Shared requirements for key residues in the antibiotic resistance enzymes ErmC and ErmE suggest a common mode of RNA recognition

Sebastian J. Rowe, Ryan J. Mecaskey, Mohamed Nasef, Rachel C. Talton, Rory E. Sharkey, Joshua C. Halliday, and Jack A. Dunkle

From the Department of Chemistry and Biochemistry, University of Alabama, Tuscaloosa, Alabama, USA

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Erythromycin-resistance methyltransferases are SAM dependent Rossmann fold methyltransferases that convert A2058 of 23S rRNA to m^6A2058. This modification sterically blocks binding of several classes of antibiotics to 23S rRNA, resulting in a multidrug-resistant phenotype in bacteria expressing the enzyme. ErmC is an erythromycin resistance methyltransferase found in many Gram-positive pathogens, whereas ErmE is found in the soil bacterium that biosynthesizes erythromycin. Whether ErmC and ErmE, which possess only 24% sequence identity, use similar structural elements for rRNA substrate recognition and positioning is not known. To investigate this question, we used structural data from related proteins to guide site-saturation mutagenesis of key residues and characterized selected variants by antibiotic susceptibility testing, single turnover kinetics, and RNA affinity binding assays. We demonstrate that residues in α4, α5, and the α5-α6 linker are essential for methyltransferase function, including an aromatic residue on α4 that likely forms stacking interactions with the substrate adenosine and basic residues in α5 and the α5-α6 linker that likely mediate conformational rearrangements in the protein and cognate rRNA upon interaction. The functional studies led us to a new structural model for the ErmC or ErmE-rRNA complex.

Posttranscriptional modification of RNA is ubiquitous throughout the three domains of life. Methylation and pseudouridylation of rRNA is widespread, whereas nucleotides in tRNA can be methylated or thiolated or contain amino acid additions (threonylation) (1). The structure, dynamics, and chemistry of RNA can be altered by posttranscriptional modification (1).

The physiological effects of RNA modification are known in many cases. In eukaryotes, the presence of m^6A in mRNA can affect splicing, translation initiation, and mRNA decay (2). Modifications in the anticodon stem loop of tRNA often preorganize the tRNA for efficient and accurate decoding on the ribosome (3–6). Most modifications of rRNA in bacteria are installed by dedicated enzymes capable of modifying a single site within rRNA. Therefore, knockout strains lacking the individual modification enzymes can be constructed to assess the effect of the modification on bacterial fitness. Typically, cells with a single rRNA modification enzyme knocked out are viable with only modest phenotypes such as slower growth or increased sensitivity to cold stress (7). However, strains with several rRNA modification enzymes knocked out can have severe phenotypes and the importance of these genes for bacterial fitness is revealed by the fact that scientists aiming to identify a minimal bacterial genome were only able to remove three rRNA modification genes (8). An exception to the scenario of single rRNA modification enzymes mediates mild phenotypic effects is observed when the rRNA modification enzyme mediates antibiotic resistance. Formation of m^4A1408 on 16S rRNA or m^6G1405 on bacterial 16S rRNA confers resistance to aminoglycoside antibiotics. Methylation of C8 of A2503 on bacterial 23S rRNA confers a multidrug-resistance phenotype known as PhLOPS for the classes of drugs resistance is conferred to: phenicols, lincosamides, oxazaolidonones, pleuromutilins, and streptogramin A (9). Formation of m^6A2058 on 23S rRNA by erythromycin-resistance methyltransferases (Erm) confers the MLSbK multidrug phenotype named for the macroline, lincosamide, streptogramin B, and ketolide antibiotics (Fig. 1A). When cells carrying these rRNA methyltransferases are exposed to antibiotics, the presence of the methylation results in robust growth instead of growth arrest (10). The genes for the methyltransferases conferring the drug-resistance phenotypes have been acquired by many pathogens, resulting in a major threat to public health (11).

Methyltransferases acting upon tRNA and rRNA must solve a specific thermodynamic and kinetic problem that arises because of the highly base-paired structures of tRNA and rRNA. Unfolding of the base-paired substrate is thermodynamically unfavorable and kinetically slow. Yet the enzymes need to achieve rates that support the rapid growth of bacteria and the demand for high-substrate specificity. DNA methyltransferases can distort B-form DNA to “flip-out” the target nucleotide, accomplishing an ~180° rotation of the nucleotide to position it into the enzyme active site (12). rRNA and tRNA methyltransferases can do the same (13, 14). Although this solves the substrate positioning problem the challenge of substrate recognition must be explained. The deep, narrow major groove of A-form RNA makes sequence-specific recognition via this part of the helix more difficult than it is for enzymes binding B-form DNA. rRNA and tRNA methyltransferases appear to solve this problem by recognizing secondary and tertiary features of the RNA, in addition to primary sequence, reducing the need to...
whether or not there are general principles guiding substrate recognition and positioning by rRNA and tRNA methyltransferases is an open question. A limited repertoire of protein folds is observed among rRNA and tRNA methyltransferases. Although methyltransferases, broadly, can be described by five classes, rRNA and tRNA methyltransferases typically fall into Class I, Rossmann fold or Class IV, SPOUT methyltransferases (15). Erm proteins possess the Rossmann fold composed of seven β-strands and a small, all α-helical C-terminal domain (Fig. 1B). Important features of this fold are a GXGAG motif following β1 and involved in SAM binding, an Asp or Glu residue following β2 also involved in SAM binding, and a motif following β4 originally annotated as DPPY that is associated with methyltransferases that modify the exocyclic amines of cytosine and adenine (Fig. S1) (16). Elaborations to the β-stranded core of the Rossmann fold direct the methyltransferase to specific RNA substrates.

An important unanswered question is whether ErmC, found in pathogens, and ErmE, an evolutionarily distant enzyme that provides resistance to a soil bacterium that biosynthesizes erythromycin, use the same mechanisms for substrate recognition and positioning. Extensive site-directed mutagenesis of ErmC has been previously performed identifying three residues whose mutation to alanine appears to abolish ErmC activity, leading to erythromycin sensitivity: Asn-101, Tyr-104, and Arg-134 located in the Rossmann fold methyltransferase domain were previously shown to be crucial for rRNA methylation. The functional role of additional ErmC and ErmE residues within this domain (marked with an asterisk) are investigated in this study. Site-directed mutants within the C-terminal domain (CTD) of ErmC have been shown to retain function in previous studies. 

**Figure 1.** Erythromycin resistance methyltransferases confer antibiotic resistance by methylating 23S rRNA using residues in and adjacent to α-helix 5. A, Erm mono- or dimethylate adenosine 2058 (A2058, E. coli numbering) of 23S rRNA sterically occluding antibiotics which bind this region of the rRNA. A secondary structure diagram of 23S rRNA is shown for helix 73 (H73) and the surrounding rRNA. Helix 73 is located adjacent to the peptidyl transferase center deep within the folded structure of the 50S subunit shown as a thumbnail image. B, the ErmC residues Asn-101, Tyr-104, and Arg-134 located in the Rossmann fold methyltransferase domain were previously shown to be crucial for rRNA methylation. The functional role of additional ErmC and ErmE residues within this domain (marked with an asterisk) are investigated in this study. Site-directed mutants within the C-terminal domain (CTD) of ErmC have been shown to retain function in previous studies. C, a radial phylogram of rRNA adenine demethylase family members indicates that although ErmC and ErmE both methylate A2058 of 23S rRNA they possess substantial sequence divergence. A scale bar indicates 1.0 substitutions per site. D, a superposition of ErmC (PDB code 1qao) and ErmE (PDB code 6nvm) reveals that Tyr-104 and Lys-164 of ErmE correspond to the crucial ErmC residues Tyr-104 and Arg-134, respectively. An additional ErmE residue of interest, Arg-171 is shown as sticks and the SAM cofactor is shown as spheres. E, cryo-EM analysis (PDB code 4adv) of the interaction of RsmA, a homolog of Erm, with the 30S ribosome suggests the face of Erm that contains α4, α5, and α6 interacts with rRNA including helix 45 (H45) the methylation site.
Arg-134 (Fig. 1B) (17, 18). We have used site-directed mutagenesis to construct two of the three corresponding mutants in ErmE (Fig. S1), and in the case of Y134A, have performed detailed in vitro studies to identify the mechanism of the erythromycin-sensitive mutant. Recently it was shown that grafting regions of ErmC, including a loop linking α5 and α6, onto Bacillus subtilis KsgA (RsmA) was sufficient to provide methylation activity on a substrate modeling the A2058 region of rRNA and resistance in vivo against low concentrations of erythromycin (19). Motivated by this result, we investigated the functional significance of the α5-α6 linker in both ErmC and ErmE, identifying residues that are required for Erm function.

Results

Previously, extensive mutagenesis of ErmC identified only three residues essential for erythromycin resistance (Asn-101, Tyr-104, and Arg-134), two of which are conserved in ErmE (Fig. 1B and Fig. S1) (17, 18). However, whether ErmE uses the same residues to recognize and methylate 23S rRNA is an open question motivated by the sequence divergence between the two proteins which possess only 24% sequence identity. Indeed, a phylogram of members of the rRNA adenine dimethylase family, PFAM PF00398, shows that the distance between ErmC and ErmE is similar to the distance between ErmC and MTF1 or RsmA which have a distinct rRNA substrate (Fig. 1C). Therefore, although ErmC and ErmE both methylate A2058 of 23S rRNA, it is reasonable to question whether they may differ mechanistically. ErmC residue Asn-101 is not conserved in ErmE, however, ErmE contains homologous residues for Tyr-104 and Arg-134, Tyr-134 and Lys-164, respectively (Fig. 1B). By comparison of ErmC to the crystal structure of the m6A DNA modifying enzyme M.TaqI bound to DNA and a recently reported structure human TFB1M bound to a RNA substrate, Tyr-104 is believed to position the substrate Ade residue in the active site via π-stacking interactions. M.TaqI does not have a similar basic residue in α5, the α-helix following β5 of the Rossmann fold, analogous to Arg-134 (Fig. 1D). However, both Escherichia coli RsmA (KsgA) and human TFB1M, members of the same protein family as Erm, do, and their structures bound to substrate suggest the basic residue on α5 forms a key interaction with RNA (Fig. 1, C and E). For example, Fig. 1E depicts the model of RsmA bound to the 30S ribosome derived from a medium-resolution cryo-EM structure (20). Although the resolution of this map does not permit modeling protein side chains, it suggests several Ca atoms of α5 are located with 5 Å of rRNA, suggesting interactions (Fig. 1E). To address whether ErmC and ErmE use similar mechanisms for substrate recognition and positioning we performed site-directed mutagenesis of the Tyr-134 and Lys-164 positions of ErmE. Recently, it was demonstrated that the ErmC region adjacent to α5 plays an important role in RNA substrate specificity (19). Therefore, we also made site-directed mutants in the region adjacent to ErmE α5, residues Gly-170 and Arg-171.

We assayed E. coli transformed with a collection of ErmE mutants in and adjacent to α5 for resistance to erythromycin finding that each Ala mutant, except G170A, was more sensitive to erythromycin (Eryα phenotype) than cells transformed with WT ErmE, which confers robust erythromycin resistance (Eryγ phenotype) (Fig. 2, A and B and Fig. S2). Erythromycin sensitivity was measured by a minimum inhibitory concentration assay. Arabinose was used to induce recombinant expression in E. coli cells possessing one of the ErmE variants. The cells were then streaked to a series of agar plates containing between 47 and 1200 μg/ml erythromycin, and scored for growth. The origin of the Eryα phenotype could be because of the Ala mutants destabilizing the folded state, leading to insoluble or rapidly degraded enzyme. To investigate this possibility, we took advantage of the fact that our ErmE expression construct contained a C-terminal MYC tag and assayed the levels of soluble protein present in cells with an anti-MYC antibody by Western blotting (Fig. 2C). For each variant associated with an Eryα phenotype, protein levels were similar to WT ErmE suggesting the phenotype is a result of a defect in protein function rather than a difference in protein amount. Triplicates of the Western blotting shown in Fig. 2C were performed and a representative blot is shown. We also used the software tool Missense3D to model the effect of each site-directed mutant on ErmE structure, finding that none of the mutations were predicted to damage the protein’s structure (21). Therefore, we attempted further mechanistic investigation of these variants.

In a first step toward understanding the mechanistic role of Tyr-134 and Arg-171, we employed a site-saturation mutagenesis protocol to identify which amino acids might support ErmE function at these positions (Fig. S3). We performed site-directed mutagenesis of our ermE expression construct with an NNK codon, where K stands for a G or T nucleotide, positioned either at position 134 or 171, generating a library of ermE variants where all 20 possible amino acids were encoded at 134 or 171. After selecting for transformants on a plate with ampicillin we transferred colonies to a plate with erythromycin and arabinose to score colonies as Eryα or Eryγ (Fig. S3). For Tyr-134 only 6 of 30 colonies transferred to the erythromycin plate were Eryγ. Sequencing of ermE in each of the Eryγ colonies revealed each contained a Tyr or Phe, although sequencing of the Eryα colonies from the ampicillin plate revealed a variety of residues but not Tyr or Phe (Fig. 3A). This result suggests that an aromatic residue capable of π-stacking with the target Ade nucleotide is critical for ErmE function, as in ErmC, but that the −OH of Tyr is not strictly required. For Arg-171, 5 of 18 colonies were Eryγ and sequencing of ermE from each colony revealed all contained Arg (Fig. 3B). Sequencing of the Eryγ Arg-171 variants revealed a variety of residues but not Arg. Taken together the observations indicate ErmE function is very sensitive to alteration of Arg-171. For example R171K, despite the fact that the mutant contains a basic residue, and R171M, which is sterically similar to Arg, do not support ErmE function. Because the site-directed mutants could destabilize folded ErmE, we again estimated the levels of ErmE variants by Western blotting and observed levels similar to WT for each variant (Fig. 3C). Representative blots are shown in Fig. 3C selected from three replicates each. These observations are consistent with the crystal structure of ErmE which shows that both Tyr-134 and Arg-171 are located on the surface of the protein facing solvent and therefore are expected to accommodate a variety of amino acids substitutions without altering protein folding (22).
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RNA methyltransferases typically contain many basic residues on the surface that interact with RNA; however, mutation of a single basic residue to Ala does not usually result in loss of function, so we were surprised by the Ery\textsuperscript{R} phenotypes of the Arg-171 variants (13). For this reason, we further investigated the activity of the Arg-171 variants. We purified seven of the Arg-171 variants and employed an \textit{in vitro} methylation assay based on \textsuperscript{3}H-SAM and \textit{in vitro} transcribed analogs of 23S rRNA (Fig. 4, \textit{A} and \textit{B}). Two analogs of 23S RNA domain V, which contains the A2058 methylation site for Erm, were used: dV, which contains WT rRNA sequence and A2058G in which the target Ade nucleotide is mutated to Gua. The A2058G substrate serves as a specificity control; because Gua does not possess the exocyclic N6 amine moiety required for methylation by Erm, any methylation of this substrate would be assumed to derive from other Ade residues in its sequence. An end point assay revealed that the mean amount of methylation by each Arg-171 variant was less than WT ErmE, however, statistical analysis indicated that the difference between R171T or R171Q and WT was not significant. For the other five variants the mean amount of methylation was significantly different from WT with the magnitude of the defect varying between 2- to 20-fold (Fig. 4C). R171V and R171I had the largest defect in methylation, perhaps indicating that introducing steric bulk around C\textsubscript{\beta} perturbed residues adjacent to Arg-171, Tyr-169 or Tyr-270, leading to an additive effect (22). In no case was product above background levels observed on the A2058G substrate, indicating that the Arg-171 variants did not reduce the substrate specificity of ErmE directing it toward other adenosines within domain V.

To further dissect the mechanistic basis of the defect in ErmE Tyr-134 and Arg-171 variants, we measured methylation kinetics under single turnover conditions with purified Y134A and R171V (Fig. 5A). These variants were chosen because we anticipated they would confer large defects \textit{in vitro}, facilitating our mechanistic investigation. We also attempted to perform these measurements with ErmE K164A, but this mutant was poorly soluble \textit{in vitro} despite its apparent solubility in cells indicated by Western blotting. We measured CD spectra for Y134A, R171V, and WT which indicated both mutants were well-folded, possessing the same secondary structure as WT (Fig. 5B). We performed time-resolved methylation assays using a 48-nucleotide synthetic RNA that mimics helix 73 of 23S rRNA a substrate design first reported by Vester and colleagues and one that is similar to the helix 73 mimic used in kinetics studies of ErmC (23). Methylation by WT ErmE resulted in nearly identical \(k_{\text{obs}}\) values when run under conditions with either limiting \textsuperscript{3}H-SAM or limiting RNA, which is expected if the amount of enzyme used, 10 \(\mu\text{M}\), is well above \(K_d\) in both cases (Fig. 5C and Table 1). By contrast, both Y134A and R171V displayed ~30- to 100-fold defects in \(k_{\text{obs}}\). This was true whether \textsuperscript{3}H-SAM was the limiting reagent or RNA was the limiting reagent, suggesting that the mutants did not have a defect in \(K_d\) for either substrate which would have been rescued when that substrate was no longer limiting. To further validate the observation that Y134A and R171V are not defective in RNA binding we performed an RNA affinity–binding assay: nanomolar \(\textsuperscript{32}\text{P}\)-labeled helix 73 analog was incubated with titrations of each ErmE variant and the fraction bound at equilibrium was determined by filtering the reactions over a nitrocellulose and Hybond membrane sandwich followed by

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{figure3.png}
    \caption{Site-saturation mutagenesis assays of ErmE Tyr-134 and Arg-171 indicate residues that are functional substitutes. \textit{A}, site-saturation mutagenesis of Tyr-134 reveals that, of the variants tested, only Y134F confers erythromycin resistance. Cyan blocks indicate variants that were observed and erythromycin sensitive (Ery\textsuperscript{S}), whereas pink blocks indicate variants that were observed and erythromycin resistant (Ery\textsuperscript{R}), as scored on plates with 1200 \(\mu\text{g/mL}\) antibiotic. A white block indicates the residue was not observed in the site-saturation mutagenesis assay. Amino acids are colored according to side chain chemistry: black, nonpolar; blue, basic; red, acidic; green, hydroxyl containing; and purple, amide containing. \textit{B}, site-saturation mutagenesis of Arg-171 reveals that, of the variants tested, only Arg confers erythromycin resistance. C, cell lysates of ErmE mutants that are Erys were subjected to Western blotting. \(M\), denotes molecular weight markers.}
\end{figure}

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{figure2.png}
    \caption{Erythromycin resistance phenotypes of ErmE site-directed mutants in and adjacent to \(\alpha\)-helix 5. \textit{A}, ErmE residues investigated are mapped onto the structure given by PDB code 6nvm. \textit{B}, growth of \textit{E. coli} transformed with ErmE was assayed on erythromycin containing LB agar plates with 1280 \textit{g/mL} antibiotic. A \textit{Y134} indicates residues that are functional substitutes.}
\end{figure}
phosphorimaging to quantitate bound RNA (Fig. S4). The affinity assay confirmed that both mutants possess a \( k_{d,\text{RNA}} \) value similar to WT (Table 1). Because the Y134A or R171V variants do not interfere with substrate binding and there is no reason to believe these residues participate directly in the \( S_{n,2} \) methylation reaction we suspect Tyr-134 and Arg-171 play a crucial role in substrate positioning.

Having confirmed that Tyr-104/134 is essential for function in both ErmC and ErmE, we turned our attention to the \( \alpha_5-\alpha_6 \) linker region of ErmC (Fig. 6A). We were motivated by recent studies that showed grafting of the \( \alpha_5-\alpha_6 \) linker from ErmC to RsmA (KsgA) contributes to altering the substrate specificity of RsmA so that it behaves like ErmC and our observations that ErmE Arg-171, located in the \( \alpha_5-\alpha_6 \) linker, is essential for rRNA methylation (19). Notably, the \( \alpha_5-\alpha_6 \) linker region of ErmC has not been previously tested by site-directed mutagenesis to identify the effects of individual amino acids on function. Therefore, we made Ala site-directed mutants at two positions of the \( \alpha_5-\alpha_6 \) linker, Thr-138 and Lys-139, and performed site-saturation mutagenesis at a third position, Asn-137. We assayed \( E. \ coli \) transformed with the ErmC variants in erythromycin-resistance assays confirming that N137V possessed an Ery\(^{+}\) phenotype but T138A and K139A did not (Fig. 6B and Fig. S5). Site-saturation mutagenesis of residue 137 revealed that primarily small, polar residues correlated with an Ery\(^{+}\) phenotype although other residues did not (Fig. 6C). We subjected four Asn-137 variants that were Ery\(^{+}\) to Western blotting to estimate protein levels relative to WT ErmC. A representative blot taken from three replicates is shown (Fig. 6D). The levels of the Asn-137 Ery\(^{+}\) variants appeared roughly similar to WT (Fig. 6D). We again used the software tool Missense3D to model the structural effect of the N137V mutation (21). Although Missense3D determined that the N137V mutation does not structurally damage the protein, it is predicted to alter the rotamers of nearby Lys-133 and Arg-140 (Fig. 6E). Therefore, the Ery\(^{+}\) phenotype associated with this mutant could arise from these structural changes. Further studies will be needed to dissect the mechanistic contribution of the \( \alpha_5-\alpha_6 \) linker to 23S rRNA methylation by ErmC, but our data suggest the linker may play an important role.

**Discussion**

To address the issue of a universal versus idiosyncratic mechanism for Erm function, we mutated ErmE residues and characterized these variants. We performed a mechanistic investigation of the Y134A mutant. Y134A possesses an 80- to 100-fold defect in methylation that is not because of a defect in RNA binding or SAM binding. This phenomenon can be explained if Tyr-134 participates in an induced-fit mechanism wherein interactions between the active site Tyr and A2058, such as
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Single-turnover kinetics and RNA affinity–binding experiments indicated the methylation defects of R171V, as in Y134A, are not explained by a defect in substrate binding, suggesting that Arg-171 could also participate in an induced fit conformational rearrangement required for catalysis. We constructed site-directed mutants of the ErmC α5-α6 linker and found that Asn-137 variants not containing a small polar residue were Ery⁺, consistent with the data from Bhujbalrao and co-workers (19) indicating an important role for this region in ErmC specificity. The experiments reported herein, taken together with previously reported functional characterizations of ErmC suggest the region of Erm proteins containing α4, α5, and the α5-α6 linker plays an essential role in RNA substrate recognition and positioning (Fig. 1).

A co-crystal structure of an Erm protein bound to RNA is currently not available. An in silico docking model of ErmC bound to a modeled 23S rRNA helix 73 was constructed by Maravic and colleagues (17) and tested by site-directed mutagenesis. A prominent feature of this model was extensive interaction of helix 73 with the cleft between the catalytic and C-terminal domains (the region below α6 in Fig. 1D). However, site-directed mutants of cleft residues did not result in substantial defects in ErmC activity (17). Recent structural data have emerged for two members of the RRAD family, a low-resolution cryo-EM structure of RsmA (KsgA) bound to the 30S ribosome and a crystal structure of TFB1M bound to helix 45 of mitochondrial 12S rRNA (Figs. 1E and 7A) (20, 24). Both structures possess a similar mode of interaction with their RNA substrate in which the helical axis of helix 45 is perpendicular to the long axis of the methyltransferase, which can be best seen in Fig. 7A. The TFB1M structure reveals that the methylated nucleotide, A937, and nucleotides adjacent to it, G934-A936 interact with the N-terminal regions of α4 and α6 (Fig. 7A). TFB1M α5 interacts with the major groove of helix 45 adjacent to G934-A937. Given that there is strong structural conservation across the RRAD family members and that the evolutionary distance between human TFB1M and E. coli RsmA (KsgA) is similar to the difference between either one of these proteins and the Erm proteins, it seems possible that Erm proteins interact with 23S rRNA helix 73 in a similar manner (Fig. 1C). To test this idea, we superpositioned ErmC or ErmE onto the structure of human TFB1M bound to helix 45 rRNA. Next, we positioned coordinates of E. coli 23S rRNA helix 73, derived from X-ray structures of the 70S ribosome, so that the riboses of C2055 and A2058 roughly superimposed with the G934 and A937 of TFB1M (Fig. 7) (25). Positioned this way, C2055-A2058 in helix 73 interact with the Erm proteins in a manner similar to that of G934-A937 interacting with TFB1M and the Erm-helix 73 pose is consistent with numerous biochemical observations. For example, this model postulates little interaction between the 3’ strand of helix 73 and Erm.

The specific features of 23S rRNA that lead to A2058 methylation by ErmE have been studied extensively by multiple complementary approaches revealing that helix 73 followed by five residues on the 3’ end is sufficient for methylation (23, 26). Within this minimal unit, the residues on the 3’ side of helix 73 play little role in methylation outside of generally maintaining the secondary structure of the helix and the 3’ side of the helix

### Approximate erythromycin MIC (μg/mL)

|     | MIC (μg/mL) |
|-----|-------------|
| wt  | > 1200      |
| K139A | > 1200    |
| N137V | 356-533   |
| T138A | > 1200      |
| wt  | > 1200      |

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**Figure 6. Site-directed mutants of the ErmC α5-α6 linker affect erythromycin resistance phenotypes.** A. ErmC residues investigated are mapped onto the structure given by PDB code 1qao. B. growth of E. coli transformed with ErmC was assayed on erythromycin containing LB agar plates to estimate the minimum inhibitory concentration (MIC) for each mutant. C. site-saturation mutagenesis reveals that only variants of ErmC with predominately small, polar residues at Asn-137 confer the Ery’ phenotype. D. cell lysates of ErmC Asn-137 mutants with an Erys phenotype were subjected to Western blotting. M. denotes molecular weight markers. E. a computational model (Missense3D) of the local, structural effects of the N137V mutant.

π-stacking, both organize the active site and position the substrate adenosine. This mechanistic role for an active site aromatic residue is common in DNA and RNA methyltransferases and seems to be a key feature of both ErmC and ErmE (13).

Because of recent data indicating the α5-α6 linker of ErmC is an important substrate specificity determinant, we investigated this region of ErmE finding that Arg-171 is important for function (Fig. 1D) (19). We performed an end point methylation assay with seven Arg-171 variants on two in vitro transcribed RNAs (Fig. 4). WT ErmE specifically methylates A2058 so assays with the A2058G transcript provided an opportunity to observe if any of the Arg-171 variants were capable of off-target methylation. Because Bhujbalrao and co-workers (19) demonstrated grafting an N-terminal loop and the α5-α6 linker of ErmC onto B. subtilis KsgA (RsmA) caused robust in vitro methylation of a helix 73 analog, we reasoned that perhaps Arg-171 variants might be Ery⁺ because of a loss of specificity, but off-target methylation was not observed.
Interaction of Purple spheres ErmC helix 73 of 23S rRNA guided by the TFB1M-RNA co-crystal structure.

directed mutagenesis by Maravi

Figure 7. Model for Erm binding to 23S rRNA. A, a crystal structure of human 125 rRNA methyltransferase (HsTFB1M) bound to an analog of helix 45 given by PDB code 6aax. B, a model of the interaction between B. subtilis ErmC helix 73 of 23S rRNA guided by the TFB1M-RNA co-crystal structure. Purple spheres indicate crucial residues that have been subjected to site-directed mutagenesis by Maravić and co-workers (17, 18). C, a model of the interaction of Saccharopolyspora erythraea ErmE with helix 73 guided by the TFB1M-RNA structure. Purple spheres indicate site-directed mutants examined in this work.

can even be substituted with locked or deoxynucleotides with little effect (26). However, elements of the 5’ side of the helix are crucial, consistent with a direct interaction of nucleotides C2055-G2057 with Erm proteins as we have modeled (Fig. 7, B and C). It was found that the presence of A2058 within a GGAHA motif (A2058 is underlined, H is any nucleotide except G) is critical for methylation and an unpaired nucleotide at C2055 is an essential secondary structure element (27). The Erm-RNA pose derived from the TFB1M-rRNA structure places C2055 sandwiched between α4, α5, and α6 and posits that α4 distorts G2056 and G2057 such that their interactions with their base-pairing partners are lost (Fig. 7, B and C). The Fig. 7 model for Erm-rRNA interactions does not explain the importance of A2059 and A2060 (not modeled in figure); however, it suggests that these nucleotides are unpaired and interacting with the N-terminal region of ErmC or ErmE.

Bhujbalrao and coworkers presented a striking result: That a chimera between two RRAD family members, ErmC and RsmA (KsgA), can be constructed by swapping discrete linker regions to alter the specificity of a housekeeping rRNA methyltransferase imparting antibiotic resistance function (19). We present results that validate the importance of the α5–α6 linker, one of the elements (referred to as Loop 12 by Bhujbalrao) that is key to the specificity switch and put forth a structural model to rationalize the linker’s importance. Bhujbalrao and coworkers also found that an N-terminal region of ErmC was important for imparting the specificity switch in RsmA. Identifying the mechanistic contribution of this protein element is an important next step in understanding how the sequence and structure of RRAD family members drive RNA specificity and perhaps will also serve as a window to understanding the ways in which elaborations to the common Rossmann fold methyltransferase structure impart specificity in other contexts.

Experimental procedures

ErmC and ErmE expression constructs

The pET-25b-ermC’ (referred to throughout as ermC) construct was kindly provided by Dr. Janus Bujnicki (17). The ermC coding region was subcloned to pBAD/Myc-His A (Invitrogen) and verified by DNA sequencing. Gene synthesis (Eurofins Genomics) was used to obtain plasmid DNA coding for ErmE given by UniProt P07287 with the C-terminal 82 amino acids, a Gly-rich low complexity sequence, removed following work by Vester and Douthwaite (28). The coding region for ermE was subcloned into pBAD/Myc-His A and verified by DNA sequencing.

Site-directed mutants were generated using pBAD/Myc-His ermC or pBAD/Myc-His ermE as template DNA in one of three ways indicated by Table S1, which also gives the sequences of oligonucleotides used in this study. QuikChange Lightning mutagenesis was carried out according to the manufacturer’s instructions. Inverse PCR was used to construct site-directed mutants by performing a PCR reaction followed by simultaneous PNK and DNA ligase reactions using New England Biolabs enzymes in T4 PNK ligase buffer at 37°C for 15 min. DpnI treatment was carried out at 37°C for 1 h and chemically competent DH5α cells were transformed and plated on LB-ampicillin (100 μg/ml). Site-saturation mutagenesis was carried out very similarly to a published protocol (29). Briefly, partially overlapping oligos containing an NNK or MNN (reverse) codon were used for whole plasmid PCR amplification. The product was purified using the Omega Bio-tek E.Z.N. A. kit and subjected to DpnI digestion at 37°C for 2–4 h. Top 10 chemically competent cells were transformed with the product and then added to 3–5 LB-ampicillin (100 μg/ml) plates that had been coated with 250 μl 0.02% w/v arabinose. Following overnight growth at 37°C the colonies, typically 50–100, from one transformation plate were scraped from the plate and the population of plasmid DNA was purified and subjected to Sanger sequencing to verify library diversity, visualized as an approximately equal ratio of all four nucleotides in the position of the gene mutated to NN (29). Colonies from the two
additional LB-Amp plates were replica plates to LB-erythromycin (1280 μg/ml) plates coated in 0.02% w/v arabinose. After 12–24 h at 37°C the LB-Ery plate was used to identify colonies as erythromycin sensitive (Ery<sup>a</sup>) or resistant (Ery<sup>r</sup>). In all cases plasmid DNA purified from single colonies was purified from overnight cultures and validated by DNA sequencing with the forward and reverse primers given in Table S1. Ery<sup>a</sup> and Ery<sup>r</sup> phenotypes were validated by additional passage of the strains on LB-Ery.

**Construction of the RRAD family phylogram**

Sequences from 14 RNA adenine dimethylase family members drawn from model organisms were aligned with Muscle and a phylogram was constructed in Geneious using Jukes-Cantor method. The phylogram was validated by additional passage of the strains and displaying significant Pearson correlation at 0.01. Sequences from 14 RNA adenine dimethylase family members were generated as follows. An LB-ampicillin (100 μg/ml) plate was coated with 250 μl of 0.02% w/v arabinose and allowed to dry. Cells from a frozen permanent culture were streaked to the plate and a 37°C overnight incubation was performed. Cells from the LB-Amp plate were further streaked to a series of LB plates, similarly coated with arabinose, each containing between 47 and 1200 μg/ml erythromycin. Following a 37°C incubation for 16 h, the lowest concentration of erythromycin able to prevent confluent growth was recorded. As indicated in Figs. 2 and 6 the MIC lies between the highest concentration of erythromycin for which growth was observed and the lowest concentration for which confluent growth was prevented.

**Erythromycin MIC estimates**

Plates indicating Ery<sup>a</sup> and Ery<sup>r</sup> phenotypes shown in Figs. 2 and 6 were generated as follows. An LB-ampicillin (100 μg/ml) was coated with 250 μl of 0.02% w/v arabinose and allowed to dry. Cells from a frozen permanent culture were streaked to the plate and a 37°C overnight incubation was performed. Cells from the LB-Amp plate were further streaked to a series of LB plates, similarly coated with arabinose, each containing between 47 and 1200 μg/ml erythromycin. Following a 37°C incubation for 16 h, the lowest concentration of erythromycin able to prevent confluent growth was recorded. As indicated in Figs. 2 and 6 the MIC lies between the highest concentration of erythromycin for which growth was observed and the lowest concentration for which confluent growth was prevented.

**Western blotting**

100 μl of overnight culture of each mutant was diluted into 10 ml LB-Amp and grown at 37°C for 2 h with shaking. Protein expression was induced with 0.02% w/v arabinose and growth was continued for 2 additional hours at 37°C. Cells were then harvested by centrifugation and incubated on ice for 30 min in buffer containing 0.1 mg/ml lysozyme. Next, cells were lysed by two freeze-thaw cycles using liquid nitrogen. The lysates were cleared by centrifugation and 20 μl of the resulting soluble protein was subjected to SDS-PAGE on a 4–20% gel and transferred to nitrocellulose membranes using standard methods. Membranes were blocked with 3% w/v BSA in TBST for 1 h, washed three times with TBST, then incubated with 1:1250 dilution of mouse monoclonal anti-MYC antibody (Invitrogen, MA1-980) in TBST for 1 h. Membranes were then probed with an anti-mouse HRP conjugate secondary antibody (Invitrogen, 31430) at 1:2500 in TBST for 1 h. Blots were developed with 1-step Ultra TMB solution (Pierce) and blot images were captured with a digital camera.

**Protein purification**

ErmE variants used in end point methylation assays were purified as follows. *E. coli* TOP10 cells harboring pBAD-ermE WT or site-directed mutants were grown in LB media at 37°C to ~0.6 A<sub>600</sub> at which point recombinant protein expression was induced with 0.02% w/v arabinose. Cell growth was continued at 37°C for 4 h, at which point the cells were harvested by centrifugation. Cells were resuspended in TAG buffer (Tris pH 7.5 50 mM, NH<sub>4</sub>Cl 250 mM, 2-mercaptoethanol 6 mM, glycerol 10% v/v) supplemented with 0.1% v/v Triton X-100 and subjected to sonication. The cell lysate was clarified by centrifugation for 20 min at 4000 × g and applied to HisPur immobilized Ni<sup>2+</sup> resin. The resin slurry was applied to a column that was washed with 15 column volumes of TAG buffer supplemented with 15 mM imidazole. Protein was eluted from the resin with a stepwise gradient of increasing imidazole over 15 column volumes. Fractions were analyzed by SDS-PAGE for purity and flash frozen until needed for assays.

ErmE variants in single turnover kinetics assays were purified by immobilized metal affinity chromatography in a manner similar to that described above followed by size-exclusion chromatography with a S75 (sephadex) column. R171V fractions consistent with monomeric ErmE were pooled, concentrated on an Amicon stirred cell with a 10,000 MWCO membranes and extensively dialyzed at 4°C into TAG50 buffer (TAG buffer containing 50 mM NH<sub>4</sub>Cl). S75 fractions of WT ErmE and Y134A consistent with a monomer were extensively dialyzed at 4°C into TAG buffer lacking 2-mercaptoethanol, concentrated on a 10,000 MWCO spin concentrator device and flash frozen until needed.

**RNA**

An oligonucleotide mimicking H73 of 23S rRNA (V48) was synthesized by Dharmacon. The V48 oligo was first reported as a substrate for ErmE by Vester and colleagues (23). For assays with limiting RNA, V48 was PAGE purified before use. A 624 nucleotide RNA (dV) mimicking domain V of *B. subtilis* 23S RNA (2036–2657) was synthesized by *in vitro* transcription. A gene fragment coding for dV plus an EcoRI and BamH1 restriction sites and a T7 promoter was obtained from Eurofins Genomics. The fragment was cloned into pUC18 by standard methods and verified by DNA sequencing. The A2058G dV variant was made by the MegaWhoP site-directed mutagenesis method using the primers given in Table S1. Both plasmids were purified RNase free by methods reported in Linpinsel and Conn (31). BamH1 linearized plasmid DNA was used for *in vitro* transcription with the Promega T7 Ribomax kit according to the manufacturer’s instructions.

**In vitro methylation assays**

Methylation assays under single turnover conditions to extract <i>k</i><sub>obs</sub> were performed as follows. Substrate solution containing V48 RNA and <sup>3</sup>H-SAM were mixed with an equal volume of Erm protein to initiate methylation. The final concentration of Erm was 10 μM in all cases. In reactions with limiting SAM, <sup>3</sup>H-SAM was present at 0.05 μM and V48 RNA was present at 10 μM. In reactions with limiting RNA, <sup>3</sup>H-SAM was present at 5 μM and V48 was present at 0.2 μM. <sup>3</sup>H-SAM was purchased from Perkin Elmer and was ~100 μCi/mm mol. Reaction conditions were 20–22°C in Tris pH 7.5 50 mM, NH<sub>4</sub>Cl 50 mM and glycerol 10% v/v. At the indicated time points 2.5–50 μl of reaction volume were removed and quenched by dilution into 45 μl of 0.5
mg/ml salmon sperm DNA followed by the addition of 450 μl of 10% TCA. After more than 30-min incubation to allow RNA precipitation, RNA was collected by vacuum filtering on a Millipore Multiscreen GF 96-well plate. The filtered RNA was washed two times in 10% TCA and once with ethanol and allowed to dry. Perkin Elmer Betaplate scintillation fluid was then added and cpm were read on a MicroBeta 2 scintillation counter. Product versus time data were fit to the expression \( y = (y_{\text{max}} - y_0) \times (1 - e^{-k_t t}) + y_0 \) using GraphPad Prism.

End point methylation assays of ErmE Arg-171 variant (Fig. 4) were carried out at ~20°C with 0.1 μM 3H-SAM, 0.8 μM dV or A2058G RNA and 0.8 μM ErmE. Reactions were quenched after 4 min; RNA was precipitated, vacuum filtered, and subjected to scintillation counting as described above.

**RNA affinity binding**

PAGE purified V48 synthetic RNA was 5′-32P labeled using [γ-32P]ATP at 3000 Ci/mmol and T4 polynucleotide kinase. Unreacted [γ-32P]ATP was removed following labeling with a G-25 spin column. Labeled V48 was heated and annealed prior to binding reactions with a 70°C incubation for 2 min followed by slow cooling to room temperature in an aluminum heat block. Serial dilutions of ErmE WT or variants were made in buffer containing Tris pH 7.5 50 mM, NH4Cl 50 mM, and glyceral 10% v/v and mixed with labeled V48 at a final concentration of 20 nM. Incubation was performed for approximately 1 h and then the amount of bound RNA was measured by a double membrane binding assay (32). Amersham Biosciences Protran 0.45 μM nitrocellulose and Amersham Biosciences Hybond N+ membranes were soaked in buffer and the nitrocellulose membrane was stacked upon the Hybond membrane in a Bio-Rad BioDot vacuum filtration device. Reactions were applied to the membrane sandwich and filtered, and the membranes were then washed with 100 μl cold buffer. The membrane sandwich was disassembled, dried, and then subjected to phosphorimaging using GE storage phosphor screens and a Typhoon imager. The fraction bound in each titration series was calculated after integration of the membrane arrays in GE ImageQuant software by dividing the nitrocellulose counts by the sum of the counts from both membranes. Data from five technical replicates were fit to the equation \( y = B_{\text{max}} x/(K_d + x) + y_0 \) in GraphPad Prism version 7 to acquire \( K_d \). Global fitting of \( B_{\text{max}} \) and \( y_0 \) was performed across mutant and WT replicates producing a \( B_{\text{max}} \sim 1.0 \).

**Data Availability**

All data described are contained in the manuscript.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: Erm, erythromycin-resistance methyltransferase.

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