positive bacteria diverge significantly when compared to ^{Ec}MraY, the specific protein–protein contacts necessary for E-sensitivity might not occur with enzymes from the former. In fact, it has been reported that the cloned *E* gene is not lytic when expressed in *Staphylococcus carnosus* (Halfmann *et al.* 1993). To assess the ability of the plasmid-based system to discriminate between MraY proteins on the basis of their interaction with E, we decided to repeat the experiments described above using plasmids carrying active and inactive alleles of the *mraY* gene from *B. subtilis* (^{Bs}*mraY*). As a first step, we tested the ability of the ^{Bs}*mraY* gene to complement a chromosomal deletion of *mraY*. In this experiment, the only source of MraY protein is from the transformed plasmid, unlike the protection assay where the chromosomal *mraY* is kept intact for testing the ability to protect by the enzymatically inactive allele. As can be seen in Figure 3, the essential

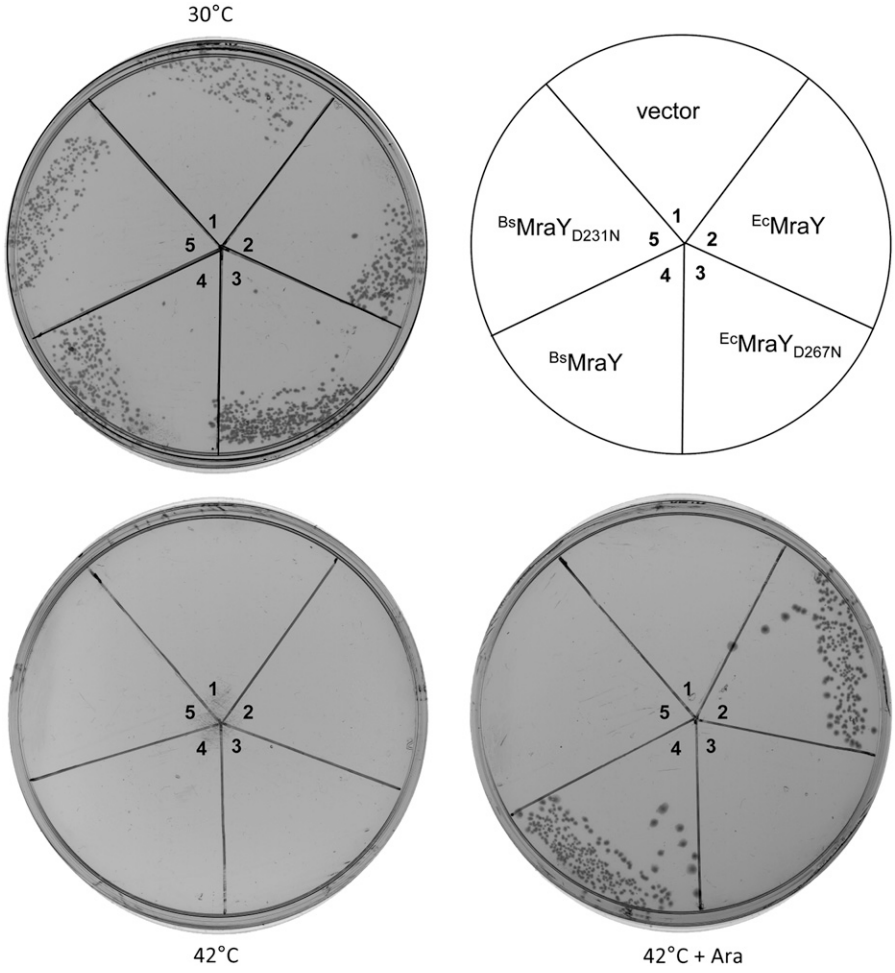

FIGURE 3.—*^{Bs}mraY* complements Δ*^{Ec}mraY*. RY3321, which has a chromosomal deletion of *mraY* and carries the wt *^{Ec}mraY* gene on a low-copy, *ts*-replicon, was transformed with the indicated plasmids and tested for growth under the indicated conditions, as described in MATERIALS AND METHODS.

function(s) of MraY in *E. coli* can be fulfilled by the *B. subtilis* enzyme. Since sequence alignment indicates that Asp231 of the ^{Bs}MraY is equivalent to the proposed catalytic Asp267 of the ^{Ec}MraY (LEHRMAN 1994; AL-DABBAGH *et al.* 2008), we tested both *^{Ec}mraY$_{D267N}$* and *^{Bs}mraY$_{D231N}$* genes for their ability to complement the *mraY* deletion. As can be seen in Figure 3, neither allele allowed cell growth at the restrictive temperature, consistent with a catalytic role for the altered aspartate residues protein.

We next examined the ability of *^{Bs}mraY* and *^{Bs}mraY$_{D231N}$* to prevent lysis of an induced λ*E lysogen. In contrast to what was observed with *^{Ec}mraY*, the ability of the active allele, *^{Bs}mraY*, to block E-mediated lysis was independent of culture density (Figure 2) suggesting that fewer molecules of the Bacillus enzyme are required to protect cells from lysis after induction of λ*E. However, overexpression of the catalytically inactive allele, *^{Bs}mraY$_{D231N}$*, afforded little or no protection against E-mediated lysis in this system. Taken together, these results suggest that the active *B. subtilis* enzyme, even at the lower level of production, can provide sufficient lipid I, despite inhibition of the host MraY by E, but the inactive ^{Bs}MraY, even at the higher level of expression, cannot titrate out E and thus allow lipid I production by the host MraY. Thus, in our protection assay, the *^{Ec}mraY* and *^{Bs}mraY* genes are useful as controls encoding proteins that, respectively, do and do not interact strongly with E. For the experiments described below, the ability of the inactivated version of any *mraY* allele to protect against lysis is taken as evidence for the ability of its product to bind E and thus spare the host MraY activity.

The E-resistant alleles of *^{Ec}mraY* encode proteins with different apparent affinities for E: The original E-resistant mutants were obtained by inducing a plasmid-borne allele of *E* and then screening the spontaneous survivors for resistance to ϕX174 (BERNHARDT *et al.* 2000). More than 99% of the survivors harbored alterations in the *E*-plasmid, and only two ϕX174^{R} alleles of *mraY* were found, ΔL172 and F288L. To increase the pool of ϕX174^{R} alleles, we used EMS mutagenesis to increase the total frequency of survivors by ~20-fold. Every phage-resistant isolate was found to have a missense change in the *mraY* gene. However, from three independent mutagenesis pools in which a total of 13 mutants were sequenced, only three more alleles were obtained, all multiple times (not shown). Together with the original mutants used to identify MraY as the target

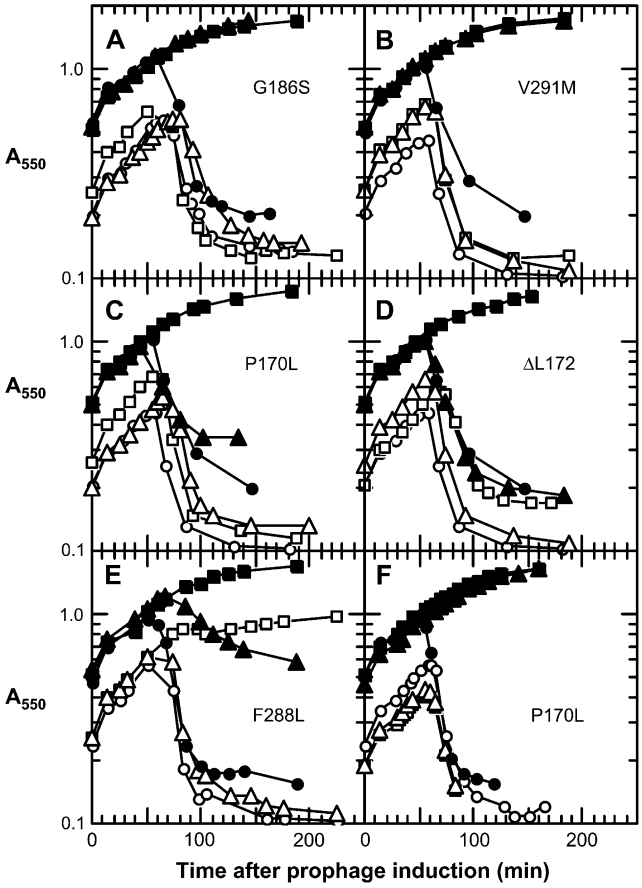

FIGURE 4.—E-resistant alleles of *mraY* show different protection against E-mediated lysis. Inductions and symbols are the same as in Figure 2, except that the indicated ^{Ec}*mraY* allele is used; in F, the prophage is λEpos. ○ and ●, pBAD30 vector; □ and ■, pBAD30 carrying *mraY*; and △ and ▲, pBAD30 carrying the inactivated gene ($mraY_{D267N}$).

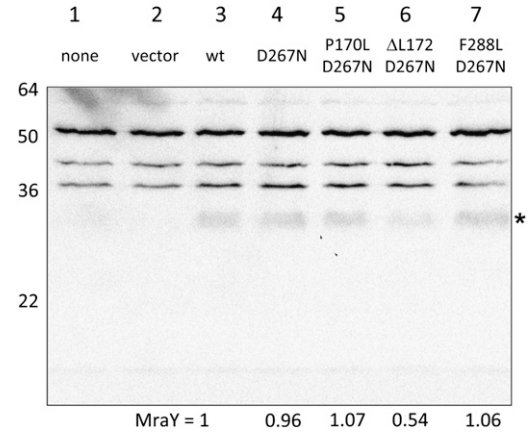

FIGURE 5.—Accumulation of MraY proteins. Membranes from induced cultures bearing no plasmid (lane 1), pBAD30 (lane 2), or pBAD30 carrying the indicated allele of ^{Ec}*mraY* (lanes 3–7) were analyzed by immunoblot with antibodies raised against a peptide of MraY. The position of molecular mass standards are indicated to the left and the relative amount of MraY, by integration of the band indicated by an asterisk, is given at the bottom of lanes 4–7, relative to the amount in lane 3.

of E, the five E-resistance mutations mapped to only 2 of the 10 TMDs of MraY (Figure 1). The clustering of these mutations in the 2 TMDs and their repeated isolation suggested that this mutant selection was at or near saturation.

We examined the behavior of the five E-resistant alleles of ^{Ec}*mraY* in our protection assay. As can be seen in Figure 4, these alleles fall into three classes. Two of these *mraY* mutants, G186S and V291M, are indistinguishable from the wild type. In their active or inactive (D267N) forms, neither protects at low culture density but both do at high culture density. Alleles encoding the P170L and ΔL172 variants protect only in their active forms and only at high culture density. Finally, the ^{Ec}MraY$_{F288L}$ protein appears to be similar to ^{Bs}MraY in that protection is observed only with the active enzyme but occurs at both low and high culture densities. Although the low levels of MraY even from the plasmid-borne alleles preclude accurate quantitation by immunoblot, nevertheless it is clear that at least for the $mraY_{P170L,D267N}$ and $mraY_{F288L,D267N}$ alleles, the amount of MraY protein in these protection experiments is as high as or higher than the parental $mraY_{D267N}$ (Figure 5).

MraY^{P170L} interacts with Epos more strongly than E: The alleles of ^{Ec}*mraY* that provide resistance to E were originally selected using a plasmid encoding the *Epos* gene (BERNHARDT *et al.* 2000). This was necessary because otherwise frequent knockout mutations in *slyD*, which encodes an abundant cytoplasmic peptidyl-prolyl isomerase required for the stability of the E protein, would overwhelm the selection for E insensitivity (MARATEA *et al.* 1985; ROOF *et al.* 1994; BERNHARDT *et al.* 2002a). The *Epos* allele does not require *slyD* for function. The protein encoded by the *Epos* gene has two missense changes, R3H and L19F (Figure 1). For this reason, we repeated the protection assays using a λEpos lysogen. The only significant difference from the experiments using the λ*E lysogen was found with the $^{Ec}mraY_{P170L}$ plasmid. While the inactive form (D267N) of ^{Ec}MraY$_{P170L}$ did not protect against lysis by E under any condition tested, it did provide complete protection against Epos at high culture densities (Figure 4). The simplest interpretation of this result is that ^{Ec}MraY$_{P170L}$ binds Epos more tightly than E.

DISCUSSION

Genetic systems for assessing *mraY* function and interaction with E: Here, we present two genetic systems for the further analysis of MraY and its interaction with E, the lysis protein of ϕX174. First, using a host with a deletion of *mraY* on the chromosome and a functional copy of *mraY* on a *ts*-replicon, we were able to test the ability of any given *mraY* allele to substitute for the

TABLE 1

The ability of plasmid-borne *mraY* alleles to protect against E-mediated lysis defines different levels of E-binding

MraY protein	Position of E-resistance mutation	Protection at $A_{550} = 0.2$	Protection at $A_{550} = 0.5$	Apparent affinity for E
MraY		No	Yes	+++
$MraY_{D267N}$		No	Yes	
BsMraY		Yes	Yes	−/+
$BsMraY_{D231N}$		No	No	
$MraY_{G186S}$	TMD5	No	Yes	+++
$MraY_{G186S,\ D267N}$		No	Yes	
$MraY_{V291M}$	TMD9	No	Yes	+++
$MraY_{D267N,\ V291M}$		No	Yes	
$MraY_{\Delta L172}$	TMD5	No	Yes	+
$MraY_{\Delta L172,\ D267N}$		No	No	
$MraY_{P170L}$	TMD5	No	Yes	+
$MraY_{P170L,\ D267N}$		No	No	
$MraY_{F288L}$	TMD9	Yes	Yes	−/+
$MraY_{D267N,\ F288L}$		No	No	

^{Ec}*mraY* gene. Somewhat unexpectedly, we found that the highly divergent ^{Bs}*mraY* gene was able to complement a chromosomal ^{Ec}*mraY* deletion. Given the high degree of divergence in the primary structures of ^{Ec}MraY and ^{Bs}MraY, this argues that the single, essential function of MraY is to convert UDP-MurNAc-pentapeptide into lipid I. It has been suggested that MraY might participate in the formation of a multienzyme complex or "machine" that is essential for the biosynthesis of peptidoglycan (Bugg *et al.* 2006; Mendel *et al.* 2006; Bouhss *et al.* 2008). While our results do not rule out this possibility, they do suggest that MraY is, at best, a peripheral and not essential for the assembly of such a machine. Finally, we found that the $^{Ec}mraY_{D267N}$ allele was unable to complement the chromosomal *mraY* deletions, providing additional support for the proposal that Asp267 is an essential residue for ^{Ec}MraY, as reported by Lloyd *et al.* (2004). That this system provides a robust, low-background readout on solid medium for functional determination makes it ideally suited for high-throughput analysis of randomly mutated *mraY*, which, to date, has been subjected only to limited site-directed mutagenesis of conserved residues (Lloyd *et al.* 2004). The adjustability of the p_{araBAD} vector, using alleles of *pcnB* to alter copy number (Lopilato *et al.* 1986) and numerous agents that exert different levels of catabolite repression also may allow this system to be used for a chemical biology approach in screening for small molecule inhibitors of MraY.

In a second type of assay, the ability of a plasmid-borne allele of *mraY* under p_{araBAD} control is tested for its ability to prevent the lysis by an induced λ*E lysogen. The chromosomal *mraY* gene of the host is left intact, so that even enzymatically inactive *mraY* alleles could be examined for their ability to protect against E-mediated lysis. When ^{Ec}*mraY* was present on the plasmid, we found that inductions of a culture in early logarithmic phase had no effect on lysis, but when cultures were allowed to grow to a higher culture density before induction, lysis was prevented. Presumably, this dependence on culture density reflects the 2.5-fold higher level of expression at high culture density from the catabolite-sensitive p_{araBAD} promoter in the plasmid vector. Essentially identical results were obtained when the $^{Ec}mraY_{D267N}$ allele was present on the plasmid. We interpret this as indicating that the inactive $MraY_{D267N}$ protein bound E and, thus, spared a smaller pool of active MraY produced from the chromosomal *mraY* gene from E-mediated inhibition. Strikingly different results were obtained using plasmids carrying ^{Bs}*mraY* or a variant encoding an inactive protein, $^{Bs}mraY_{D231N}$. First, the protection seen with ^{Bs}*mraY* was independent of culture density, suggesting that the ^{Bs}MraY protein protected against E-mediated lysis, even when present at low levels, by catalyzing the formation of lipid I. Furthermore, in contrast to what was seen with $^{Ec}mraY_{D267N}$, induction of $^{Bs}mraY_{D231N}$ did not block lysis at either high or low cell densities. Together, these results indicate that the ^{Bs}MraY protein has very low affinity, if any, for E. Thus, the protection assay we have developed is a genetic tool that can be used to distinguish between genes encoding MraY proteins with differing apparent affinities for E.

Next, we assessed the behavior of five ^{Ec}*mraY* alleles that provide resistance to ϕX174 in our protection assay (Figure 4; Table 1). Two of these alleles were previously identified (Bernhardt *et al.* 2000), while the isolation of the other three is described in this study. The protection assay allowed these five alleles to be divided into three classes, on the basis of their apparent affinity for E. The G186S and V291M variants behaved identically to

wild-type ^{Ec}*mraY*, in that their catalytically active and inactive forms protect at the higher expression level, but not at the lower expression level (Figure 4, A and B). In contrast, the F288L mutant was indistinguishable from ^{Bs}*mraY*, protecting at low expression when catalytically active but unable to protect in the catalytically inactive form even when produced at the higher level (Figure 4E, open squares). We interpret this as indicating that F228L abrogates or severely reduces E binding, because the active form can provide lipid I even in the presence of excess E and an excess of its inactive form does not titrate E. The other two alleles, P170L and ΔL172, exhibited an intermediate behavior, since their active forms resemble wt ^{Ec}*mraY* in requiring the higher expression level to prevent lysis, but their inactive forms, like ^{Bs}MraY$_{D231N}$, cannot titrate E when overexpressed (Figure 4, C and D, solid triangles). Thus, in this interpretation, P170L and ΔL172 would have an intermediate affinity for E. All five of the ϕX174^{R} alleles of *mraY* were initially isolated by selecting for cells that survived the induction of the cloned *Epos* gene, which has two changes, R3H and L19F, relative to wild-type *E*. Thus, it was satisfying to find that the protection assay indicated that one of the ϕX174^{R} variants, ^{Ec}MraY$_{P170L}$, interacts more strongly with Epos than it does with E. Although preliminary in nature, this observation might indicate that one or both of the residues altered in Epos interacts with Leu170 of ^{Ec}MraY$_{P170L}$. Moreover, since all five E-resistance mutations map to predicted TMDs 5 and 9 of MraY, it is tempting to speculate that they interact directly with the single TMD of E. In the continued absence of any structural information about MraY, using a genetically malleable probe like E may be an effective way to make progress toward mechanistic understanding. From this perspective, we note that the detailed topology that we have presented for MraY (Figure 1) differs from that proposed by Bouhss *et al.* (1999) in the positions of the TMDs, most specifically to allow the sites that give rise to E resistance to be contained within domains predicted to span the bilayer.

Implications for the mechanism of E inhibition: The ability of ^{Bs}*mraY* to complement chromosomal Δ*mraY* casts doubt on the notion that MraY plays an integral role in the formation of a multiprotein machine required for murein synthesis and suggests, instead, that its sole essential role is to catalyze the formation of lipid I. This perspective is also inconsistent with the model proposed by Mendel *et al.* (2006) where E acts by binding MraY and preventing its incorporation into such a complex. Our results do put constraints on models for the E-mediated inhibition of MraY. First, the ability of modest increases in expression of *mraY* to block E-mediated lysis indicates that E does not function catalytically, like some bacteriocins of approximately the same size. Host lysis by λ*E occurs in approximately the same time scale after infection as occurs with ϕX174, so the level of E produced is likely to be comparable in the two cases. This suggests that ϕX174 does not produce E in large excess over its target, MraY, presumably because the phage never encounters situations where the level of MraY is dramatically different. Together, the protection conferred by the catalytically inactive protein in sparing the chromosomal MraY and the existence of three classes of E-resistant MraY mutants may indicate that the ϕX174^{R} alleles of *mraY* encode proteins with different affinities for E. In this view, the F288L mutant is resistant because it binds E poorly, whereas the G186S and V291M mutants bind E with an affinity that is not distinguishable, at least in our assay, from that of the wt protein. These classes resemble the different classes of inducer insensitivity that have been observed in the Lac repressor, in which some mutations block inducer binding but others interfere with the inducer-mediated conformational change (Pace *et al.* 1997). It will be interesting to exploit this system to select *E* mutants that overcome the *mraY* mutations, with the aim of using allele-specific suppression to map out point-to-point interactions between E and MraY.

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