# Ribosome protection by antibiotic resistance ATP-binding cassette protein

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Edited by Peter B. Moore, Yale University, New Haven, CT, and approved April 11, 2018 (received for review February 23, 2018)

**The ribosome is one of the richest targets for antibiotics. Unfortunately, antibiotic resistance is an urgent issue in clinical practice. Several ATP-binding cassette family proteins confer resistance to ribosome-targeting antibiotics through a yet unknown mechanism. Among them, MsrE has been implicated in macrolide resistance. Here, we report the cryo-EM structure of ATP form MsrE bound to the ribosome. Unlike previously characterized ribosomal protection proteins, MsrE is shown to bind to ribosomal exit site. Our structure reveals that the domain linker forms a unique needle-like arrangement with two crossed helices connected by an extended loop projecting into the peptidyl-transferase center and the nascent peptide exit tunnel, where numerous antibiotics bind. In combination with biochemical assays, our structure provides insight into how MsrE binding leads to conformational changes, which results in the release of the drug. This mechanism appears to be universal for the ABC-F type ribosome protection proteins.**

ribosome protection | antibiotic resistance | ABC-F | MsrE | protein synthesis

More than one-half of the antibiotics in clinical use target bacterial ribosome and protein synthesis, particularly the elongation step (1). The peptidyl-transferase center (PTC) and the adjacent nascent peptide exit tunnel (NPET) in the ribosomal large subunit are the key players in protein elongation, with functions in catalyzing the peptide bond formation and the emergence of the nascent chain, respectively. PTC-targeting antibiotics, such as chloramphenicol, group A streptogramins, lincosamides, and pleuromutilins, inhibit protein synthesis by interfering with the correct positioning of the tRNA substrates (1, 2). In contrast, macrolides and group B streptogramins bind to a site within the NPET adjacent to the PTC and immediately before the constriction point at which ribosomal proteins (r-proteins) L4 and L22 narrow the tunnel width to approximately 10 Å (3, 4).

The primary mechanism of macrolide action is believed to be the context-specific inhibition of peptide bond formation rather than the indiscriminate obstruction of nascent chain passage through the NPET. Namely, ribosome-profiling analyses have revealed that translation of most genes proceeds past the first six to eight codons and can be arrested at any point during the translation when the ribosome encounters specific short-sequence motifs (5–7). The problematic sequence motifs are confined to the nascent peptide residues in the PTC, not the peptide segment in contact with the macrolide further down the NPET (5). Therefore, it appears that the general mode of macrolide action involves selective inhibition of peptide bond formation between specific combinations of donor and acceptor substrates. In some cases, the macrolide-induced and leader peptide-mediated translational arrest is used to regulate the expression of downstream macrolide resistance methyltransferase genes, such as *ermB* and *ermC* (8–12).

Structural characterization of the erythromycin-ErmBL leader peptide-ribosome complex reveals that the drug redirects the path of the peptide in the tunnel and leads to conformational changes in PTC and tRNA substrates unable to participate in the peptide bond formation that underlies translation arrest (10, 11). However, certain oligopeptides are believed to lead to drug resistance by "flushing out" the macrolides while passing through the NPET (13, 14). Therefore, the fate of the macrolide-bound ribosome is determined by the dynamic interactions among the bound drug, the PTC, and the sequence specificity of the emerging oligopeptide chain (5, 15). It should be noted, however, that macrolides could induce ribosomal arrest by allosterically altering the PTC even without forming significant contacts with the nascent chain, demonstrating the existence of a functional link between the NPET and the PTC (16).

A wide range of mechanisms mediate antibiotic resistance, one of the greatest threats to public health and food security worldwide. In the case of macrolides, various bacterial species are intrinsically insensitive due to chromosomal mutations in ribosomal genes causing reduced drug-binding efficiency (17, 18). The most common acquired resistance mechanism is the posttranscriptional methylation of the 23S rRNA by methyltransferases (e.g., Erm family), which also results in decreased drug-binding efficiency (1,

## Significance

ARE ABC-F genes have been found in numerous pathogen genomes and multi-drug resistance conferring plasmids. Further transmission will challenge the clinical use of many antibiotics. The development of improved ribosome-targeting therapeutics relies on the elucidation of the resistance mechanisms. Characterization of MsrE protein bound to the bacterial ribosome is first of its kind for ARE ABC-F members. Together with biochemical data, it sheds light on the ribosome protection mechanism by domain linker-mediated conformational change and displacement leading to drug release, suggesting a mechanism shared by other ARE ABC-F proteins. These proteins present an intriguing example of structure-function relationship and a medically relevant target of study as they collectively mediate resistance to the majority of antibiotic classes targeting the peptidyl-transferase center region.

Author contributions: L.Y. and Y.-G.G. initiated the project; Y.-G.G. directed the project; W.S., V.K., Y.D., R.E., A.S., B.S.T.L., and S.K.S. performed the experiments; W.S., V.K., R.E., and Y.-G.G. analyzed the data, determined the structure, and built the model; A.S.W.W. and J.S. helped with data collection; and W.S., V.K., R.E., and Y.-G.G. wrote the manuscript with input from all members.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.



Data deposition: The atomic coordinates and cryo-EM map have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID code 5ZLU) and Electron Microscopy Data Bank (accession no. EMD-6934), respectively.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1803313115/-/DCSupplemental.

Published online April 30, 2018.

19). Ribosome protection mechanism, by which the drug is actively removed from the ribosome, has recently become of great interest.

The ATP-binding cassette F (ABC-F) family proteins confer resistance to a number of clinically relevant antibiotics targeting the ribosome PTC/NPET region (20). These proteins are collectively referred to as antibiotic resistance (ARE) ABC-F proteins (21). Unlike other ARE ABC family members that are shown to actively pump drugs out of the cells, ARE ABC-F proteins lack the transmembrane domain characteristic to transporters and are believed to confer antibiotic resistance via ribosomal protection mechanism by interacting with the ribosome and displacing the bound drug (17, 20, 22–24). ARE ABC-F proteins have been classified into three groups based on antibiotic resistance: (*i*) Msr homologs (macrolides and streptogramin B), (*ii*) Vga/Lsa/Sal homologs (lincosamides, pleuromutilins, and streptogramin A), and (*iii*) OptrA homologs (phenicols and oxazolidinones) (20) (*SI Appendix*, Fig. S1). Notably, these proteins compose two ATP-binding domains connected by a linker of various lengths, which appears to be crucial for the efficiency and specificity of antibiotic resistance (20). However, an understanding of the molecular mechanism of how ARE ABC-F proteins interact with ribosomes to mediate antibiotic resistance requires a high-resolution structure of the complex.

Here we report the cryo-EM structure of the ARE ABC-F protein MsrE bound to the bacterial ribosome at 3.6-Å resolution. Note that the Msr homologs (divided into four classes: A, C, D, and E) have the longest domain linkers among the ARE ABC-F members (*SI Appendix*, Fig. S1). Furthermore, we have previously identified a *Pseudomonas aeruginosa* clinical isolate carrying the *msrE* gene obtained through horizontal gene transfer (NCBI accession ID: CP020704), which highlights the importance of the ARE ABC-F proteins in clinical practice. Therefore, our findings offer insight into the ribosomal protection mechanism underlying antibiotic resistance of ABC-F proteins, and demonstrate the huge potential for the future antibacterial drug development to counteract this resistance.

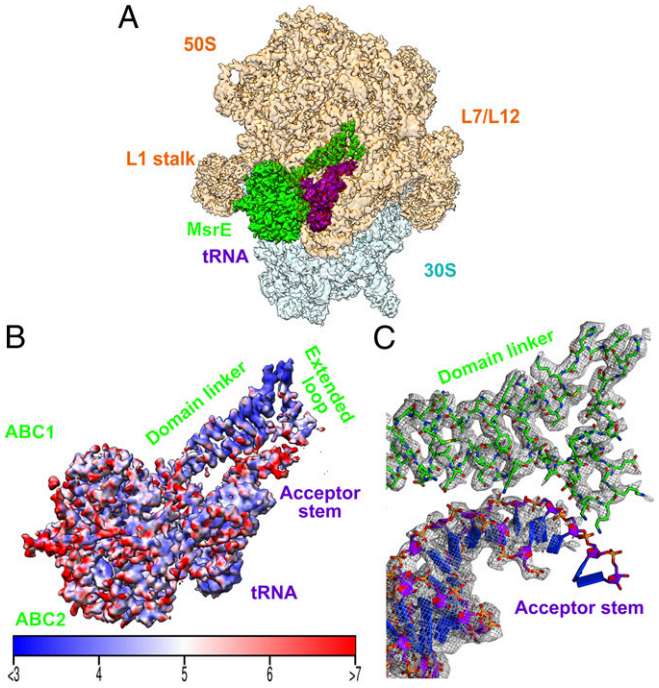


**Fig. 1.** Cryo-EM structure of the MsrE-ribosome complex. (*A*) Electron density of the overall complex. Ribosome 50S and 30S subunits are shown in pale orange and cyan, respectively. The electron density of 50S is made partially transparent to reveal the densities corresponding to MsrE protein (green) and tRNA (purple). (*B*) Local resolution of MsrE and tRNA shown in same orientation as in *A*. (*C*) Cryo-EM electron density of the MsrE domain linker extended loop and tRNA acceptor stem region.

## Results

**MsrE Rescues AZM Affected Translation.** The *msr* genes have mostly been identified in staphylococci, streptococci, and enterococci, and have recently spread to *P. aeruginosa* (18). We have previously observed that the exogenous expression of MsrE protein from its putative promoter significantly increases the azithromycin (AZM; a second-generation derivative of erythromycin) resistance of *P. aeruginosa* laboratory strains. Furthermore, the expression of MsrE from the arabinose-inducible promoter $P_{BAD}$ confers AZM resistance to *Escherichia coli* in a dose-dependent manner (*SI Appendix*, Table S1). The induction of MsrE expression by 0.2% arabinose increases the minimum inhibitory concentration of AZM by 16-fold compared with the uninduced condition. Overexpression of MsrE does not significantly affect the fitness of *E. coli*, as revealed by the comparison of growth curves of *E. coli*/$P_{BAD}$-*msrE* in the presence of 0.2% arabinose and 0.2% glucose (which reduces leaky expression from the arabinose-inducible promoter) (*SI Appendix*, Fig. S2). Moreover, purified MsrE protein rescues the AZM-inhibited translation process in a dose-dependent fashion, as shown by our in vitro transcription/translation assay (*SI Appendix*, Fig. S3*A*). These results clearly support a ribosomal protection mechanism for MsrE and ARE ABC-F proteins in general, which is also in agreement with the recent bacteriological and biochemical studies on VgaA and LsaA proteins (ARE ABC-F members conferring resistance to lincosamides, pleuromutilins, and streptogramin A) (*SI Appendix*, Fig. S1) (20, 22).

**Structure of Ribosome-Bound MsrE.** Purified MsrE protein can bind to *Thermus thermophilus* ribosome with a stoichiometry of 1:1 (*SI Appendix*, Fig. S3*B*). The addition of AZM, $tRNA^{fMet}$, and its corresponding mRNA did not significantly affect the binding stoichiometry. Furthermore, the complex was reconstituted in the presence of ATP or its nonhydrolyzable analog AMP-PNP (*SI Appendix*, Fig. S3*B*), indicating that ATP hydrolysis is not required for MsrE ribosome binding.

We next determined the cryo-EM structure of the MsrE-ribosome complex with an mRNA and its cognate P-tRNA at 3.6-Å resolution (Fig. 1*A* and *SI Appendix*, Fig. S4). Local resolution analysis revealed that the PTC/NPET of the 50S and the decoding center region of the 30S, as well as the tRNA anticodon stem loop and MsrE domain linker region, could be visualized at approximately 3.5-Å resolution. The local resolution was lower for the globular domains of the MsrE protein and the acceptor stem of the tRNA (Fig. 1 *B* and *C*), indicating higher structural flexibility.

As no structures of ARE ABC-F proteins are available, the ribosome-bound MsrE model is of great interest. The MsrE protein has a needle-like overall structure, with two ABC transporter domains (ABC1 and ABC2) carrying highly conserved nucleotide-binding sites (NBSs) (residues 37–44 and 329–336) assembled as its base and a domain linker (residues 180–279) that form two long crossed helices connected by an extended loop) assembled as the needle and tip (Fig. 2*A*). Both NBSs reveal electron density for the bound nonhydrolyzable ATP analog (AMP-PNP) (Fig. 2 *B* and *C*).

Despite structural similarity to the ribosome-bound ATP form of the non-ARE ABC-F protein EttA (25), ATP hydrolysis appears to play a different role in the functioning of MsrE. ATP hydrolysis is essential for the release of EttA from the ribosomes, as revealed by the finding that its deficient mutant (double substitutions E188Q/E470Q of the catalytic residues) affects cell growth by inhibiting protein synthesis through trapping ribosomes (26). In contrast, the cell growth of *E. coli* is not significantly affected by overexpression of the corresponding ATP hydrolysis deficient MsrE mutant (E104Q/E413Q) (*SI Appendix*, Fig. S2). However, the MsrE E104Q/E413Q mutant in vitro ribosome binding (*SI Appendix*, Fig. S3*B*) and in vivo AZM resistance (*SI Appendix*, Table S2) efficiencies are significantly reduced, indicating that ATP hydrolysis is crucial for mediating macrolide resistance.

Analyzing the aforementioned mutations one by one reveals that both residues contribute to antibiotic resistance, but the

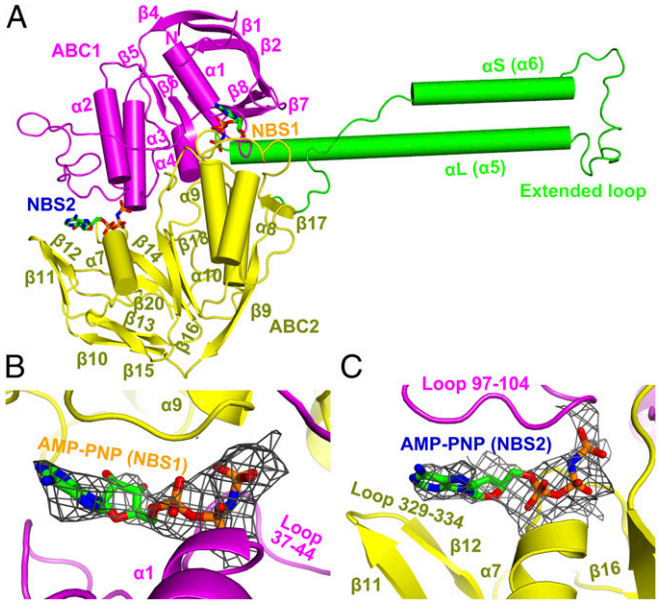


**Fig. 2.** Structure of the ribosome-bound MsrE protein. (*A*) MsrE protein shown in cartoon with secondary structure elements of ABC1 (magenta), ABC2 (yellow), and domain linker (green) labeled. The nonhydrolyzable ATP analog AMP-PNP is shown bound to NBSs NBS1 and NBS2. (*B* and *C*) Close-up views of the MsrE NBS1 (*B*) and NBS2 (*C*) with the cryo-EM electron density map shown for AMP-PNP.

one in NBS1 (E413Q) has a more significant effect (*SI Appendix*, Table S2). In line with this, ATPase activity has been reported to be crucial for ARE ABC-F protein VgaA, since mutation of the catalytic glutamines results in abolished antibiotic resistance in vivo (27) and rescue activity in the in vitro translation system (20). It was recently reported that the hydrolytically inactive *Staphylococcus haemolyticus* $VgaA_{LC}$ and *Enterococcus faecalis* LsaA mutants inhibit peptidyl transferase activity of the ribosome in a reconstituted translation system (28).

The long and flexible domain linker is a conserved feature in the ARE ABC-F family and likely provides an explanation for the lack of available structures. The sole identified crystal structure of ABC-F protein is the *E. coli* EttA (non-ARE) mentioned above (26), which has a significantly shorter linker region. Furthermore, the EttA crystal structure reveals a dimer formation mediated by the linker that might have facilitated the crystallization, yet the monomer state is favored in solution and is likely the active form of EttA (26). MsrE likely also functions as a monomer in cells, as revealed by molecular weight analysis. Surprisingly, in our structure, the entire backbone of the MsrE domain linker can be traced, demonstrating two crossed helices, longer α5, and shorter α6 (referred to as αL and αS, respectively) connected by an extended loop (Fig. 2*A*), with the majority of the amino acid side chains visualized in the electron density map (Fig. 1*C*).

**MsrE Binds to Ribosomal E-Sites.** MsrE is held in a position known as the ribosomal exit (E) site (Fig. 3*A*). The ABC1 domain of MsrE faces the L1 stalk of 50S, whereas the ABC2 domain contacts the 30S head (h41 and h42 of 16S rRNA and the r-protein S7), the r-protein L5 in the P-site finger region of 50S, and the elbow of P-tRNA (Fig. 3*B*). In contrast to the few ribosome contacts observed for the two ABC domains, the elongated domain linker establishes extensive contacts with ribosomes as it stretches in parallel with the acceptor arm of P-tRNA toward the NPET (Fig. 3*C*). The region at the interface between ABC1 and the linker (foremost the residues Glu191 and His194) contacts the H68 of 23S rRNA, which is involved in ribosomal intersubunit bridge as well as E-site formation and is important for translation activity (29) (Fig. 3*C*). Starting from the middle of domain linker and heading toward the terminal loop, MsrE αS helix (Lys264-Thr250 region) forms extensive contacts with H74 (A2435-U2438 and C2064-C2065 regions), H80 (G2252-G2253), and H93 (C2601) of 23S rRNA (mainly the backbone) (Fig. 3*C*). From the P-tRNA side, the residues Pro275 (conserved in the Msr subfamily) and Glu276 at the C terminus of the domain linker interact with the P-site tRNA elbow region (D-loop nucleotides 17–19) (Fig. 3*C*). The residues Arg217, Lys216, and Gln214 in αL directly contact the acceptor stem of P-tRNA (Fig. 3*C*). The orientation and detailed interactions of the extended loop in the MsrE domain linker with ribosome are discussed below.

Taken together, the foregoing findings indicate that MsrE interacts with the ribosome mainly through nonspecific interactions,

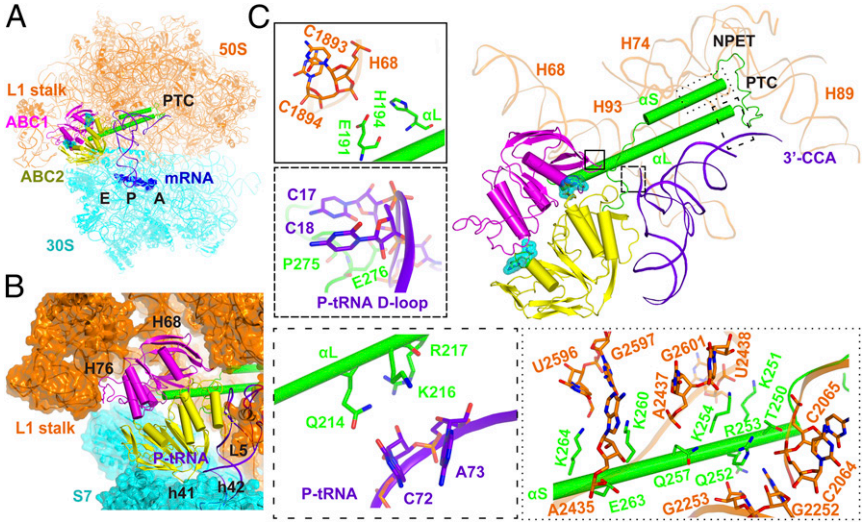


**Fig. 3.** Structure of the MsrE-ribosome complex. (*A*) Overall structure of the MsrE-ribosome complex. The 50S and 30S subunits are shown in orange and cyan, respectively, and the tRNA and mRNA are in purple and blue, respectively. (*B*) Interactions of the MsrE ABC1 domain with the 50S subunit L1-stalk (23S rRNA helices H68 and H76) and of the ABC2 domain with the 30S subunit (16S rRNA helices h41 and h42 and r-protein S7), r-protein L5, and tRNA. (*C*) MsrE domain linker helical region interactions with 50S subunit 23S rRNA and P-site tRNA.

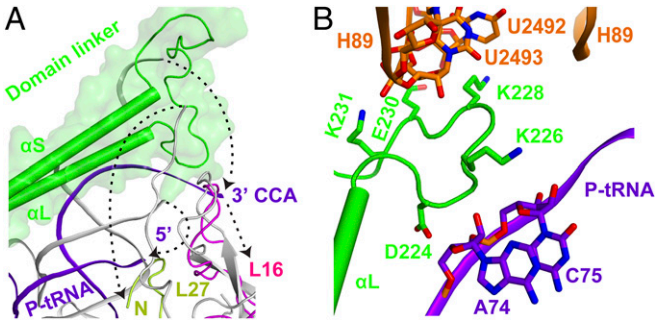


**Fig. 4.** Major conformational changes in ribosomes surrounding the MsrE domain linker. (*A*) Comparison of the acceptor stem of the P-site tRNA (purple), the N-terminal domain of r-protein L27 (forest green), and the r-protein L16 (violet) in the MsrE-ribosome complex and in the post-peptidyl transferase state ribosome (gray) (30). Conformational changes are highlighted with arrows. (*B*) The shifted 3′ CCA of MsrE-ribosome complex tRNA interacts with the MsrE extended loop. The extended loop residues form bilateral interactions with both the amino acid arm of P-tRNA and H89 of 23S rRNA located at the PTC.

in agreement with its cross-species specificity. Therefore, MsrE can likely function on drug-affected ribosomes across a variety of bacterial species, making it an intriguing target for investigations of antiresistance strategies.

MsrE binding causes dramatic conformational changes in both the ribosome and the P-tRNA. Overall, the 30S subunit of the MsrE-bound ribosome is rotated counterclockwise with respect to the 50S subunit by 2.5 Å, with its head rotated by 3.9° (*SI Appendix*, Fig. S5*A*) compared with the post-peptidyl transfer state ribosome (30, 31). Concurrently, MsrE affects the positioning of the P-tRNA; the acceptor stem shifts by 30 Å toward a site usually occupied by the acceptor stem of the fully accommodated A-tRNA (Fig. 4*A* and *SI Appendix*, Fig. S5*B*), and the anticodon stem loop moves by approximately 5 Å coupled to the ribosome rotation (*SI Appendix*, Fig. S5*B*) but is seen to maintain the codon–anticodon interaction with its cognate mRNA (*SI Appendix*, Fig. S5*C*). Other notable changes include a more open positioning of the L1 stalk and displacement of the N-terminal extension of the r-proteins L27 and L16 from their usual positions in close proximity to the PTC to accommodate the MsrE protein (Fig. 4*A*). Since the N-terminal extension of L27 is known to be crucial for tRNA binding and ribosome functioning (32–34), the MsrE-induced conformation is likely transient, and the original state is restored on MsrE dissociation from the ribosome to resume normal translation.

While both MsrE and EttA belong to the ABC-F family and bind to ribosome E-sites, their interactions with the ribosomes vary significantly. MsrE has the elongated domain linker but lacks the insertions called "arm" and "toe" observed in EttA (*SI Appendix*, Fig. S6*A*). The EttA arm makes additional contacts with the L1 stalk, resulting in an even more open form than that seen in the present structure (*SI Appendix*, Fig. S6*B*). Note that EttA arm has been shown to restrict the ribosome and tRNA dynamics required for translation elongation in response to the availability of ATP (25). The EttA toe region interacts with the r-protein L5 and positions it away from the 30S (*SI Appendix*, Fig. S6*B*). The different positions of MsrE and EttA in the ribosome likely reflects their diverse functions.

**MsrE Extended Loop Displaces AZM from Ribosome.** As mentioned above, the extended loop of the MsrE domain linker binds deep into the PTC/NPET region and causes deformation of the r-protein L16, the N-terminal extension of the r-protein L27, and the acceptor stem of P-tRNA (Fig. 4*A*). Unexpectedly, we observed the positions of the acceptor stems of both classical A- and P-tRNA occupied by the domain liker of MsrE (*SI Appendix*, Fig. S5*B*). The shifted P-site tRNA acceptor stem is likely stabilized by interactions with MsrE residues Asp224–Lys226 (Fig. 4*B*). Furthermore, the residues Arg241, Leu242, and His244 at the tip insert deep even beyond the PTC, with their side chains approaching the macrolide-binding site (Fig. 5 *A*–*C*). In particular, the Leu242 clashes (within 1.8 Å) with AZM when our structure is compared with that of AZM bound to the ribosome (2, 3) (Fig. 5*C*).

The 23S rRNA residue A2062, located in the NPET, is believed to relay the drug-induced stalling signal to the PTC, as mutations of this nucleotide can eliminate stalling (35). The base of A2062 protrudes into the tunnel lumen in drug-free ribosomes (30), whereas it moves closer to the tunnel wall as macrolide and nascent peptide chain fill the tunnel (9, 35). Such a movement would clash with MsrE His244 residue, however (Fig. 5*C*). Instead, A2062 nucleotide is reoriented in the tunnel lumen in the presence of MsrE compared with that in drug-free ribosomes (Fig. 5*C*).

On AZM binding to ribosomes, the nucleotide U2506 was observed to shift toward AZM and to be involved in its binding, corroborating the role of U2506 in macrolide drug action (2, 3). In contrast, the U2506 in the present complex is seen to undergo a significant conformational change away from both the PTC and the macrolide-binding site (Fig. 5*C*). Furthermore, the neighboring U2504, which has been implicated in determining the species-specificity of several PTC A-site–binding antibiotics (e.g., tiamulin) (36), shifts away by approximately 2 Å (Fig. 5*C*). Simultaneously, the next nucleotide, A2503, whose identity was found to be critical for programmed translational arrest (8, 9) and whose C8 methylation by Cfr methyltransferase is known to cause resistance to some macrolides (37, 38), is also deformed from AZM-binding positioning (*SI Appendix*, Fig. S7*A*). These conformational changes in the A-site side of PTC appear to be caused by MsrE extended-loop tip residues, especially Arg241 (Fig. 5*C*). As for the P-site side, U2585, a key player in PTC activity, is displaced away from PTC (>90° flip and a 5-Å movement), consequently forming a stacking interaction with A2602, which is also rearranged from its conformation in ribosomes with AZM (2, 3) and without AZM (30) (Fig. 5*C*).

The inherent flexibility of the universally conserved residues U2506 and U2585 at active sites is essential for their pivotal role in peptide transfer (30, 39, 40). For instance, the 180° flip of U2585 caused by the ErmCL nascent chain and macrolide interplay in the NPET prevents the stable binding and accommodation of A-site tRNA, leading to inhibition of peptide bond formation and the translation of downstream macrolide resistance protein ErmC (41). The movement of U2585 caused by macrolide erythromycin binding is apparently accompanied by repositioning of A2602 (16). The function of the universally conserved A2602, essential for peptidyl-tRNA hydrolysis to release nascent peptide, is highly dependent on its positioning (42). Here the residue Lys233 in MsrE is likely involved in stabilizing the reorientation of A2602 (Fig. 5*B*).

Our mutagenesis study on MsrE revealed that mutation of Arg241, Leu242, or His244 to Ala results in a significantly diminished ability of MsrE to confer AZM resistance in vivo (*SI Appendix*, Table S2) and to rescue AZM-affected translation in vitro (Fig. 5*D*). This finding demonstrates their crucial role in mediating macrolide resistance, which is consistent with our structural information. Furthermore, truncation of this extended loop region (residues Lys216–Lys254, Δ loop) or even mutation of only the two residues (Arg241Ala/His244Ala) completely abolished its ability to mediate AZM resistance (Fig. 5*D* and *SI Appendix*, Table S2). This finding demonstrates that the extended loop region, particularly the two residues Arg241 and His244, is essential for displacing AZM from its binding site through strong interactions with ribosomes. Interestingly, Lys233, Arg241, Leu242, and Lys246 are universally conserved in the Msr subfamily, whereas His244 is found only in MsrE and MsrD, substituted by small residues Ala and Ser in MsrA and MsrC, respectively (*SI Appendix*, Fig. S1). The Arg241Ala and His244Ala mutations or even loop deletion had no significant effect on the ribosome binding efficiency (*SI Appendix*, Fig. S3*B*), but did render the MsrE protein unable to recover the AZM-affected translation in vitro (Fig. 5*D*).

Finally, we tested whether binding of MsrE could displace AZM and release it from the ribosome. Quantification of AZM copelleting with ribosomes revealed that incubation of the AZM-ribosome complex with MsrE indeed reduced the amount of AZM

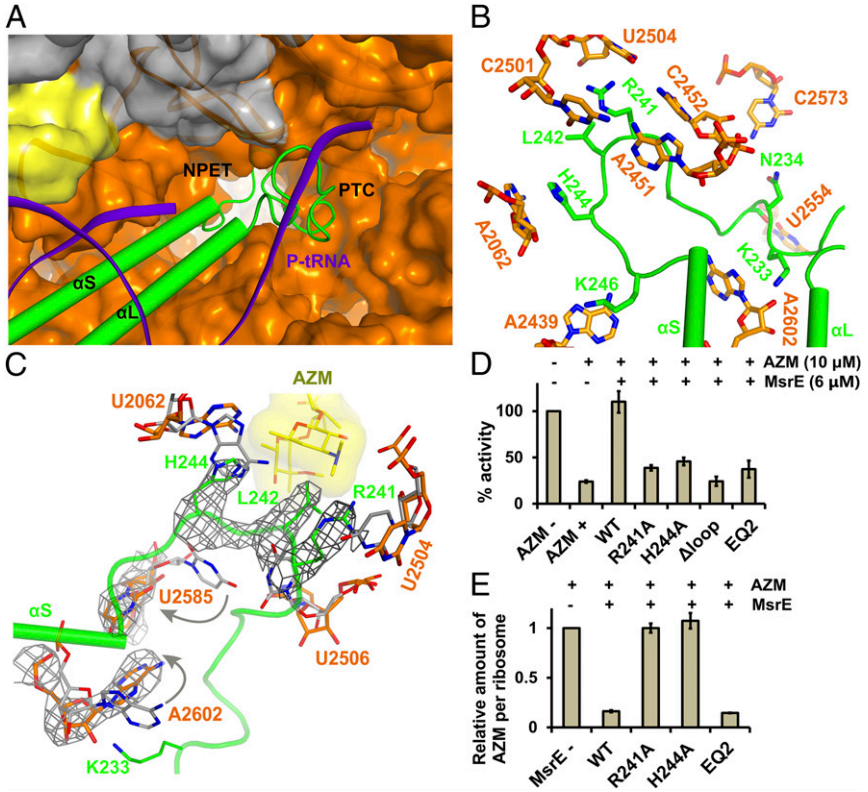


**Fig. 5.** Structure and function of the MsrE domain linker extended loop. (*A*) The MsrE domain linker extended loop projects into the PTC/NPET region. The 50S subunit is shown in surface representation, and the P-tRNA acceptor stem is shown in cartoon representation. (*B*) Orientation of the MsrE extended loop and surrounding key PTC residues. (*C*) Conformational changes in the PTC and macrolide-binding site in the MsrE-ribosome complex compared with the post-peptidyl transfer state ribosome (gray) (30). Conformational changes are indicated by arrows. For reference, the AZM-binding mode is shown based on AZM-ribosome X-ray crystal structure (3). (*D*) Effect of WT and mutant MsrE proteins on *E. coli*-derived in vitro transcription/translation assay inhibited by AZM. Results are means of three independent repeats; error bars represent SDs. The uninhibited condition served as a standard. EQ2, MsrE(E104Q/E413Q) mutant. (*E*) Effect of MsrE on AZM displacement from ribosomes. Ribosomes treated with AZM were incubated with WT and mutant MsrE proteins preincubated with AMP-PNP and ATP, respectively. Ribosomes were pelleted through a sucrose cushion, and AZM was quantified using mass spectrometry. Results are means of three MS assays; error bars represent SDs. The no MsrE addition condition served as a standard.

associated with ribosomes (Fig. 5*E*). Furthermore, our data demonstrate the importance of the loop residues Arg241 and His244, as mutating either to Ala resulted in complete loss of AZM release activity (Fig. 5*E*). Curiously, when the lesser binding efficiency of the ATP hydrolysis-deficient mutant E104Q/E413Q (*SI Appendix*, Fig. S3*B*) is taken into account, its drug displacing effect is comparable to that of WT MsrE (Fig. 5*E*). Therefore, the role of ATP hydrolysis in MsrE resistance activity is likely due to its importance in turnover.

Regardless of the size of the macrolactone ring, all macrolides are oriented in the ribosomal tunnel in a similar manner involving a hydrogen bond between their desosamine hydroxyl and the N1 atom of A2058 in the 23S rRNA (2, 3). Furthermore, the binding of macrolides to ribosomes is stabilized by the tight hydrophobic packing of the lactone ring against the conserved U2611 and A2057, as well as the ionic interaction between desosamine and the phosphate group of G2505. In addition to the aforementioned notable changes for several crucial PTC residues on MsrE binding (Fig. 5*C*), some minor changes are observed in 23S rRNA (U2609 and U2611 on one side and A2058 and A2057 on the other side) and r-protein L22 (Arg90 at the tip of elongated hairpin) involved in formation of the macrolide-binding site in the tunnel (*SI Appendix*, Fig. S7*A*). As a result, the tunnel around the macrolide- binding site widens by approximately 1.5–2 Å.

Taken together, the MsrE extended loop mediated allosteric relay of changes to the PTC and NPET in the vicinity of the macrolide-binding site likely contributes to the dislodgement of AZM from the tunnel. Consistent with this notion, many nucleotide modifications mediating antibiotic resistances do not involve direct interactions with the drug, but instead involve reshaping of the binding pocket to release the drug.

It appears that the composition and length of the extended loop correlate with antibiotic resistance profiles of ARE ABC-F proteins (*SI Appendix*, Fig. S1). Indeed, detailed mutational analysis of VgaA linker residues identified a short stretch of residues 212–220 whose composition determines the efficiency as well as the specificity of antibiotic resistance (27, 43). Based on sequence alignment (*SI Appendix*, Fig. S1), this short stretch corresponds to the region Arg241-Leu242, the importance of which can be rationalized by our structural information. The diverse nature of ARE ABC-F domain linker extended loops correlating with the differences in their drug specificities suggests that bacteria have evolved a plethora of mechanisms to protect the ribosome from PTC- and NPET-targeting antibiotics.

## Discussion

We propose the following mechanism for MsrE, which appears to be universally conserved for ARE ABC-F proteins that confer resistance to translation elongation inhibitors, which trap the ribosome with a tRNA in the P-site (1). The ATP form MsrE recognizes the stalled ribosome with a peptidyl-tRNA in P-site and a bound AZM blocking the NPET. MsrE subsequently binds to the ribosomal E-site, with its domain linker inserting into the PTC/NPET region and stabilizing the P-tRNA to prevent its drop-off. To accommodate the MsrE extended loop into the PTC region, the tRNA is shifted away from 50S, and its acceptor stem is bent toward the A-site. The extended loop approaches the bound AZM

and causes its release allosterically through a combinative effect of structural displacement and ribosomal conformational changes taking place in PTC and NPET. The drug likely leaves the ribosome through the PTC rather than the NPET given the structural constrictions, especially when the nascent chain is present.

Interestingly, a comparison of the present complex, the ErmBL nascent peptide trapped ribosome complex (11), and the post-peptidyl transfer ribosome structure (30) reveals that the conformation of the P-site tRNA is similar in the latter two but undergoes a significant conformational shift when MsrE is bound to the ribosome (Fig. 4*A*). There is a possible passageway for the nascent chain of the P-tRNA distorted by MsrE given the available space and structural flexibility around this area in the present complex (*SI Appendix*, Fig. S7*B*); however, understanding the detailed interactions of MsrE with the nascent chain of P-tRNA requires a high-resolution structure of MsrE-bound ribosome with a P-tRNA carrying a nascent chain. ATP hydrolysis on MsrE likely drives its two ABC domains apart into a conformation that is no longer compatible with ribosome binding, thereby triggering the release of MsrE. With MsrE and the drug released, peptidyl tRNA is presumably returned to the P/P-site, and the nascent chain can likely proceed to the tunnel without any obstacles, so that the translation can resume. The nascent chain likely blocks drug rebinding, and MsrE rebinding is hindered by deacylated tRNA progression into the E-site when translation proceeds, as has been proposed for EttA (26). In a sense, ARE ABC-F proteins are similar to ribosome protection proteins TetM and TetO, which are homologous to EF-G and bind to the ribosome A-site to displace tetracycline from ribosomes, leading to drug resistance (44, 45).

## Materials and Methods

The MsrE-ribosome complex was reconstituted and used for cryo-EM grid preparation. Grids were analyzed with an FEI Titan/Krios cryo-transmission electron microscope (Thermo Fisher Scientific) operated at 300 kV. Data were acquired automatically in movie mode as sets of 20 frames at a nominal magnification of 75,000×. Particles picking and data processing were done in Relion 2.0. A total of 310,270 particles were used for reference-free 2D classification, and nonribosome particles were removed. The remaining 186,654 particles were used for 3D classification using empty 70S ribosome as a reference. Finally, 127,778 particles with homogenous density for MsrE and P-tRNA were used for final reconstruction with statistical movie processing. Final reconstruction yielded a map of 3.6-Å resolution, as determined using the gold standard Fourier cell correlation criteria in Relion (*SI Appendix*, Fig. S4). The MsrE model was built using a sequence from *P. aeruginosa* PASGNDM699. Initial docking of the 50S, 30S, and tRNA (Protein Data Bank ID code 5AA0) structures and the MsrE model into cryo-EM maps was performed in Chimera. Structures were subsequently rigid-fitted manually and refined in Coot. More details on the study methodology are provided in *SI Appendix, Materials and Methods*.

**ACKNOWLEDGMENTS.** This work was supported by the Singapore National Research Foundation (Grant NRF-RF2009-RF001-267, to Y.-G.G.) and the Ministry of Education of Singapore (Tier II Grants MOE2014-T2-1-083 and MOE2017-T2-1-106, to Y.-G.G., and MOE2016-T2-1-010, to L.Y.).

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