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## **ABC-F proteins in mRNA translation and antibiotic resistance.**

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**Abstract**

The ATP binding cassette protein superfamily comprises ATPase enzymes which are, for the most part, involved in transmembrane transport. Within this superfamily however, some protein families have other functions unrelated to transport. One example is the ABC-F family, which comprises an extremely diverse set of cytoplasmic proteins. All of the proteins in the ABC-F family characterized to date act on the ribosome and are translation factors. Their common function is ATP-dependent modulation of the stereochemistry of the peptidyl transferase center (PTC) in the ribosome coupled to changes in its global conformation and P-site tRNA binding geometry. In this review, we give an overview of the function, structure, and theories for the mechanisms-of-action of microbial proteins in the ABC-F family, including those involved in mediating resistance to ribosome-binding antibiotics.

**Keywords:** ABC superfamily, ABC-F protein family, mRNA translation, translation regulation, antibiotic resistance, ATPase.

**Abbreviations:**

PTC : peptidyl transferase center

NPET : nascent peptide exit tunnel

A/P/E-sites : ribosomal Acceptor (A), peptidyl (P), exit (E) sites

Cryo-EM : Cryo-electron microscopy

## 1. Introduction

The ATP Binding Cassette protein (ABC) superfamily is one of the largest protein families in all kingdoms of life. As of April 2019, the Pfam database reported 681,506 sequences with the ABC\_tran (PF00005) sequence profile, making it the largest superfamily in the database. The fundamental molecular function of ABC domains is to dimerize upon ATP binding to form an “ATP-sandwich dimer”. This canonical structure can be either a homodimer formed by two identical ABC domains or a heterodimer formed by two non-identical but homologous ABC domains located either in the same polypeptide or in different polypeptides. Every ABC domain has Walker A and B motif ATP-binding/hydrolysis motifs homologous to those in the F1 ATPase, the AAA<sup>+</sup> ATPases, and superfamily I and II helicases [1]. The hallmark of the ABC superfamily is a five-residue “Signature Sequence” or “C motif”, most frequently LSGGQ, which resides in a subdomain comprising a bundle of three  $\alpha$ -helices (ABC $\alpha$  subdomain) that is a unique and defining feature of ABC domains [2] (see **supplementary figure 1**). This motif in one ABC domain forms a composite ATP-binding site with the Walker A/B motifs from a second ABC domain, and the canonical ATP-sandwich dimer structure referred to above has two such ATP-binding sites in a symmetrical or pseudo-symmetrical conformation.

As illustrated by the other articles in this special edition of Research in Microbiology, the vast majority ABC superfamily proteins are transporters and have ABC domains associated with transmembrane domains. Nevertheless, there are a very large number of cytoplasmic proteins within the superfamily that are not involved in transport but still have ABC ATP-binding domains with canonical architecture. These proteins include the DNA repair enzymes UvrA [3, 4] and Rad50 [5], the eukaryotic translation factors eEF3 [6] and ABCE1/RLI1 [7-10], and the much larger ABC-F protein family. ABC-F proteins represent the largest family of soluble proteins in the ABC superfamily, and all of them that have been functionally

characterized to date, show some involvement in the protein synthesis process. The available data suggest that all ABC-F proteins are likely to be involved in the modulation of the stereochemistry at the PTC in the ribosome, and some of them are more specifically microbial antibiotic resistance factors that act on antibiotics that bind near this region of the ribosome. These so-called antibiotic resistance (ARE) proteins in the ABC-F family are often encoded on mobile elements that facilitate their spread and expansion. [11, 12] ARE ABC-F proteins confer resistance to most of the antibiotics that bind to the 50S subunit of the ribosome, including the widely used macrolides and ketolides [13-20]. They mediate resistance against the clinically crucial antibiotic Linezolid [21], which is used in the treatment of vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* nosocomial strains[22]. It was first proposed that ARE ABC-F proteins function as antibiotic exporters, but recent publications make it clear that they act directly on the ribosome [14, 15, 18, 19, 23].

## 2. The ABC-F proteins family

ABC-F proteins comprise the most widespread family of soluble proteins within the ABC superfamily. They have two tandem ABC domains in a single polypeptide chain separated by a 60-to-100 residue linker sequence [24, 25] that is the defining feature of the ABC-F family. This linker, now called the P-site tRNA Interaction Motif (PtIM) for reasons explained below, was originally identified by the Pfam database [26] as a unique and conserved domain based on large-scale automated sequence analyses performed before any structural data were available for ABC-F proteins. The Pfam curators designated this domain ABC\_tran\_Xtn (PF12848).

The first reported x-ray structure of an ABC-F protein, the Energy-dependent Translational Throttle A, or EttA, combined with the cryogenic electron microscopy (cryo-EM) image reconstruction of EttA in complex with a 70S ribosome (both described below)

demonstrated that the PF12848 domain linking the tandem ABC domains in ABC-F proteins comprises an  $\alpha$ -helical hairpin containing an inter-helical loop or “Tip” of variable length [25, 27]. Because the cryo-EM structure showed direct binding of this  $\alpha$ -helical hairpin to a tRNA in the peptidyl-tRNA-binding (P) site of the ribosome, this domain was named P-site tRNA Interaction Motif (PtIM). All of the ABC-F proteins that have been structurally characterized to date show the PtIM domain adopting an equivalent  $\alpha$ -helical hairpin structure and making an equivalent P-site tRNA interaction.

ABC superfamily proteins from several other families have an architecture with two tandem ABC domains and interact with ribosomes, but they lack the PtIM domain, which is a unique and defining feature of proteins in the ABC-F family. Noteworthy examples are the ABC-E and eukaryotic Elongation Factor 3 (eEF-3) families, which interact with the ribosome very differently than ABC-F proteins and have unrelated biochemical functions. The ABC-E protein binds in the aminoacyl-tRNA-binding (A) site on the ribosome [9], while the eEF-3 protein binds above the tRNA exit (E) site [28]. These structural observations reaffirm the principle that presence of the PtIM domain is a good landmark for the ABC-F protein family because it allows reliable discrimination of homologous proteins that interact with the ribosome differently and have unrelated functions. In this review, we will include as members of the ABC-F family only proteins containing a PtIM domain identifiable through sequence-profiling analysis. While this approach does create a risk of excluding ABC proteins with very strongly diverged PtIM domains, it has the advantage of systematically excluding proteins from structurally distinct families that have different functional interactions with ribosomes, including ABC-E, eEF-3 and its homolog New1, and the *E. coli* protein Ribosomal-Bound ATPase (RbbA) [29, 30], a membrane-bound ABC protein that interacts with the ribosomes.

Phylogenetic studies of ABC protein families are difficult due to technical factors related to the pattern of sequence conservation in the larger superfamily. When distantly

related and therefore widely diverged ABC proteins are compared, the ABC domains generally share the strongest homology. However, the level of sequence identity is still relatively weak when comparing ABC domains from functionally diverged ABC-F proteins, which significantly complicates efforts to assess their phylogenetic relationships. The average percent identity levels between the ABC domains in different ABC-F proteins is generally under 30%, while the percent identity between ABC domains from completely different protein families within the superfamily can be as high as 25%. Rudimentary statistical theory indicates that, given an expected probability that 30% of the residues will be identical between two domains  $\sim 225$  residues in length, the expected value of the standard deviation in percent identity is  $\pm 3\%$  (*i.e.*,  $(p*(1-p)*length)^{-1/2}/length$ ), meaning the mathematical uncertainty in percent identity between the ABC domains in two different ABC-F families is similar in magnitude to the expected difference compared to ABC domains from other branches of the ABC superfamily. Therefore, simple pairwise alignment procedures are unreliable in assessing relationships between different ABC-F proteins, and sequence profiling methods of some kind must be used to make such assessments. The most straightforward and reliable approach to identifying ABC-F family proteins is thus based on the presence or absence of the PtIM as assessed using sequence profiling.

According to this classification principle, ABC-F proteins are found in all eukaryotes and the vast majority of eubacteria, but are absent in archaeobacteria (**Fig. 1**). Interestingly, another soluble ABC protein family involved in translation, the ABC-E family is present only in archaeobacteria and eukaryotes. The ABCE1 protein family has only one representative per organism which functions as ribosome-recycling factors in those two kingdoms [7-10], while in bacteria this function is carried out by the Ribosome-Recycling Factor protein family [31], which is unrelated to the ABC superfamily [32]. The functions of the ABC-F protein family could be fulfilled by distinct evolutionary units in archaeobacteria.

Four representatives of the ABC-F family are present in *Escherichia coli* K12, two in *Saccharomyces cerevisiae*, five in *Arabidopsis thaliana*, and three in *Homo sapiens* (**Fig. 1**). The algae *Chlamydomonas reinhardtii* has five ABC-F proteins, and one of these (A8JCJ9) has an N-terminal chloroplast transit peptide, as does one of the ABC-F proteins in *A. thaliana* (Q9FJH6), suggesting that an ABC-F protein is necessary for chloroplast function. Consistent with such a role, two ABC-F proteins are encoded in the genome of the cyanobacteria *Synechocystis*. Minimal genomes, such as the synthetic *Mycoplasma mycoides* strain Syn 1.0, generally encode an ABC-F protein (MMSYN1\_0118), suggesting an important function for the protein [33], but ABC-F genes are generally not essential for eubacterial viability, and *Mycoplasma mycoides* strain Syn 3.0 with a more reduced genome does not encode any ABC-F protein [34].

More than 25 different ABC-F protein paralogue groups can be identified in eubacteria. The four paralogues encoded in the *E. coli* genome (EttA, YbiT, YheS and Uup) all seem to modulate the activity of the ribosome, but deletion of each of these non-essential genes produces a different phenotype. *Bacillus subtilis* encodes five ABC-F proteins (**Fig. 1A**) that remain functionally uncharacterized with the exception of VmlR, an ARE ABC-F that mediates antibiotic resistance [18].

Along with the PtIM, two other structural features not found in other ABC superfamily proteins are found in many, although not all, ABC-F proteins. The first is the Arm motif, an  $\alpha$ -helical hairpin inserted into ABC $\alpha$  subdomain in the N-terminal ABC domain at the primary contact site between ABC domains and the transmembrane domains in ABC transporters. The arm instead contacts the L1 stalk in the 50S ribosomal subunit in ABC-F complexes with ribosomes (as described further below). The second ABC-F-specific structural feature is the C-terminal domain (CTD) that that was first characterized in the protein Uup [35-37]. This small domain, identified by Pfam as ABC\_tran\_CTD (PF16326), forms a two-stranded  $\alpha$ -helical



coiled-coil that can bind DNA [36] and RNA [18].

### 3. ABC-F translation factors

While ABC-F proteins have been implicated in multiple cellular stress responses, all of their functions that have been rigorously characterized to date are related to protein synthesis [15, 18, 19, 23-25, 38-41]. Therefore, it is likely that the proteins members of this family essentially function as translation factors. In eukaryotes, deletion or overexpression of the ABC-F genes creates pleiotropic phenotypes. The gene encoding the ABC-F protein ARB1 is essential in the yeast *Saccharomyces cerevisiae*, and its depletion impairs ribosome biogenesis, the encoded protein is also shuttling from the nucleus to the cytoplasm [39, 42]. The other yeast ABC-F protein, GCN20 is involved in regulating translation initiation in response to amino-acid starvation [38, 43, 44] but this activity is mediated by an N-terminal domain and remains unchanged when the ABC domains are deleted. The GCN20 homologue in plants is involved in the oxidative stress response [45]. Human ABCF1 (ABC50) participates in the initiation of translation [40, 46] and promotes translational accuracy [47], and it has also been proposed to participate in an alternative translation-initiation mechanism at N6-methyladenosine [48]. These functions are all related to translation, but recent observations suggest that ABCF1 also functions as an E2 Ubiquitin-Conjugating enzyme that regulates the inflammatory response in macrophages [49]. ABCF1 is believed to be an important regulator of the innate immune response to viral DNA and RNA [50]. The ABCF2 protein has been linked to bacterial infection [51] as well as cancer [48], but its role in these processes is unclear. One of the five ABC-F proteins (A8JC09) in the algae *C. reinhardtii* is known to be associated with the flagella apparatus, but its function also remains unknown [52].

*E. coli* EttA, the first ABC-F protein to have its structure and detailed biochemical function elucidated, functions as a translational regulatory factor that controls synthesis of the

first peptide bond in a nascent protein dependent on cellular ADP:ATP ratio [27]. The crystal structure of *E. coli* EttA was used to design mutants for the seminal studies establishing its function. One such mutant contained dual glutamate-to-glutamine mutations in the catalytic bases following the Walker B motifs in both ABC domains (Glu-188 and Glu-470), which prevents ATP hydrolysis and traps EttA in a closed ATP-bound conformation [53]. This EQ<sub>2</sub> mutant was used to prove that EttA functionally interacts with ribosomes *in vivo* and *in vitro*. Enzymological experiments together with cryo-EM image reconstruction demonstrated that EttA binds in the E-site of 70S ribosome initiation complexes (70 ICs) bearing the acylated initiator tRNA (*i.e.*, fMet-tRNA<sup>fMet</sup>) in the P-site [27]. Expression of EttA-EQ<sub>2</sub> in *E. coli* inhibits growth due to inhibition of protein synthesis [27]. In a series of *in vitro* translation assays conducted in presence of EttA, increasing the concentration of ADP inhibits the synthesis of the first peptide bond in a reporter peptide, while addition of ATP restores its synthesis [25]. Furthermore, in equivalent assays performed in the presence of ATP, EttA slightly accelerated the formation of the first peptide bond in the nascent protein [27], and the EQ<sub>2</sub>-mutations that lock EttA in the ATP-bound state trap ribosome complexes containing the resulting dipeptide. Based on these results, the authors proposed that EttA can regulate the first committed step in the synthesis of a new protein based on ADP/ATP concentration ratio. Consistent with this model, *E. coli* cells with the *ettA* gene knocked out show a severe decline in competitive fitness in stationary phase cultures that have exhausted the metabolic resources in the growth medium [25].

*In vivo* expression of EQ<sub>2</sub> mutant variants of YbiT, YheS and Uup, the three *E. coli* EttA paralogues, reduces growth rate and inhibits protein synthesis [24], but at different levels for the different proteins. This suggests that all of the ABC-F paralogues in *E. coli* functionally interact with ribosomes but they may have different affinities or recognize different functional states. Furthermore, overexpression of Uup can rescue the cold-sensitive phenotype of an *E.*

*coli* strain deleted of the gene encoding BipA, a protein involved in the assembly of the 50S ribosome subunit [24], and these observations also support functional interactions with the ribosome. Deletion of the *uup* gene in *E. coli* compromises bacterial competitiveness [54], indicating that, like EttA, it is important for cellular fitness. Deletion of the *uup* gene in *E. coli* also increases the frequency of precise excision of transposons *Tn10* and *Tn5* [55-57]. The activity of Uup in promoting excision requires the ATPase activity and the CTD, which is known to interact with DNA [55, 57], but it is unknown whether this activity reflects a translational regulatory effect dependent on ribosome interaction or involves a different activity or interaction of Uup.

#### 4. ABC-F proteins mediating antibiotic resistance

A large proportion of clinically useful antibiotics act on the protein synthesis machinery by direct interaction with the ribosome. They affect translation by interfering with the high-fidelity decoding mechanism via several different mechanisms. Aminoglycosides bind to the 16S ribosomal RNA (rRNA) in the 30S subunit to prevent (orthosomycins) or impair (tetracyclines) the binding of aminoacyl-tRNA to the A-site. Macrolides, ketolides, lincosamides, oxazolidinones, streptogramins, pleuromutilins, and amphenicols all target the PTC or nascent peptide exit tunnel (NPET) to block polypeptide chain elongation [58, 59].

PTC-targeting antibiotics, such as chloramphenicol, group A streptogramins, lincosamides, pleuromutilins and oxazolidinones, inhibit protein elongation by preventing peptide bond formation and/or the translocation of tRNA from the aminoacyl-tRNA-binding (A) to the P site on the ribosome [59]. The prevalence of PTC-interacting antibiotics is believed to reflect, first, the large number of crevices in this structure allowing binding of small molecules with high affinity and, second, the fact that the PTC needs a high degree of structural flexibility to function efficiently. This second factor implies any interference with its flexibility

by drug binding or by mutations causing drug resistance are likely to hamper the speed and/or accuracy of translation, meaning any rescue mutations within the PTC are likely to seriously reduce fitness [60]. NPET-targeting antibiotics, such as macrolides, ketolides, and group B streptogramins, bind at a short distance from the PTC. Binding of these antibiotics obstructs the NPET and restricts the placement of the nascent chain in the tunnel, slowing down or even stopping its progression. Peptide chain elongation is stopped in a context-specific manner that depends on the nature of the nascent chain and the structure of the drug [61]. There is now a rich body of evidence indicating that peptide synthesis inhibition by chloramphenicol and oxazolidinones is influenced by the nature of the amino acids forming a peptide bond in the PTC [62]. Therefore, antibiotics targeting the PTC/NPET region can be viewed as translation modulators rather than simply translation inhibitors.

The ARE ABC-F proteins confer resistance to growth-inhibiting effects of these translation modulators (**Fig. 1B**). Cross-resistance mediated by individual ARE ABC-F protein to different antibiotics correlates with spatial overlap in their binding sites (**Table 1**). Three distinct resistance phenotypes can be distinguished: (i) the PhO phenotype which is defined by resistance to phenicols and oxazolidinones and exemplified by the Optr(A) and Pox(A) proteins; (ii) the MKS<sub>B</sub> phenotype which is defined by resistance to macrolides, ketolides, and group B streptogramins and exemplified by Msr proteins; and (iii) the PLS<sub>A</sub> phenotype which is defined by resistance to pleuromutilins, lincosamides, and group A streptogramins which is exhibited by multiple variant proteins arising from distinct bacterial lineages [24]. To date, only some ARE ABC-F proteins mediating the PLS<sub>A</sub> resistance phenotype have been found to be chromosomally encoded in all strains of a eubacterial species. Examples include the *vmlR* gene in *B. subtilis*, the *lsaA* gene in *E. faecalis*, and the *sala* gene in *Staphylococcus sciuri* [63]. Neither *lsaA* nor *vmlR* has yet been found in other bacterial species [16], presumably because they are resident genes that are not carried by mobile genetic elements such as plasmids or

transposons. In contrast, *vga* related gene can disseminate easily from strain to strain because they are carried by mobile genetic elements (**Fig. 2**). Similarly, for ARE ABC-Fs mediating the PhO and MKS<sub>B</sub> phenotypes encoded by *optr(A)* or *pox(A)* genes and *msr* related genes, respectively (**Fig 1B**), there are many examples of horizontal gene transfer that have been reported [11, 17, 64-67] (**Fig. 2**). The accepted nomenclature for ARE ABC-F genes carried on a mobile genetic element is to put in parentheses and not italicizing the letter representing the class to which the gene belongs [68].

There is one case reported in the literature in which a proto-resistance gene encoding an ABC-F protein has evolved by a single mutation to confer significant antibiotic resistance. A transition mutation (C1349T) in the *eatA* gene of *E. faecium* produces an amino acid substitution (Thr450Ile) within the Walker B motif of its ABC2 protein domain. This mutation results in an increase of antibiotic resistance (32-fold for lincomycin, 16-fold for dalfopristin and 64-fold for tiamulin) [69]. The mutant variant of the *eatA* gene containing this single critical antibiotic-resistance-generating mutation is called *eatA<sub>v</sub>*. This example demonstrates the remarkably easy way some bacteria can switch from susceptibility to resistance against antibiotics targeting the 50S ribosomal subunit.

The phylogenetic tree of bacteria is informative to predict which species and strains may encode functional ARE ABC-F proteins that have not yet been characterized (**Fig. 1B**). Actinobacteria are the source of most of the antibiotics to which ARE ABC-F proteins confer resistance. Except for oxazolidinones which are synthetic drugs, all the others are natural products produced by strains that have biosynthesis loci and also resistance loci that can contain genes that mediate self-immunity via multiple molecular mechanisms. VarM, LmrC and OleB are well-known examples of ARE ABC-F proteins likely to confer resistance and thereby self-immunity in strains producing streptogramins, lincomycin and oleandomycin, respectively (**Fig. 1B**). Whole genome sequencing data have revealed genes encoding ARE ABC-F proteins

in many different phylogenetic lineages [24], and these proteins could potentially confer resistance to novel inhibitors or modulators of the 50S ribosomal subunit function. In this context, the ARE ABC-F proteins Tva(A) and Tva(B) from *Brachyspira spp.* are noteworthy candidates to be evaluated for potential functions mediating antibiotic resistance [70].

The first strong evidence that Vga(A) confers resistance by acting on the ribosome was presented by Lenart *et al.* [14], while direct interaction of both Vga(A) and LsaA with the ribosome was established by the biochemical study of Sharkey *et al.* [15]. These authors demonstrated that addition of each of the purified ARE ABC-F proteins to *in vitro* translation reactions [15] relieves some of the inhibition of protein synthesis produced by lincomycin or virginiamycin M antibiotics.

The mechanism by which several ARE ABC-F proteins confer antibiotic resistance is the subject of several recent studies that used a combination of biochemical and structural analysis to unambiguously demonstrate the role of these proteins in protecting protein synthesis by ribosomes. Biochemical and structural studies of Msr(E) and VmlR bound to ribosomes from *E. coli* and *B. subtilis*, respectively, have confirmed that protein-specific structural interactions are required to generate resistance, but these studies have also stressed a great number of similarities in their interactions with ribosomes. Both proteins bind in the E site and extend their PtIMs into the PTC/NPET in the 23S rRNA of the 50S subunit in a similar geometry to the ABC-F protein EttA. However, the PtIMs in Msr(E) and Vml(R) are significantly longer and extend further into the PTC/NPET than that of EttA, which is a not an ARE protein, and the increased length of the PtIM in Msr(E) and Vml(R) contributes to their abilities to dislodge drugs bound in this region of the ribosome [15, 18, 19, 23], as discussed further below. The CTD of VmlR and Vga(A) is required for these proteins to confer antibiotic resistance *in vivo* [13, 18].

Other important issues regarding ARE ABC-F protein function remain unresolved. The

two available structures of ribosome complexes do not provide clear answers as to how differences in the length and amino acid composition of the Tip of the PtIM alter specificity to different antibiotics. It penetrates more or less deeply into the PTC/NPET in the two available structures, and mutagenesis studies have begun to evaluate the impact of key amino acid substitutions in the Tip on the resistance specificity of Vga(A) [14] and VmlR [18] (as summarized below). These studies have to yet identify any conserved sequence signatures associated with the PLS<sub>A</sub> resistance phenotype conferred by these proteins, so it is not yet possible to predict the antibiotic resistance profiles for the multiple variants of the VmlR, Vga, and Sal proteins recently identified in whole genome sequences of Gram-positive bacteria.

While all ARE ABC-F proteins are likely to have similar mechanisms-of-action, the details of how they dislodge antibiotics from bacterial ribosomes are not yet understood. Discussion of this topic is presented in the section 7 (**ABC-F mechanism-of-action**). Another issue concerns rebinding of the ejected antibiotic to the ribosome after the ARE ABC-F protein dissociates from the E site, which is required for the ribosome to continue translation. An obligate connection with a dedicated antibiotic efflux system required for efficient functioning has repeatedly been proposed [20, 71, 72]. The possible necessity of coupling to an efflux system is suggested by the genetic linkage of some ARE ABC-F genes with genes encoding protein of the Major Facilitator Superfamily [73]. These types of linkage are observed for: *msr(D)* with *mef(E)* in streptococcal mobile genetic elements, *optr(A)* with *fexA* in enterococcal plasmid, and for *poxt(A)* with *fexB* in staphylococcal plasmid (**Fig. 2**).

The results on the EatA protein summarized above demonstrate that a non-ARE ABC-F protein can naturally mutate to gain antibiotic resistance function [69]. Conceivably, the evolutionary precursor of the ARE ABC-Fs could have been involved in mediating translation in the presence of small molecules that bind the PTC/NPET to reshape the cellular translational landscape [74]. Gram-positive bacteria, which produce antibiotics, could naturally have

evolved ARE ABC-F proteins from such precursor proteins in order to provide self-immunity, and horizontal gene transfer may have disseminated this efficient antibiotic resistance mechanism to all the bacterial phyla.

## 5. ABC-F protein structure

The first structures of an ABC-F protein were reported in 2014, when two coupled papers reported the x-ray crystal structure of *E. coli* EttA together with a Cryo-EM structure of this protein bound to a 70S IC [25, 27]. The 2.4 Å crystal structure contained a nucleotide-free (*apo*) dimer in its asymmetric unit. The authors showed that purified EttA participates in a reversible but slow monomer-dimer equilibrium in which the protein is predominantly a dimer at the 240 μM concentration used for crystallization but a monomer at the ~7-20 μM concentration found *in vivo*. In the crystallographically observed dimer, the N-terminal ABC domain (ABC1) in one protomer interacts with the C-terminal ABC domain (ABC2) from the other protomer and *vice-versa*. In contrast, the ~8 Å cryo-EM structure showed an EttA monomer (**Fig. 3A**) in which ABC1 and ABC2 in the same protomer interact with one another to encapsulate two Mg-ATP molecules in their mutual interface in a geometry equivalent to that observed for the functional ATP-bound conformations of many other proteins in the ABC superfamily. The ATP-bound conformation of EttA was trapped by introducing the EQ<sub>2</sub> mutations (described above) required to obtain stable binding of EttA to the ribosome. In the domain-swapped dimer observed in the crystal structure of EttA, the PtIM adopts a conformation incompatible with ribosome binding, leading the authors to hypothesize that the dimer represents an inactive storage form of the protein or a crystallographic artifact. The *apo* structure of the dimer nonetheless provides a model for the ABC1-ABC2 interaction geometry in the absence of bound ATP, which suggests that ATP hydrolysis will produce a 41° rotation between the tandem ABC domains around an axis that runs roughly between the two ATP



molecules bound in their mutual interface in the active monomeric form of the protein (**Fig. 3A**). This inference is consistent with the function of a wide variety of evolutionarily related proteins as ATP-dependent mechanical clamps.

The two tandem ABC domains in EttA (**Fig. 3A**) and other ABC-F proteins share the canonical structure comprising three subdomains that is present in all ABC superfamily ATPases: (i) an F1-ATPase-like  $\alpha/\beta$  Core subdomain with a complex topology conserved in many other ATPases including F1 and the AAA+ ATPase superfamily (as noted above); (ii) an N-terminal antiparallel  $\beta$ -sheet (ABC $\beta$ ) subdomain that forms a rigid ATP-binding module together with the F1-like Core subdomain, and (iii) a 60-100 residue  $\alpha$ -helical (ABC $\alpha$ ) subdomain containing the ABC superfamily Signature Sequence, canonically LSGGQ but generally LSGGE in proteins, inserted within the primary sequence of the F1-like Core subdomain [18, 23, 25, 75]. The Walker A motif in the F1-like Core is a phosphate-binding loop or “P-loop” that ligates the phosphates of ATP, while Walker B motif in this subdomain is a hydrophobic  $\beta$ -strand terminating in an aspartic acid that contributes to ligating the Mg<sup>++</sup> cofactor of ATP [18, 25, 53]. A short “ $\gamma$ -phosphate linker” at the N-terminus of the ABC $\alpha$  subdomain holds it in a rigid geometry relative to the ATP-binding core when the  $\gamma$ -phosphate group of ATP is bound in the active site, but the ABC $\alpha$  subdomain rotates relative to the ATP-binding core in the nucleotide-free and ADP-bound conformations of ABC domains [18, 23, 25, 75]. The “ $\gamma$ -phosphate linker” has also been called the “Q-loop” because a conserved glutamine at its N-terminus mediates the stabilizing contact to the  $\gamma$ -phosphate group of ATP [2, 53].

As described above, the functional ATPase active sites in ABC-F proteins are composite structures in which the ATP substrate is encapsulated between the Walker A/B motifs in the F1-like Core of one ABC domain and the LSGGE Signature Sequence in the

ABC $\alpha$  subdomain of the other ABC domain [25, 53]. ABC superfamily domains are designed to enable two such composite active sites to form simultaneously in an ATP-sandwich dimer structure, which in the case of ABC-F proteins is a heterodimer like that formed by the ABC1/ABC2 domains in ribosome-bound EttA monomer shown in **Fig. 3A** [27]. ATP hydrolysis weakens the interaction energy between the tandem ATPase domains and may also exert a transient force pushing them apart. Most ABC superfamily proteins have other interactions that tend to pull the ABC domains apart except when ATP is bound in to active sites at their mutual interface [25, 27, 53, 76]. ATP binding therefore generally causes a clamp-like closure of the interface between the ABC domains, while hydrolysis generally causes them to rotate apart as schematized in **Fig. 3A**.

The ABC $\alpha$  subdomains in EttA both have short ribosome-interacting motifs added compared to the canonical subdomain structure observed in most superfamily members. The Arm, alluded to above as being present in many ABC-F proteins, is an ~40 residue  $\alpha$ -helical hairpin inserted into the ABC $\alpha$  subdomain in ABC1; it interacts with the L1 protein in the 50S ribosomal subunit. The Toe, an ~12-residue antiparallel  $\beta$ -hairpin inserted into the ABC $\alpha$  subdomain in ABC2, interacts with the L5 protein in the large ribosomal subunit. ABC2 in EttA also contains an ~15-residue C-terminal extension (CTE) that adopts an extended conformation that packs between the ABC $\alpha$  subdomain in ABC1 and bases in the 23S rRNA spanning helices 76-78 in the L1 stalk of the 50S subunit.

The PtIM forms an  $\alpha$ -helical hairpin that covalently links the tandem ABC domains in EttA and other ABC-F proteins (**Fig. 3 & supplementary Fig. 1**). The first  $\alpha$ -helix in the PtIM, which is located at the C-terminus of ABC1, represents an extension of the final  $\alpha$ -helix present in most ABC superfamily ATPase domains and appears to be rigidly attached to ABC1 based on the available structural information. The Tip of the PtIM which links the two  $\alpha$ -

helices forming the hairpin, makes stereospecific contacts near the peptidyl-transferase center (PTC) in the ribosome. The second  $\alpha$ -helix in the PtIM, which is significantly shorter than the first, is connected to ABC2 by an approximately 20-residue protein segment that adopts an extended conformation. This linker at the C-terminus of the PtIM exhibits a significantly higher degree of flexibility than the N-terminus of the first  $\alpha$ -helix in the PtIM at its point of attachment to the C-terminus of ABC1. The PtIM is therefore likely to move like a rigid body with ABC1 during ATP-dependent conformational changes in ABC-F proteins.

An x-ray crystal structure has been determined at 2.3 Å resolution for wild-type human ABCF1 (ABC50) bound to unhydrolyzed ATP [75]. Both ATP-binding sites in this structure are open, meaning the bound ATP molecules contact the Walker A/B motifs in the F1-like binding cores of each ABC domain but not the Signature Sequences in the opposite ABC domain. Compared to the proper catalytic ATP-sandwich dimer conformation, the ABC domains have rotated away from one another in the same general direction observed in the *apo* structure of EttA but not to the same extent. The authors of the human ABCF1 structure propose that their structure with partially closed ATP-binding sites represents a “pre-activated” conformation that does not close fully to form the composite ATP-binding sites at the ABC1-ABC2 interface until bound to the ribosome. While this inference seems reasonable based on conformational changes previously observed in the ABC domains of some transmembrane transporters [77, 78], further research will be required to evaluate its validity because the conformation of the ABC domains observed in their single human ABCF1 structure could potentially be biased by crystal-packing interactions.

Cryo-EM structures have been determined at 3.5 Å and 3.6 Å, respectively, for the ATP-bound conformations of the ARE ABC-F proteins VmlR [18] and Msr(E) [23] complexed with 70S ribosomes bearing a tRNA in their peptidyl-tRNA-binding (P) sites, and these structures show that the architecture and conformation (**Fig. 3B-C**) of these proteins as well as

their ribosome interactions (**Fig. 4**) are all very similar to those of EttA. These ARE ABC-F proteins both lack the Arm and Toe motifs, but their tandem ABC domains otherwise have exactly the same architecture (**Figs. 3B-C and supplementary Fig. 1**) as EttA. Their PtIMs are both significantly longer than the PtIM in EttA, which produces very different stereochemical interactions with the peptidyl-transferase center (PTC) in the ribosome (as discussed below and shown in **Fig. 5**). The C-terminal extension after ABC2 in EttA is missing in Msr(E) and replaced in VmlR by a longer extension that includes the CTD (ABC\_tran\_CTD/PF16326 – highlighted in **Fig. 4A& 4C**).

Superimposing the ABC1 domains in VmlR and Msr(E) on the ABC1 domain in EttA demonstrates that the ABC1-ABC2 interaction geometries are very similar in the ATP-bound conformations of all three proteins (**Fig. 3B**). The small differences observed in the ABC1-ABC2 interaction angles in the structures parallels the variations seen in the ATP-bound conformations of many proteins in the ABC superfamily [76]. However, the ABC1 domain alignment reveals larger variations in the orientations of their PtIMs, reflecting differences in the packing interactions between the C-terminus of the ABC1 domain and the N-terminus of the first  $\alpha$ -helix in the PtIM (**Fig. 3B**). The analyses presented below of the ribosome-bound structures of these ABC-F proteins suggests that the observed variation in PtIM orientation contributes to their different mechanisms-of-action on the ribosome. Superimposing the 23S RNA in the corresponding ribosome complexes demonstrates that the PtIM in all three ABC-F proteins adopt very similar conformations (**Fig. 3C**), and they bind in an equivalent geometry at the same docking site in the 50S ribosomal subunit (discussed below and shown in **Figs. 4-5**). The observed differences in the packing angle of the PtIM relative to ABC1 (**Fig. 3B**) thus changes the orientation and the ribosome interactions of both of the tandem ABC domains in the different ABC-F proteins (**Fig. 4A**) as well as their interactions with the tRNAs bound to the peptidyl-tRNA-binding (P) site (**Fig. AC & 3C**).

## 6. Ribosome interactions of ABC-F proteins

Analyses of the available structural and functional data suggest that ABC-F proteins generally couple specific stereochemical changes in the PTC to changes in both P-site tRNA interaction geometry and the global conformation of the 70S ribosome (**Figs. 4-5**). The cryo-EM structures of the ATP-bound conformations of EttA, VmlR, and Msr(E) demonstrate that all of these ABC-F proteins bind in a similar geometry in the E site of 70S ribosomal complexes harboring a tRNA in the adjacent P site (**Fig. 4A**). The same structural regions in all three proteins bind to the same segments of both the ribosome and the P-site tRNA, but, remarkably, there is no significant sequence conservation in the segments of the ABC-F proteins mediating these contacts. Despite the lack of sequence conservation, the PtIM from all three ABC-F proteins binds in an equivalent location and orientation pointing into the PTC on the 23S rRNA in the 50S ribosomal subunit (**Fig. 4B**). However, the local packing interactions and binding geometry vary significantly at every other conserved contact site, producing small but significant differences in the global conformation of the 70S ribosome (**Fig. 4A**) and substantially larger differences in the location and orientation of the tRNA bound in its P site (**Fig. 3C, 4C, & 6B**). The most dramatic difference observed in the three available ABC-F/ribosome protein complexes is the location of the CCA acceptor stem of the P-site tRNA, which is located in the PTC in the proper geometry for peptide-bond formation in the EttA complex but shifted 30-35 Å away from the PTC in the VmlR and Msr(E) complexes, where it moves into space occupied by the A site amino-acyl tRNA in actively translating ribosomes. Notably, the E-site binding translation factor Elongation Factor P (EF-P), which promotes efficient synthesis of stall-prone proline sequences, stabilizes a very similar conformation of the P-site tRNA at both its CCA acceptor stem in the PTC the 50S subunit and its anticodon loop in the decoding center in the 30S subunit as EttA, even though EF-P is

structurally unrelated to the ABC-F proteins and maintains the L1 stalk in the 50S subunit in a dramatically different orientation [79-81].

Given the well-established conformational properties of ABC superfamily domains (*e.g.*, as schematized in **Fig. 3A**), ATP binding and hydrolysis by ABC-F proteins is likely to produce dynamic changes in the stereochemistry of the PTC and the interaction geometry of tRNAs bound in either the P site or A site. ATP binding is known to drive closure of the interface between the ABC domains in many well-studied superfamily members [76]. This observation indicates that their lowest energy conformational state generally involves a rotational orientation between the ABC domains that opens the ATP-binding sites at their interface, which implies that domain closure upon ATP binding creates an elastic strain in the protein conformation. This strain will generate forces that push the proteins back into their conformational ground-state upon ATP-hydrolysis. The cryo-EM structure of ribosome-bound EttA-EQ<sub>2</sub> shows that both of its ABC domains make direct contacts in the ribosomal E site when both of its active sites encapsulate ATP in the fully closed conformation. Therefore, ATP hydrolysis by ribosome-bound EttA is likely to exert some degree of force between the 50S and 30S ribosomal subunits, and the observed geometry of the ATP-bound ribosome complex suggests that this force will push the ribosomal subunits apart and towards a more negative rotation state [25]. Based on reasoning explained below, ATP hydrolysis may also change conformation of the PtIM. It is noteworthy that both ATP-binding sites in the ABC-F proteins are exposed to solvent on the external surfaces of their ribosome complexes, making it plausible for ADP/ATP exchange to occur while the ABC-F proteins remain bound in the E site. However, the evidence available to date supports their ATP-bound conformations have higher ribosome-binding affinity than the other nucleotide states, suggesting release from the ribosome may follow ATP hydrolysis prior to ADP release and re-binding of ATP [18, 24, 25].

In all of the available ribosome-bound ABC-F structures, the PtIM docks in an

equivalent geometry into a cradle formed by helices 68, 75, and 93 proximal to the PTC in the 23S rRNA in the 50S subunit (**Fig. 4B**). The other ABC-F protein contacts to the ribosome vary in geometry in the different complexes, but, as indicated above involve the same conserved ribosomal substructures (**Figs. 4A-B**): (i) the ABC $\beta$  subdomain in ABC1 binds to helix 68 in the 23S RNA; (ii) the ABC $\alpha$  subdomain and Walker B helix in the F1-like Core subdomain in ABC1 bind to region encompassing helices 76, 77 and 78 in the L1 stalk of the 23S RNA; (iii) the Arm motif in EttA ABC1 binds to the L1 protein in the 50S ribosomal subunit; (iv) the ABC $\beta$  subdomain in ABC2 binds to region surrounding helix 42 in the 16S RNA in the 30S subunit; (v) the ABC $\alpha$  subdomain in ABC2 binds to the elbow of the P-site tRNA; and (v) the C-terminal region of the F1-like Core subdomain in ABC2 binds to the S7 protein in the 30S subunit. The C-terminal extensions of EttA and VmlR make non-conserved contacts to the ribosome, with the short extension in EttA contacting helix 78 in the 23S RNA and the  $\alpha$ -helical hairpin domain formed by the C-terminal extension in EttA contacting the S7 protein in the 30S subunit.

The conserved binding location/orientation of the stem of the PtIM (proximal to the ABC domains) consistently positions its Tip proximal to the PTC in the 50S subunit of the ribosome. However, the length of the PtIM varies substantially between different ABC-F proteins, resulting in very different conformational and stereochemical effects at the PTC (**Fig. 5**). The Tip of EttA, which has among the shortest PtIMs in the ABC-F family, directly contacts helices 74 and 93 in the 23S RNA and protein L27 as well as the CCA acceptor stem of the P-site tRNA, and this last interaction ostensibly hold the CCA in optimal geometry for peptide-bond formation (**Fig. 5C**). The PtIMs in VmlR and Msr(E) are significantly longer. This adaptation lengthens the  $\alpha$ -helical hairpin region so that it overlaps with the proper docking site of the CCA acceptor stem in the P-site tRNA and makes direct contacts to the PTC and the entrance of the NPET (**Fig. 4C-D**). These contacts are very extensive in the case of

Msr(E) (**Fig. 4D**), which has among the longest PtIMs in the ABC-F family (**Table 1 & supplementary Fig.1**).

ATP hydrolysis by the ABC-F proteins in the ribosome-bound conformation may lead to disruption of its  $\alpha$ -helical hairpin structure. The first  $\alpha$ -helix in this structure seems to be rigidly attached to ABC1, as alluded to above (**Fig. 3B**), while the short linker following the C-terminus of the PtIM also seems to be tightly packed in the interface between the ABC $\alpha$  and F1-like Core subdomains in ABC2. A significant mutual rotation between ABC1 and ABC2 following ATP hydrolysis, which occurs in the well-characterized ABC superfamily proteins, will thus tend to pull apart the  $\alpha$ -helical hairpin in the PtIM, which would dynamically alter the interactions of its Tip with the PTC and also possibly the conformation of the PTC. In this context, it is noteworthy that 36 residues in the PtIM including its second  $\alpha$ -helix are disordered in the crystal structure of human ABCF1 in which the ABC domains have rotated apart to open the interfacial ATP-binding sites [75].

## 7. ABC-F mechanism-of-action

The cryo-EM structures of ribosome-bound ABC-F proteins provide a foundation for understanding their mechanisms-of-action. A detailed hypothesis has been developed for the mechanism by which EttA prevents formation of the first peptide bond in a nascent polypeptide chain in the presence of ADP but promotes it in the presence of ATP [25]. This hypothesis proposes that the ATP-bound conformation of EttA stabilizes the proper catalytic conformation of the CCA stem of the aminoacylated initiator tRNA<sup>fMet</sup> in the PTC, while the ADP-bound conformation of EttA is different due to changes in ABC1-ABC2 interaction geometry and stabilizes instead a catalytically incompetent conformation of the CCA acceptor stem. The first part of this hypothesis has been confirmed by the cryo-EM structure described above (**Fig.**



5C), while the second part remains untested. An explicit direct-drug-displacement hypothesis has been advanced to explain the mechanism by which ARE ABC-Fs produce resistance to ribosome-directed antibiotics. This mechanism (outlined below) may contribute to antibiotic resistance, but seems unlikely to account fully for the antibiotic-resistance activity of ARE ABC-Fs. One baseline issue that any mechanistic hypothesis must address is that the location of ARE ABC-F binding in the E site of the ribosome blocks release of the P site tRNA. Therefore, the ARE ABC-Fs must be released from the ribosome, which presumably happens upon ATP hydrolysis, in order to allow protein elongation to continue, and their release will allow rebinding of the ejected translation-inhibiting antibiotic. Therefore, the kinetics of drug binding to the ribosome and the impact of ribosome dynamics on this process are critical factors in establishing effective resistance, but these processes are difficult to study experimentally. The following mechanistic hypotheses are consistent with the available data including importantly the observation of a P-site tRNA but an empty A site in all of the available structures of ribosome complexes of ATP-bound ARE ABC-F proteins:

*Direct drug displacement.* This hypothesis proposes that binding of the Tip of the PtIM to the PTC/NPET physically displaces antibiotics that interact with this region of the ribosome. Displacement could be mediated either by a steric clash between the PtIM and the antibiotic, or alternatively, binding of the PtIM could induce a conformational change in the PTC/NPET that allosterically disrupts energetically important structural contacts to the antibiotic. The direct-drug-displacement hypothesis has been tested using structure-based mutagenesis experiments on VmlR that gave mixed results [18]. Introducing the F237A mutation into its PtIM greatly reduces the size of the sidechain that penetrates most deeply into the PTC in the cryo-EM structure of ribosome-bound VmlR. This mutation was predicted to eliminate steric clashes with several antibiotics to which VmlR confers resistance and also to reduce the magnitude of the relatively small allosteric conformational changes in the PTC/NPET inferred

to occur upon VmlR binding. The F237A mutation reduced resistance to virginiamycin, which was predicted to have the strongest steric clash with the mutated residue, but not to several other antibiotics that bind at overlapping sites in the PTC/NPET. Notably, F237A did not reduce resistance to lincomycin, which binds at the deepest site in the NPET among the tested antibiotics and should have no steric interaction with F237A-VmlR. Structural analyses predict this mutant protein should still produce 1-2 Å conformational shifts in two nearby bases in the PTC that directly contact bound lincomycin (nucleotides A2450-C2451 in *E. coli* numbering). However, especially because antibiotic binding itself generally produces some allosteric conformational changes in the PTC/NPET, it is unclear if the small conformational change produced by F237A-VmlR would significantly reduce lincomycin binding affinity. While direct drug displacement still might contribute to ARE ABC-F activity, it is not clearly demonstrated yet.

*Dynamic destabilization of drug binding.* This hypothesis proposes that the physiological interaction of the CCA acceptor stem of the P-site tRNA stabilizes antibiotic binding in the PTC/NPET by restricting dynamic fluctuations in these structures that weaken antibiotic binding. In this case, the displacement of the CCA stem driven by ARE ABC-F binding would disrupt its stabilizing effect on the PTC/NPET and thereby increase dynamic fluctuations in this region of the ribosome that promote antibiotic release. This mechanism does not invoke any specific stereochemical interaction between the PtIM and the bound antibiotic or any specific allosteric conformational change in the PTC/NPET, making it consistent with the molecular promiscuity of ARE ABC-F action, *i.e.*, the ability of diverse PtIM Tip sequences to produce resistance to antibiotics with diverse chemical structures that share proximal binding sites in the PTC/NPET but few other molecular properties.

*Drain-snake displacement of drug binding.* This hypothesis proposes that ARE ABC-Fs effectively pull antibiotics out of their interaction sites in the PTC/NPET by dragging the

nascent polypeptide chain past them (**Fig. 7**). Binding of an ATP-bound ARE ABC-F to a ribosome bearing a peptidyl-tRNA in the P site will result in the nascent polypeptide chain attached to that tRNA being pulled out of the NPET during the 30-35 Å displacement of its CCA acceptor stem away from the PTC, as observed in the cryo-EM structures of VmlR and Msr(E) bound to ribosomes (**Figs. 4C & 5D**). This pulling action on the nascent polypeptide chain would exert a force on an antibiotic bound in the NPET to dislodge it from its binding site using a molecular mechanism analogous to the mechanical mechanism used by a plumber employing a flexible metal “snake” to dislodge debris from a clogged drain. Following release of the ARE ABC-F from the E site induced by ATP hydrolysis, the nascent polypeptide chain can retract back into the NPET when the P site tRNA returns to its normal location in which its CCA acceptor stem positions the peptidyl-tRNA linkage in the PTC ready for the next round of peptide bond synthesis. If the antibiotic is not displaced from the PTC/NPET during the first round of ARE ABC-F binding and release, this process can be repeated until the antibiotic is released in a repetitive process. This mechanism is also consistent with the molecular promiscuity of ARE ABC-F action because it relies on frictional force between the nascent polypeptide and the ribosome-bound antibiotic rather than specific stereochemical interactions with either the antibiotic or the PTC/NPEN.

*Alternative A/P site tRNA translocation to disrupt drug binding.* This more speculative variant of the drain-snake hypothesis would enable ARE ABC-Fs to act on antibiotics that block translocation of the peptidyl-tRNA from the A site to the P site after peptide bond synthesis in the PTC. If an ARE ABC-F binds to a ribosome with an empty P site bearing a peptidyl-tRNA in the A site, the affinity of the ARE ABC-F for tRNA could help translocate the A-site tRNA to the P site, perhaps in concert with the action of Elongation Factor G. Because the P-site tRNA conformation stabilized by the ARE ABC-F involves a large displacement of the CCA acceptor stem away from the PTC (**Figs. 4C & 5D**), this alternative A-to-P site translocation

process would be accompanied by the same drain-snake-like action described above pulling the nascent polypeptide attached to the tRNA out of the NPET, which could dislodge antibiotics bound in the PTC/NPET. During the standard protein elongation process on the ribosome, the P site will be occupied by a deacylated tRNA whenever a peptidyl-tRNA is bound in the A site. However, antibiotics blocking tRNA translocation from the A site to the P site could result in an empty P site if deacylated tRNA can slowly dissociate spontaneously from the P site of the antibiotic-stalled ribosome, enabling ARE ABC-F binding to a ribosome with a peptidyl-tRNA in the A site and an empty P site. Alternatively, due to the interaction of ARE/ABC-F proteins with P-site tRNA (**Figs. 3C, 4C, & 5**), binding of one of these proteins to a ribosome with a deacylated tRNA in the P site could help remove that tRNA from the ribosome when the ARE ABC-F is released from the E site following ATP hydrolysis. Such a novel and as yet unproven P-site tRNA release activity for an ABC-F protein would not directly contribute to antibiotic resistance, but it could open up the P site to enable execution of the alternative A/P site tRNA translocation mechanism proposed above to dislodge antibiotics that block peptidyl-tRNA translocation from the A site.

These four mechanisms could potentially all contribute in parallel to the resistance conferred by ARE ABC-Fs to antibiotics that interact with the PTC/NPET of the ribosome, which increases the challenges involved in designing experiments to test them. Importantly, all four mechanisms fundamentally rely on the ability of the PtIM to dynamically modulate the stereochemistry of the PTC and its interactions with the CCA acceptor stem of the P-site tRNA during reversible, ATP-controlled binding of ABC-F proteins in the E site of the ribosome.

## **8. Conclusions**

Numerous studies have shown that proteins of the ABC-F family are translation factors that bind in the ribosomal E site, interact with the PTC of the ribosome, and thereby influence

peptide-bond formation and mRNA translation. However, for many ABC-F proteins and especially for Uup, the results pointing to a function related to mRNA translation by the ribosome have not been connected to the phenotype observed when the corresponding gene is deleted. Except for the models provided by EttA and the ARE ABC-Fs mediating antibiotic resistance, the physiological functions of ABC-F proteins remain unclear. Elucidating these functions and understanding more clearly the exact mechanisms-of-action of ABC-F proteins on the ribosome are the current challenges for related research programs. ABC-F proteins with their two ATP binding sites can be viewed as mediator of information storage in the cell [82] by using one ATP site for mechanical work and the other to monitor the state of the ribosome. This perspective may be helpful for understanding more deeply their roles in living organisms.

It is interesting to note that multiple distinct and distantly related protein families within the ABC superfamily function as translation factors, and more proteins in the superfamily function in translation than in any molecular process other than transmembrane transport. Given that mRNA translation is more fundamental to the chemistry of life on earth and may have evolved earlier than phospholipid membranes [83, 84], it is reasonable to wonder if the translation factors in the superfamily evolved from the transmembrane transporters or *vice versa*. In this context, it is noteworthy that one domain in ribosomal protein S13 is homologous to the ABC $\alpha$   $\alpha$ -helical subdomain that is the defining feature of ABC superfamily domains, raising the possibility that the superfamily could potentially have an evolutionary origin related to mRNA translation.

### **Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this article.

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### Reference list

- [1] Cavanaugh LF, Palmer AG, 3rd, Gierasch LM, Hunt JF. Disorder breathes life into a DEAD motor. *Nature structural & molecular biology* 2006;13:566-9.
- [2] Davidson AL, Dassa E, Orelle C, Chen J. Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiology and molecular biology reviews* : MMBR 2008;72:317-64, table of contents.
- [3] Pakotiprapha D, Samuels M, Shen K, Hu JH, Jeruzalmi D. Structure and mechanism of the UvrA-UvrB DNA damage sensor. *Nature structural & molecular biology* 2012;19:291-8.
- [4] Jaciuk M, Nowak E, Skowronek K, Tanska A, Nowotny M. Structure of UvrA nucleotide excision repair protein in complex with modified DNA. *Nature structural & molecular biology* 2011;18:191-7.
- [5] Lammens K, Bemeleit DJ, Mockel C, Clausing E, Schele A, Hartung S, et al. The Mre11:Rad50 structure shows an ATP-dependent molecular clamp in DNA double-strand break repair. *Cell* 2011;145:54-66.
- [6] Skogerson L, Wakatama E. A ribosome-dependent GTPase from yeast distinct from elongation factor 2. *Proceedings of the National Academy of Sciences of the United States of America* 1976;73:73-6.
- [7] Pisarev AV, Skabkin MA, Pisareva VP, Skabkina OV, Rakotondrafara AM, Hentze MW, et al. The role of ABCE1 in eukaryotic posttermination ribosomal recycling. *Molecular cell* 2010;37:196-210.
- [8] Khoshnevis S, Gross T, Rotte C, Baierlein C, Ficner R, Krebber H. The iron-sulphur protein RNase L inhibitor functions in translation termination. *EMBO reports* 2010;11:214-9.
- [9] Becker T, Franckenberg S, Wickles S, Shoemaker CJ, Anger AM, Armache JP, et al. Structural basis of highly conserved ribosome recycling in eukaryotes and archaea. *Nature* 2012;482:501-6.

- [10] Barthelme D, Dinkelaker S, Albers SV, Londei P, Ermler U, Tampe R. Ribosome recycling depends on a mechanistic link between the FeS cluster domain and a conformational switch of the twin-ATPase ABCE1. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:3228-33.
- [11] Roberts MC. Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS microbiology letters* 2008;282:147-59.
- [12] Kerr ID, Reynolds ED, Cove JH. ABC proteins and antibiotic drug resistance: is it all about transport? *Biochemical Society transactions* 2005;33:1000-2.
- [13] Jacquet E, Girard JM, Ramaen O, Pamlard O, Levaique H, Betton JM, et al. ATP hydrolysis and pristinamycin IIA inhibition of the *Staphylococcus aureus* Vga(A), a dual ABC protein involved in streptogramin A resistance. *J Biol Chem* 2008;283:25332-9.
- [14] Lenart J, Vimberg V, Vesela L, Janata J, Balikova Novotna G. Detailed mutational analysis of Vga(A) interdomain linker: implication for antibiotic resistance specificity and mechanism. *Antimicrobial agents and chemotherapy* 2015;59:1360-4.
- [15] Sharkey LK, Edwards TA, O'Neill AJ. ABC-F Proteins Mediate Antibiotic Resistance through Ribosomal Protection. *MBio* 2016;7:e01975.
- [16] Deng F, Wang H, Liao Y, Li J, Fessler AT, Michael GB, et al. Detection and Genetic Environment of Pleuromutilin-Lincosamide-Streptogramin A Resistance Genes in *Staphylococci* Isolated from Pets. *Frontiers in microbiology* 2017;8:234.
- [17] Fan R, Li D, Fessler AT, Wu C, Schwarz S, Wang Y. Distribution of *optrA* and *cfr* in florfenicol-resistant *Staphylococcus sciuri* of pig origin. *Veterinary microbiology* 2017;210:43-8.
- [18] Crowe-McAuliffe C, Graf M, Huter P, Takada H, Abdelshahid M, Novacek J, et al. Structural basis for antibiotic resistance mediated by the *Bacillus subtilis* ABCF ATPase VmlR. *Proceedings of the National Academy of Sciences of the United States of America* 2018;115:8978-83.
- [19] Murina V, Kasari M, Hauryliuk V, Atkinson GC. Antibiotic resistance ABCF proteins reset the peptidyl transferase centre of the ribosome to counter translational arrest. *Nucleic acids research* 2018;gky050-gky.
- [20] Tatsuno I, Isaka M, Masuno K, Hata N, Matsumoto M, Hasegawa T. Functional Predominance of *msr(D)*, Which Is More Effective as *mef(A)*-Associated Than *mef(E)*-Associated, Over *mef(A)/mef(E)* in Macrolide Resistance in *Streptococcus pyogenes*. *Microb Drug Resist* 2018;24:1089-97.
- [21] Antonelli A, D'Andrea MM, Brenciani A, Galeotti CL, Morroni G, Pollini S, et al. Characterization of *poxTA*, a novel phenicol-oxazolidinone-tetracycline resistance gene from an MRSA of clinical origin. *Journal of Antimicrobial Chemotherapy* 2018;73:1763-9.
- [22] Leach KL, Brickner SJ, Noe MC, Miller PF. Linezolid, the first oxazolidinone antibacterial agent. *Annals of the New York Academy of Sciences* 2011;1222:49-54.
- [23] Su W, Kumar V, Ding Y, Ero R, Serra A, Lee BST, et al. Ribosome protection by antibiotic resistance ATP-binding cassette protein. *Proceedings of the National Academy of Sciences* 2018.
- [24] Murina V, Kasari M, Takada H, Hinno M, Saha CK, Grimshaw JW, et al. ABCF ATPases Involved in Protein Synthesis, Ribosome Assembly and Antibiotic Resistance: Structural and Functional Diversification across the Tree of Life. *Journal of molecular biology* 2018.

- [25] Boel G, Smith PC, Ning W, Englander MT, Chen B, Hashem Y, et al. The ABC-F protein EttA gates ribosome entry into the translation elongation cycle. *Nature structural & molecular biology* 2014;21:143-51.
- [26] Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, et al. The Pfam protein families database. *Nucleic acids research* 2012;40:D290-301.
- [27] Chen B, Boel G, Hashem Y, Ning W, Fei J, Wang C, et al. EttA regulates translation by binding the ribosomal E site and restricting ribosome-tRNA dynamics. *Nature structural & molecular biology* 2014;21:152-9.
- [28] Andersen CB, Becker T, Blau M, Anand M, Halic M, Balar B, et al. Structure of eEF3 and the mechanism of transfer RNA release from the E-site. *Nature* 2006;443:663-8.
- [29] Kiel MC, Aoki H, Ganoza MC. Identification of a ribosomal ATPase in *Escherichia coli* cells. *Biochimie* 1999;81:1097-108.
- [30] Babu M, Aoki H, Chowdhury WQ, Gagarianova A, Graham C, Phanse S, et al. Ribosome-dependent ATPase interacts with conserved membrane protein in *Escherichia coli* to modulate protein synthesis and oxidative phosphorylation. *PloS one* 2011;6:e18510.
- [31] Kiel MC, Kaji H, Kaji A. Ribosome recycling: An essential process of protein synthesis. *Biochemistry and molecular biology education : a bimonthly publication of the International Union of Biochemistry and Molecular Biology* 2007;35:40-4.
- [32] Kaji A, Kiel MC, Hirokawa G, Muto AR, Inokuchi Y, Kaji H. The fourth step of protein synthesis: disassembly of the posttermination complex is catalyzed by elongation factor G and ribosome recycling factor, a near-perfect mimic of tRNA. *Cold Spring Harbor symposia on quantitative biology* 2001;66:515-29.
- [33] Gibson DG, Glass JI, Lartigue C, Noskov VN, Chuang RY, Algire MA, et al. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 2010;329:52-6.
- [34] Hutchison CA, 3rd, Chuang RY, Noskov VN, Assad-Garcia N, Deerinck TJ, Ellisman MH, et al. Design and synthesis of a minimal bacterial genome. *Science* 2016;351:aad6253.
- [35] Carlier L, Haase AS, Burgos Zepeda MY, Dassa E, Lequin O. Secondary structure and NMR resonance assignments of the C-terminal DNA-binding domain of Uup protein. *Biomolecular NMR assignments* 2012.
- [36] Burgos Zepeda MY, Alessandri K, Murat D, El Amri C, Dassa E. C-terminal domain of the Uup ATP-binding cassette ATPase is an essential folding domain that binds to DNA. *Biochimica et biophysica acta* 2010;1804:755-61.
- [37] Carlier L, Haase AS, Burgos Zepeda MY, Dassa E, Lequin O. The C-terminal domain of the Uup protein is a DNA-binding coiled coil motif. *Journal of structural biology* 2012;180:577-84.
- [38] Garcia-Barrio M, Dong J, Ufano S, Hinnebusch AG. Association of GCN1-GCN20 regulatory complex with the N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation. *The EMBO journal* 2000;19:1887-99.
- [39] Dong J, Lai R, Jennings JL, Link AJ, Hinnebusch AG. The novel ATP-binding cassette protein ARB1 is a shuttling factor that stimulates 40S and 60S ribosome biogenesis. *Mol Cell Biol* 2005;25:9859-73.
- [40] Paytubi S, Wang X, Lam YW, Izquierdo L, Hunter MJ, Jan E, et al. ABC50 promotes translation initiation in mammalian cells. *J Biol Chem* 2009;284:24061-73.



- [41] Coots RA, Liu XM, Mao Y, Dong L, Zhou J, Wan J, et al. m(6)A Facilitates eIF4F-Independent mRNA Translation. *Molecular cell* 2017;68:504-14 e7.
- [42] Gomez-Herreros F, Margaritis T, Rodriguez-Galan O, Pelechano V, Begley V, Millan-Zambrano G, et al. The ribosome assembly gene network is controlled by the feedback regulation of transcription elongation. *Nucleic acids research* 2017;45:9302-18.
- [43] Vazquez de Aldana CR, Marton MJ, Hinnebusch AG. GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2 alpha kinase GCN2 in amino acid-starved cells. *The EMBO journal* 1995;14:3184-99.
- [44] Sattlegger E, Hinnebusch AG. Polyribosome binding by GCN1 is required for full activation of eukaryotic translation initiation factor 2{alpha} kinase GCN2 during amino acid starvation. *J Biol Chem* 2005;280:16514-21.
- [45] Han TT, Liu WC, Lu YT. General control non-repressible 20 (GCN20) functions in root growth by modulating DNA damage repair in Arabidopsis. *BMC plant biology* 2018;18:274.
- [46] Tyzack JK, Wang X, Belsham GJ, Proud CG. ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner. *J Biol Chem* 2000;275:34131-9.
- [47] Stewart Joanna D, Cowan Joanne L, Perry Lisa S, Coldwell Mark J, Proud Christopher G. ABC50 mutants modify translation start codon selection. *Biochemical Journal* 2015;467:217-29.
- [48] Bao L, Wu J, Dodson M, Rojo de la Vega EM, Ning Y, Zhang Z, et al. ABCF2, an Nrf2 target gene, contributes to cisplatin resistance in ovarian cancer cells. *Molecular carcinogenesis* 2017;56:1543-53.
- [49] Arora H, Wilcox SM, Johnson LA, Munro L, Eyford BA, Pfeifer CG, et al. The ATP-Binding Cassette Gene ABCF1 Functions as an E2 Ubiquitin-Conjugating Enzyme Controlling Macrophage Polarization to Dampen Lethal Septic Shock. *Immunity* 2019;50:418-31 e6.
- [50] Lee MN, Roy M, Ong SE, Mertins P, Villani AC, Li W, et al. Identification of regulators of the innate immune response to cytosolic DNA and retroviral infection by an integrative approach. *Nature immunology* 2013;14:179-85.
- [51] Hua Y, Yan K, Wan C. Clever Cooperation: Interactions Between EspF and Host Proteins. *Frontiers in microbiology* 2018;9:2831.
- [52] Pazour GJ, Agrin N, Leszyk J, Witman GB. Proteomic analysis of a eukaryotic cilium. *The Journal of cell biology* 2005;170:103-13.
- [53] Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, et al. ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. *Molecular cell* 2002;10:139-49.
- [54] Murat D, Goncalves L, Dassa E. Deletion of the Escherichia coli uup gene encoding a protein of the ATP binding cassette superfamily affects bacterial competitiveness. *Res Microbiol* 2008;159:671-7.
- [55] Hopkins JD, Clements M, Syvanen M. New class of mutations in Escherichia coli (uup) that affect precise excision of insertion elements and bacteriophage Mu growth. *Journal of bacteriology* 1983;153:384-9.
- [56] Reddy M, Gowrishankar J. Characterization of the uup locus and its role in transposon excisions and tandem repeat deletions in Escherichia coli. *Journal of bacteriology* 2000;182:1978-86.

- [57] Murat D, Bance P, Callebaut I, Dassa E. ATP hydrolysis is essential for the function of the Uup ATP-binding cassette ATPase in precise excision of transposons. *J Biol Chem* 2006;281:6850-9.
- [58] Arenz S, Juette MF, Graf M, Nguyen F, Huter P, Polikanov YS, et al. Structures of the orthosomycin antibiotics avilamycin and evernimicin in complex with the bacterial 70S ribosome. *Proceedings of the National Academy of Sciences of the United States of America* 2016;113:7527-32.
- [59] Wilson DN. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nature reviews. Microbiology* 2014;12:35-48.
- [60] Polacek N, Mankin AS. The ribosomal peptidyl transferase center: structure, function, evolution, inhibition. *Critical reviews in biochemistry and molecular biology* 2005;40:285-311.
- [61] Sothiselvam S, Neuner S, Rigger L, Klepacki D, Micura R, Vazquez-Laslop N, et al. Binding of Macrolide Antibiotics Leads to Ribosomal Selection against Specific Substrates Based on Their Charge and Size. *Cell reports* 2016;16:1789-99.
- [62] Vazquez-Laslop N, Mankin AS. Context-Specific Action of Ribosomal Antibiotics. *Annual review of microbiology* 2018;72:185-207.
- [63] Hot C, Berthet N, Chesneau O. Characterization of sal(A), a novel gene responsible for lincosamide and streptogramin A resistance in *Staphylococcus sciuri*. *Antimicrobial agents and chemotherapy* 2014;58:3335-41.
- [64] He T, Shen Y, Schwarz S, Cai J, Lv Y, Li J, et al. Genetic environment of the transferable oxazolidinone/phenicol resistance gene *optrA* in *Enterococcus faecalis* isolates of human and animal origin. *The Journal of antimicrobial chemotherapy* 2016;71:1466-73.
- [65] Hao W, Shan X, Li D, Schwarz S, Zhang SM, Li XS, et al. Analysis of a *poxtA*- and *optrA*-co-carrying conjugative multiresistance plasmid from *Enterococcus faecalis*. *The Journal of antimicrobial chemotherapy* 2019.
- [66] Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, et al. ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. *The Journal of antimicrobial chemotherapy* 2012;67:84-90.
- [67] Sadowy E. Linezolid resistance genes and genetic elements enhancing their dissemination in enterococci and streptococci. *Plasmid* 2018;99:89-98.
- [68] Roberts JA, Kruger P, Paterson DL, Lipman J. Antibiotic resistance--what's dosing got to do with it? *Crit Care Med* 2008;36:2433-40.
- [69] Isnard C, Malbruny B, Leclercq R, Cattoir V. Genetic basis for in vitro and in vivo resistance to lincosamides, streptogramins A, and pleuromutilins (LSAP phenotype) in *Enterococcus faecium*. *Antimicrobial agents and chemotherapy* 2013;57:4463-9.
- [70] Card RM, Stubberfield E, Rogers J, Nunez-Garcia J, Ellis RJ, AbuOun M, et al. Identification of a New Antimicrobial Resistance Gene Provides Fresh Insights Into Pleuromutilin Resistance in *Brachyspira hyodysenteriae*, Aetiological Agent of Swine Dysentery. *Frontiers in microbiology* 2018;9:1183.
- [71] Ambrose KD, Nisbet R, Stephens DS. Macrolide efflux in *Streptococcus pneumoniae* is mediated by a dual efflux pump (*mel* and *mef*) and is erythromycin inducible. *Antimicrobial agents and chemotherapy* 2005;49:4203-9.

- [72] Nunez-Samudio V, Chesneau O. Functional interplay between the ATP binding cassette Msr(D) protein and the membrane facilitator superfamily Mef(E) transporter for macrolide resistance in *Escherichia coli*. *Res Microbiol* 2013;164:226-35.
- [73] Reddy VS, Shlykov MA, Castillo R, Sun EI, Saier MH, Jr. The major facilitator superfamily (MFS) revisited. *The FEBS journal* 2012;279:2022-35.
- [74] Seip B, Innis CA. How Widespread is Metabolite Sensing by Ribosome-Arresting Nascent Peptides? *Journal of molecular biology* 2016;428:2217-27.
- [75] Qu L, Jiang Y, Cheng C, Wu D, Meng B, Chen Z, et al. Crystal Structure of ATP-Bound Human ABCF1 Demonstrates a Unique Conformation of ABC Proteins. *Structure* 2018;26:1259-65.e3.
- [76] Locher KP. Mechanistic diversity in ATP-binding cassette (ABC) transporters. *Nature structural & molecular biology* 2016;23:487-93.
- [77] Korkhov VM, Mireku SA, Locher KP. Structure of AMP-PNP-bound vitamin B12 transporter BtuCD-F. *Nature* 2012;490:367-72.
- [78] Oldham ML, Chen J. Crystal Structure of the Maltose Transporter in a Pretranslocation Intermediate State. *Science* 2011;332:1202-5.
- [79] Hanawa-Suetsugu K, Sekine S, Sakai H, Hori-Takemoto C, Terada T, Unzai S, et al. Crystal structure of elongation factor P from *Thermus thermophilus* HB8. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101:9595-600.
- [80] Blaha G, Stanley RE, Steitz TA. Formation of the first peptide bond: the structure of EF-P bound to the 70S ribosome. *Science* 2009;325:966-70.
- [81] Huter P, Arenz S, Bock LV, Graf M, Frister JO, Heuer A, et al. Structural Basis for Polyproline-Mediated Ribosome Stalling and Rescue by the Translation Elongation Factor EF-P. *Molecular cell* 2017;68:515-27.e6.
- [82] Boel G, Danot O, de Lorenzo V, Danchin A. Omnipresent Maxwell's demons orchestrate information management in living cells. *Microbial biotechnology* 2019;12:210-42.
- [83] Kamat NP, Tobe S, Hill IT, Szostak JW. Electrostatic Localization of RNA to Protocell Membranes by Cationic Hydrophobic Peptides. *Angewandte Chemie (International ed. in English)* 2015;54:11735-9.
- [84] Schrum JP, Zhu TF, Szostak JW. The origins of cellular life. *Cold Spring Harbor perspectives in biology* 2010;2:a002212.

**Legends to figures**

**Figure 1: ABC-F phylogeny.** **A)** Cladogram labeled with Swissprot species codes including for reference two non-ABC-F family proteins containing tandem ABC domains (ABCE1/RLi from *S. cerevisiae* and EF3 from both *C. reinhardtii* and *S. cerevisiae*). The cladogram shows all ABC-F proteins from *Mycoplasma mycoides* (MYCLM) *E. coli* (ECOLI), *B. subtilis* (BACSU), *Synechocystis* (SYNY3), *C. reinhardtii* (CHLRE) *S. cerevisiae* (YEAST), *A. thaliana* (ARATH), and *H. sapiens* (HUMAN). **B)** Phylogeny of the antibiotic resistance (ARE) proteins in the ABC-F family. The class of the antibiotic to which each ARE ABC-F protein mediates resistance is shown on the right of the cladogram. All of the ABC-F family proteins encoded by *E. coli* K12 and two non-ABC-F family proteins containing tandem ABC domains (ABCE1/RLi and EF3) are shown for reference.

**Figure 2: Examples of mobile genetic elements disseminating ARE ABC-F proteins.** GenBank accession numbers are given in parenthesis on the right of each element.

**Figure 3: The architecture and conformation of ATP-bound ABC-F proteins observed in cryo-EM structures of ribosome complexes.** **(A)** Cartoon representation of the structure of ribosome-bound EttA (PDB-ID 3J5S) with its ABC1 and ABC2 domains colored in light and darker shades of magenta, respectively, and its P-site tRNA interaction motif (PtIM) colored in an intermediate shade of magenta. The bound ATP molecules are shown in black in stick representation. EttA is inferred to undergo a 41° rotation around the blue axis upon ATP hydrolysis. **(B)** A superposition of the ARE ABC-F proteins VmlR (cyan, PDB-ID 6HA8) and Msr(E) (green, PDB-ID 5ZLU) on EttA (magenta) based on least-squares alignment of their ABC1 domains in PyMOL. The color scheme varies from light for ABC1 to dark for

ABC2 with the PtIM in an intermediate shade. (C) The same proteins colored in the same way superimposed based on least-squares alignment of the 23S rRNAs in their ribosome-bound cryo-EM structures. The P-site tRNAs in these structures are shown in matching colors (purple for EttA, blue for VmlR, and green for Msr(E)), while the residues forming the peptidyl transferase center (PTC) and nascent peptide exit tunnel (NPET) in the EttA-bound ribosome are shown in yellow sphere representation.

**Figure 4: Orientation and positioning of the PtIMs and P-site tRNAs in ABC-F bound ribosome complexes.** (A) The EttA, VmlR, and Msr(E) proteins are aligned based on least-squares superposition of the 23S rRNAs in the respective cryo-EM structures and colored as in Fig. 4. The rRNAs are shown in transparent cartoon representation in matching colors (magenta, cyan, and green for the EttA, VmlR, Msr(E) complexes, respectively), with the 23S rRNA from the 50S subunit shown in a darker shade than the 16S rRNA from the 30S subunit. Residues in the PTC/NPET in the EttA-bound ribosome are again shown in yellow sphere representation. (B) Zoomed-in view of the P sites and PTCs in the same alignment of the same structures showing all elements in the 23S rRNAs that contact the PtIM, the P site tRNA has been excised. The black labels represent standard numbering of the helices in *E. coli* rRNA. (C) Another zoomed-in view of the same alignment of the same structures also showing space-filling models of the P sites tRNAs in the EttA (purple), VmlR (blue), and Msr(E) (green) structures. The black numbers preceded by capital “H” label helices in the 23S rRNAs that contact the PtIMs, while the gray numbers preceded by lower-case “h” label helices in the 16S rRNAs that contact the tRNAs.

**Figure 5: The Tip of the PtIM in ABC-F proteins binds directly to the acceptor stem of P-site tRNAs and to the PTC to modulate tRNA-binding geometry and PTC stereochemistry.**

Zoomed-in views of the PTCs in the same cryo-EM structures shown in **Fig. 5** colored in the same way. Panel **A** shows the point of view and orientation of the zoomed-in-views on top and the sequences of the PtIM Tip for each ABC-F. The CCA acceptor stems of the interacting P-site tRNAs are shown in stick representation, while the backbones of the remainder of these tRNA molecules are shown in ribbon representation. The tRNAs bound in the EttA, VmlR, and Msr(E) structures are shown in purple, blue, and green, respectively. The interacting segments of the 23S rRNAs (black labels) and proteins (gray labels) from the 50S ribosomal subunit are shown in transparent cartoon representation, and the PTC from the EttA structure is shown in yellow space-filling representation. Panel **B** shows a superposition of the three structures based on least-squares alignment of the 23S rRNAs, while panels **C-D** show identical views of each of the individual structures.

**Figure 6: Schematic diagram illustrating the drain-snake hypothesis for the mechanism of drug clearance from the PTC/NPET by ARE ABC-F proteins.** Coloring is equivalent to Figs. 4-6, with the 23S rRNA and Msr(E) protein from the cryo-EM structure of their complex shown in cyan, the PTC highlighted in yellow space-filling representation, and the P site tRNA before and after Msr(E) binding shown in purple and blue, respectively. An approximate model of a nascent polypeptide chains is shown in red space-filling representation, while the crystallographically observed position of the antibiotic lincomycin (PDB id 5hkv) shown in brown space-filling representation. This model proposes that ARE ABC-F protein binding and release drives movements of the P site tRNA and attached nascent polypeptide that clear antibiotics from the P site via a mechanism equivalent to the use of a mechanical “snake” to pull debris out of a clogged drain.

**Table 1. Phenotypes and characteristics of ARE ABC-F proteins with a confirmed role in antibiotic resistance in eubacteria that do not produce antibiotics themselves**

| Resistance profile  | ARE ABC-F lineage | 50S drug-binding site                       | ABC1 "Arm"     | PtiM tip | CTD           |
|---|-------------------|---|----------------|----------|---------------|
| PhO   | Optr(A)           |   | Yes            | Shortest | Yes (longest) |
| (phenicols and oxazolidinones)                                      | Pox(A)*           | PTC A-site                                  | Yes            | Shortest | Yes           |
|   | Vga variants      |   | No and shorter | Long     | Yes           |
| PLS <sub>A</sub> (pleuromutilins, lincosamides and streptogramin A) | Lsa variants      | PTC A-site overlapping with P-site and NPET | Yes (shorter)  | Medium   | No            |
|   | SalA              |   | Yes            | Short    | No            |
|   | VmlR              |   | No             | Medium   | Yes           |
| MKS <sub>B</sub>  |                   |   |                |          |               |
| (macrolides, ketolides and streptogramin B)                         | Msr variants      | NPET  | No             | Longest  | No            |

\* The *pox(A)* gene cloned into three heterologous hosts does not produce a 2-fold increase in the minimum inhibitory concentration (MIC) of various tetracyclines (Antonelli *et al.*, 2018), so it cannot be considered to confer resistance to this class of antibiotics.

Figure 1

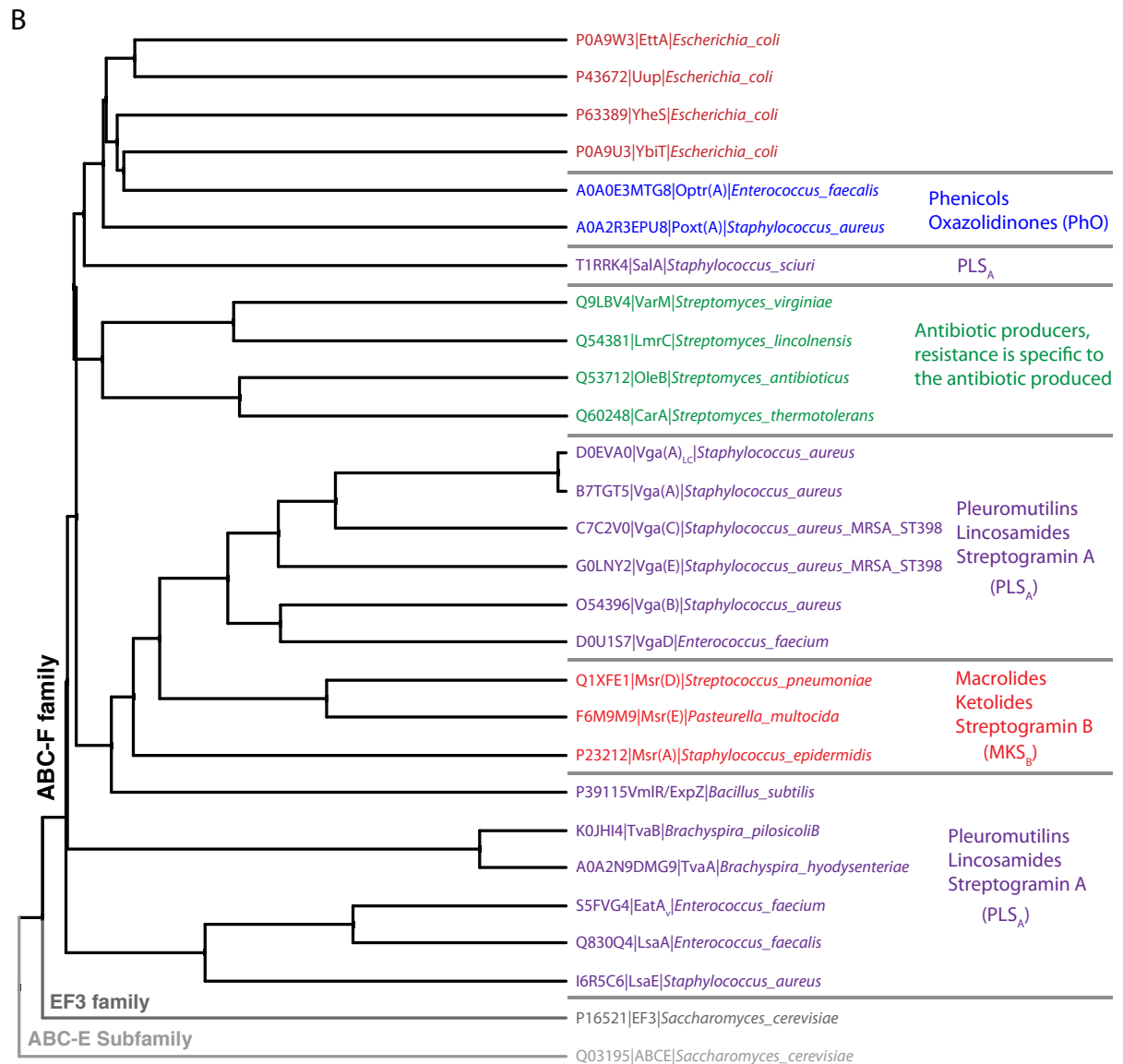
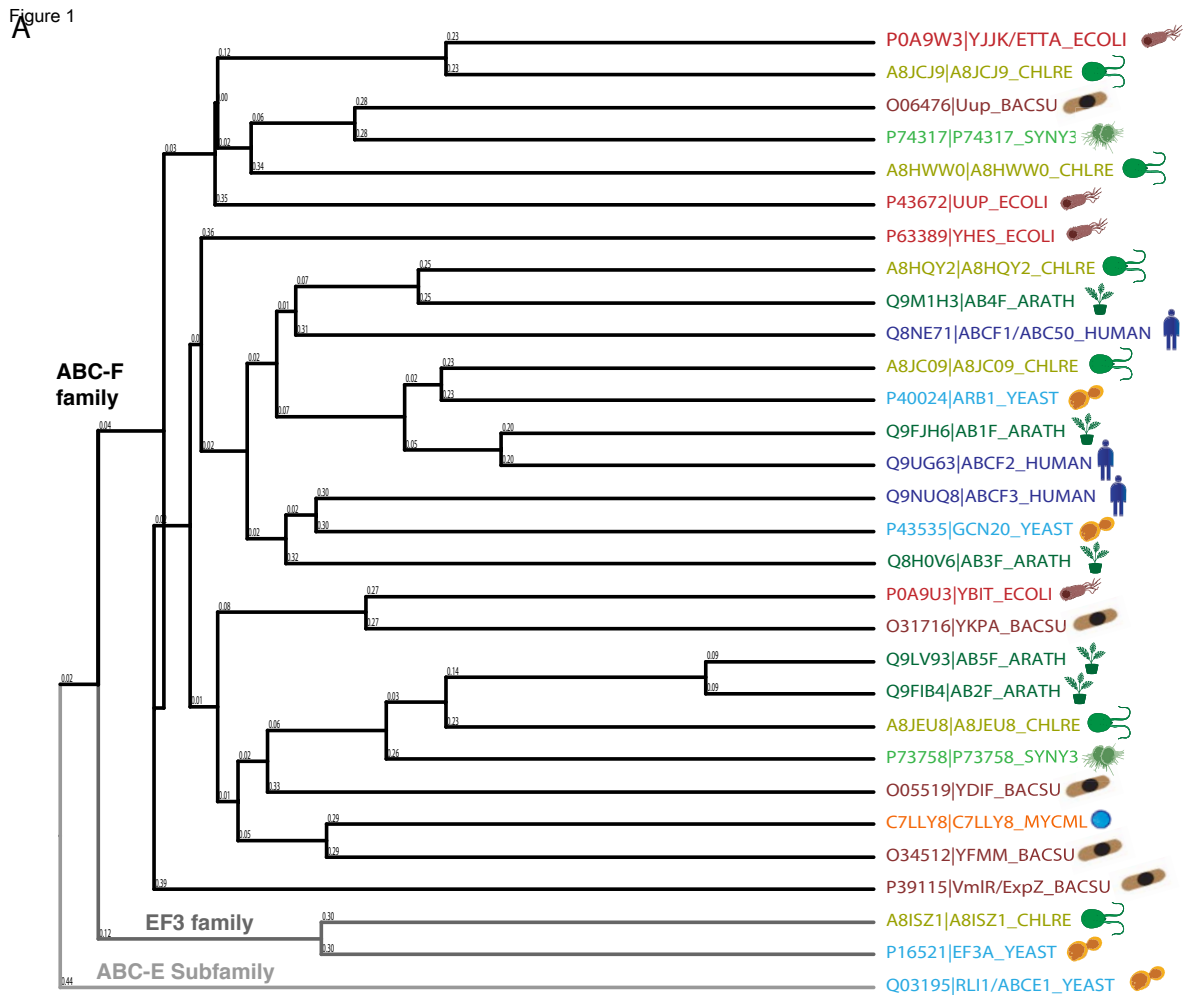


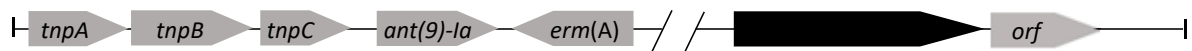


Figure 2

a) Small plasmid pVGA that disseminates *vga(A)* (FJ207465)



b) Transposon Tn6133 that disseminates *vga(E)* (FR772051)



c) Large conjugative plasmid pE349 that disseminates *optr(A)* (KP399637)



d) Large plasmid pE076 that disseminates *poxt(A)* (MK140642)

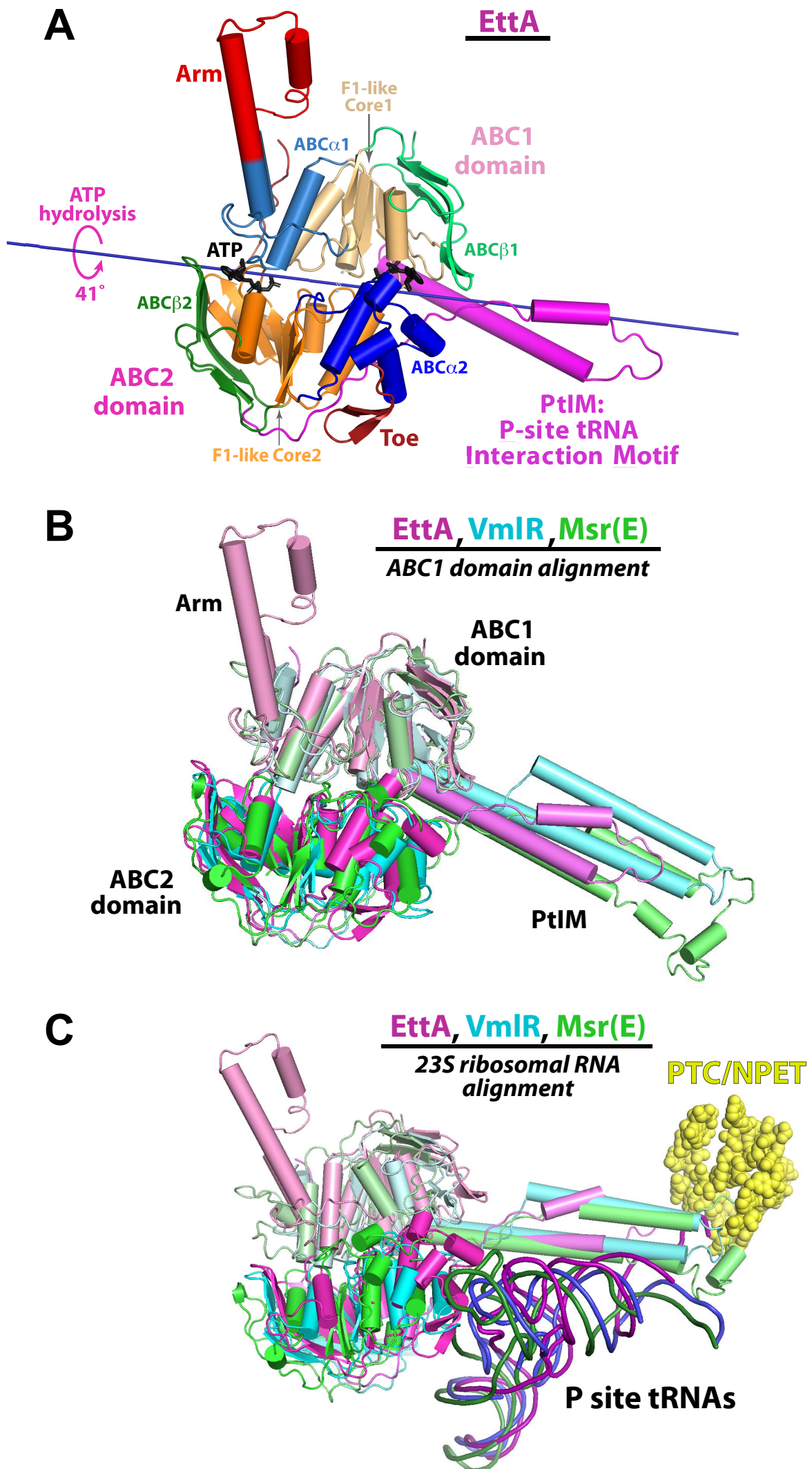


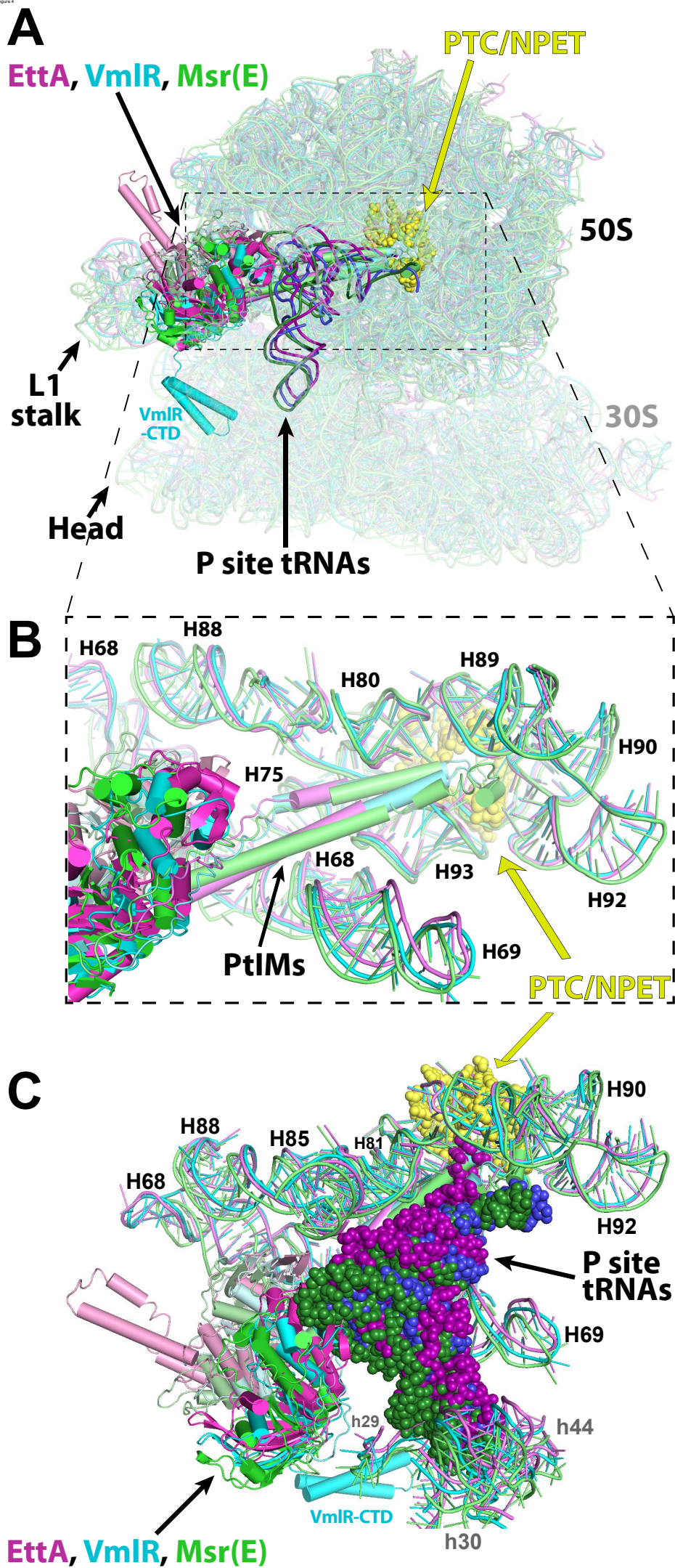
e) Conjugative prophage  $\Phi$ 1207.3 that disseminates *msr(D)* (AY657002)

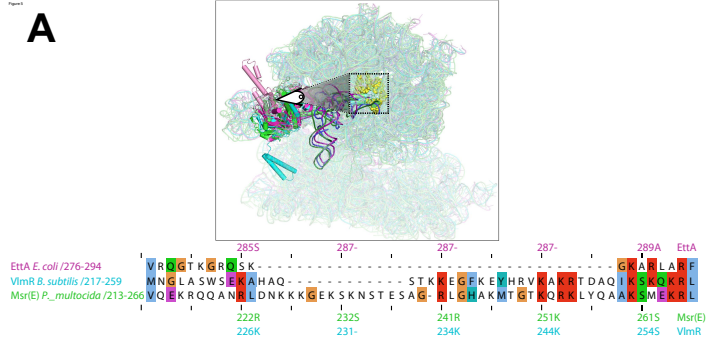
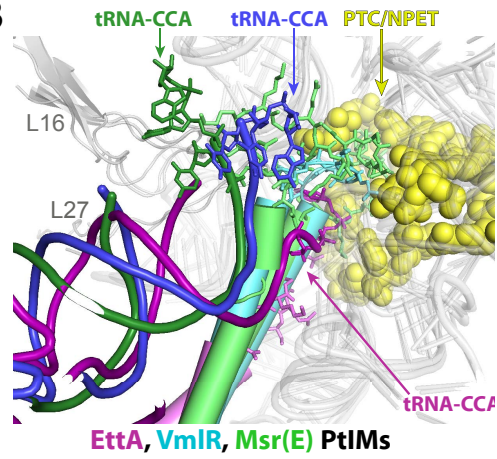
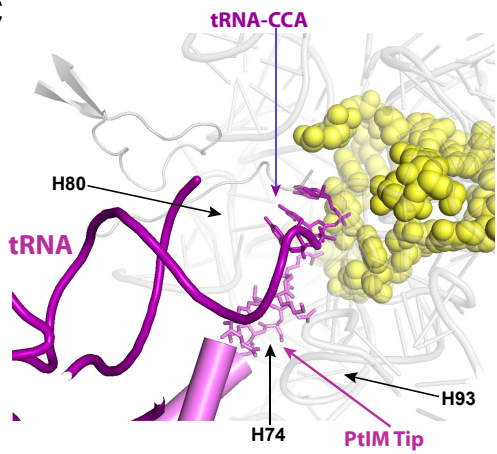
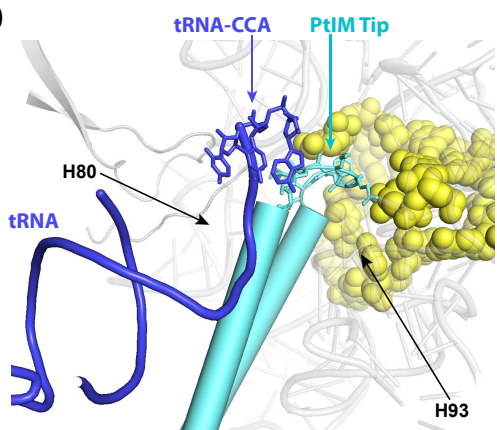


f) Xer-dependent module that disseminates *msr(E)* (KY617771)







**A****B****C****D****E**