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Target protection as a key antibiotic resistance mechanism

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Abstract

Antibiotic resistance is mediated through several distinct mechanisms, most of which are relatively well-understood and the clinical importance of which has long been recognised. Until very recently, neither of these statements was readily applicable to the class of resistance mechanism known as target protection, a phenomenon whereby a resistance protein physically associates with an antibiotic target to rescue it from antibiotic-mediated inhibition. In this Review, we summarize recent progress in understanding the mechanisms involved in target protection and its clinical importance. In particular, we describe the current state of knowledge regarding the molecular basis of the known TP systems, emphasizing the fact that TP does not involve a single, uniform mechanism – but is brought about in several, mechanistically-distinct ways.

[H1] Introduction

Bacteria have evolved an array of mechanisms that enables them to resist the inhibitory action of antibiotics, a phenomenon that is eroding our ability to manage bacterial infections[1]. Understanding the molecular details of these resistance mechanisms is not only of fundamental interest, but can also offer strategic intelligence to inform the rational development of novel therapeutic approaches to evade or block resistance. As befits a mature field of study, the beginnings of which predate the clinical deployment of penicillin[2], by now there exists an extensive body of knowledge regarding the nature of different mechanistic classes of antibiotic resistance, including drug efflux and degradataion as well as target modification and mutation[3,4]. One class of resistance mechanism that has long lagged behind the others — both in terms of perceived clinical impact and mechanistic understanding — is a phenomenon known as target protection.

Target protection involves the physical association of a resistance protein ('target protection protein') with an antibiotic target to rescue the function of the latter from antibiotic-mediated inhibition. In contrast to the more familiar mechanism of target modification, whereby the interaction between the resistance protein and the target need in principle occur only once and results in chemical alteration of the latter, target protection does not involve permanent modification of the target. Instead, direct interaction between the target protection protein and the target is required to effect resistance[4]. Target protection was first recognized as a mechanism of antibiotic resistance in the context of tetracycline resistance ~30 years ago[5,6], and for some considerable time thereafter this remained the only clearly documented example. Consequently, target protection has generally been considered little more than an unusual foot-note alongside the better-known mechanisms by which bacteria resist antibiotics. Certainly, it was believed to have limited impact in terms of mediating clinically significant resistance to antibiotics, and literature descriptions of antibiotic resistance mechanisms often fail to mention it and/or fail to distinguish it from target modification.

However, recent work has now revealed that target protection is a key mechanistic player in clinically significant antibiotic resistance that affects diverse classes of antibacterial drugs and is prevalent in bacterial pathogens. Furthermore, whereas the target protection mechanism of tetracycline resistance involves direct displacement of the drug from the target (see below), structural and functional characterization of other target protection systems has revealed modes of protection that are mechanistically distinct from this canonical example. In fact, target protection can be divided into three distinct types with respect to the underlying protection mechanism (**Fig. 1**): by sterically removing the drug from the target; by inducing conformational changes within the target that allosterically dissociate

the drug from the target, or by inducing conformational changes within the target that restore functionality despite the presence of the bound antibiotic. In this Review, we examine the major recent developments that have improved our understanding of the nature and importance of this mechanistic class, with the emphasis on the molecular detail of their action.

[H1] Tetracycline ribosomal protection proteins

Members of the tetracycline class of antibiotics inhibit bacterial translation by binding to the 30S ribosomal subunit and interfering with delivery of the incoming aminoacyl-tRNA by elongation factor Tu (EF-Tu) during the elongation phase of protein synthesis[7]. Tetracyclines achieve this by binding to helix 34 of the 16S rRNA at a position that overlaps with the anticodon loop of the aminoacyl-tRNA when accommodated at the A-site of the decoding centre[8-11]. Bacterial resistance to this class can result through diverse mechanisms, although two mechanistic types predominate as a cause of clinically significant resistance in pathogens: active efflux of the antibiotic and target protection. Tetracycline ribosomal protection proteins (TRPPs) mediate target protection, and 13 distinct TRPP classes have been described to date[12] of which Tet(O) and Tet(M) are the best-characterized[7,13]. Genes encoding TRPPs are found in a diverse range of Gram-negative and Gram-positive pathogens[14]. TRPPs represent the major cause of tetracycline resistance in Gram-positive pathogens, and *tet*(M) is by far the most prevalent tetracycline resistance determinant in clinical isolates of streptococci[15,16], staphylococci[17,18] and enterococci[19,20].

Tet(M) and Tet(O) are closely related GTPases with sequence homology (<25% identity) to translation elongation factor-G (EF-G), and thus seem to represent EF-G paralogs that have evolved the specialized ability to rescue translation in the presence of tetracyclines[7]. Indeed, structural studies have shown that Tet(O) and Tet(M) bind to the ribosome in manner analogous to EF-G[21-24]. However, whereas EF-G recognises the ribosome in a pre-translocational state (that is, the A-site and P-site are occupied by tRNAs), Tet(O) and Tet(M) bind to a post-translocational state (that is, the P-site and E-site are occupied, with the A-site being free owing to the presence of tetracycline)[21-24]. The first, low-resolution (16 Å), cryo-electron microscopy structure of Tet(O) bound to the ribosome led to the suggestion that this TRPP indirectly drives the removal of tetracycline from the target by inducing a local disturbance in helix 34 (Ref. [21]) However, subsequent higher-resolution (3.9 Å to 9.6 Å) structures have established that both Tet(O) and Tet(M) directly overlap the tetracycline binding site on the ribosome[22-24] (**Fig. 2**), thereby mediating resistance through direct physical displacement of the drug. In all of these structural studies, the TRPP was trapped on the ribosome using non-hydrolysable GTP analogs, consistent with earlier biochemical studies indicating that GTP hydrolysis is required for factor dissociation rather than

drug release[25,26]. The most recent and best-resolved structure revealed that a conserved proline residue located at the tip of loop III of domain IV of Tet(M) is located directly within the tetracycline-binding site where it interacts with nucleotide C_{1054} of the 16S rRNA[24] (**Fig. 2**). Based on changes in chemical reactivity to RNA-modifying agents it has been proposed that TRPPs alter the conformation of nucleotides within the drug-binding site (for example, C_{1054}), which disfavors immediate rebinding of the drug as well as promoting subsequent delivery of the aa-tRNA by EF-Tu[7,13]. As the conformation of C_{1054} seems identical between the available Tet(M)-bound and tetracycline-bound 70S ribosome structures[10,24], any such alterations within the drug-binding site must occur upon dissociation of the TRPP from the ribosome. Thus, conformational changes within the TRPP that are associated with GTP hydrolysis may not only facilitate dissociation from the ribosome but could induce conformational change within the drug-binding site that persists following TRPP dissociation.

Although TRPPs mediate resistance to classic tetracyclines, they have little or no effect on the activity of third-generation compounds, such as tigecycline and omadacycline[10,27,28]. The ability of these drugs to overcome the action of Tet(M) is not solely attributable to their increased affinity of ribosome binding relative to tetracycline, as the activity of other tetracycline analogs (for example, azacycline) that exhibit comparable affinity is also impaired by Tet(M)[10]. Potentially, the C9-moiety of the third generation tetracyclines that enhances ribosome binding also sterically hinders access of residues within loop III of domain IV of Tet(M) to nucleotide C_{1054}, thereby preventing the TRPP from dislodging the drug from its binding site (**Fig. 2**). In line with this idea, Tet(M) variants selected through laboratory evolution to mediate tigecycline resistance carry substitutions within this very loop[29].

Target protection involving direct physical displacement of the drug from its target by the protection protein, exemplified by the TRPPs, can be referred to as type I target protection (**Fig. 1**).

[H1] Antibiotic resistance ABC-F proteins.

In 2016, antibiotic resistance (ARE) ABC-F proteins have been shown to bind the bacterial ribosome to protect it from translation inhibitors, which has further strengthened the notion that target protection is a mechanism of clinically significant resistance[30]. The long-standing controversy surrounding the mechanism of resistance of these proteins, and their importance in resistance to a broad range of clinically deployed antibiotics against Gram-positive pathogens, have recently been reviewed[31] and will not be revisited here. This group of proteins constitutes a major source of clinical resistance to almost all antibacterial drug classes that target the 50S subunit of the ribosome (lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins and streptogramins of groups A and B), and collectively

mediates resistance to a broader range of structurally distinct antibiotic classes than any other family of resistance factors.

ABC-F proteins lack the transmembrane portions found in most other members of the ATP-binding cassette (ABC) superfamily, and instead comprise two ABC domains that are separated by a linker region[30-32]. This linker has been designated the P-site tRNA-interaction motif (PtIM)[33,34], although amongst the ARE ABC-F proteins, it is also referred to as the antibiotic resistance domain (ARD)[35]. Three major categories of ARE ABC-F proteins can be differentiated on the basis of their antibiotic specificity: Vga, Lsa, Sal and Vml belong to the first category, and they mediate resistance to streptogramins of group A, lincosamides and sometimes pleuromutilins; Msr-type proteins mediate resistance to streptogramins of group B and macrolide antibiotics (and sometimes ketolides); and Optr and Poxt mediate resistance to oxazolidinones and phenicols[31,32,36-38]. Structures of representatives from the first two groups (VmlR from *Bacillus subtilis* and MsrE from *Pseudomonas aeruginosa*) in complex with the ribosome have revealed that these proteins bind into the E-site, with their ARDs reaching into the peptidyltransferase centre (PTC) [35,39,40] (**Fig. 3**). To access the PTC, these proteins induce a conformational change within the P-site tRNA, shifting the acceptor arm out of the PTC and towards the ribosomal A-site[35,39]. The loop located at the tip of the ARD varies considerably in length and sequence between ARE ABC-F proteins, and mutations within this region can affect antibiotic specificity[31,35,39,41,42]. When bound to the ribosome, a residue (Phe_{237}) within the ARD loop of VmlR sterically overlaps the binding site of PTC-inhibiting antibiotics, such as lincosamides, streptogramins of group A and pleuromutilins [35], consistent with the reported resistance spectrum of this protein (**Fig. 3**)[35,43]. However, an engineered VmlR variant in which Phe_{237} was replaced with Ala — a substitution that would be predicted to remove the overlap — still conferred resistance to lincosamides and pleuromutilins [35], which suggests that the steric overlap is not critical for VlmR-mediated removal of these antibiotics from the ribosome. By contrast, this same VlmR variant lost the ability to mediate resistance to virginiamycin M[35], which implies that an important steric component exists for removal of streptogramins of group A. In the case of MsrE, the ARD loop is longer and reaches deeper into the ribosomal exit tunnel, where Leu_{242} of MsrE overlaps with the binding site of macrolides and streptogramins of group B [39] (**Fig. 3**). Substitution of Leu_{242} to Ala leads to near-complete loss of ability to mediate azithromycin resistance, which suggests a strong steric component to the mechanism of macrolide removal from the ribosome[39]. Whether MsrE functions sterically or allosterically on streptogramins of group B remains to be determined. Taken together, these observations imply that the precise nature of the target protection mechanism of ARE ABC-F proteins depends not only on the target protection protein itself, but can also vary amongst the targeted antibiotic class. Thus, in some

cases (for example, MsrE-mediated macrolide resistance) ARE ABC-F proteins seem to function via a type I target protection mechanism through direct physical displacement of the antibiotic from the target, analogous to that seen for the TRRPs. In other cases (for example, VmlR-mediated resistance to lincosamides and pleuromutilins) resistance is the result of an indirect, allosteric mechanism of antibiotic removal — termed type II target protection (**Fig. 1**).

ATP hydrolysis by the ARE ABC-F proteins does not seem to be required for antibiotic release, but it is required for dissociation of the resistance protein from the ribosome[35,37,39]. Following dissociation, the allosteric changes induced in the ribosome by the ABC-F protein may persist to prevent immediate rebinding of the drug, as seen for the TRPPs. Nevertheless, any such induced conformational change is unlikely to be retained throughout the process of accommodating the next incoming aminoacyl-tRNA and the subsequent translocation step, which could mean that the ABC-F proteins must rebind the ribosome after each translation elongation cycle to ensure effective target protection in the presence of the antibiotic[31,44]. However, this is not necessarily the case. For the Msr proteins that mediate resistance to macrolides, one could envisage a situation in which re-accommodation of short peptidyl-tRNA into the exit tunnel would enable continued translation that, in turn, could mask the macrolide-binding site and thereby prevent drug rebinding. Likewise, for proteins such as Vga, Lsa, Sal or Vml that mediate resistance to translation initiation inhibitors (for example, streptogramins of group A, lincosamides and pleuromutilins), re-accommodation of initiator fMet-tRNA at the PTC would enable peptide bond formation with the incoming aminoacyl-tRNA to create an elongation complex that is refractory to the action of these antibiotics. However, these ideas require experimental corroboration.

The molecular basis for the antibiotic specificity of ARE ABC-F proteins will also require further study. For example, VmlR mediates resistance to streptogramins of group A, lincosamides and pleuromutilins, but not to oxazolidinones and phenicols, even though all of these classes have binding sites at the PTC that overlap with each other and with the ARD of VmlR[35]. Similarly, MsrE mediates resistance to streptogramins of group B and macrolide antibiotics, but not streptogramins of group A, lincosamides, pleuromutilins, oxazolidinones and phenicols, despite the overlap in binding site of these classes[39]. A potential explanation for this specificity could relate to the functional state of the ribosome that becomes trapped by these antibiotic classes; whereas streptogramins of group A, lincosamides, pleuromutilins interfere with translation initiation[45-49], oxazolidinones and phenicols predominantly target elongation[50]. Thus, the majority of ribosomes stalled by oxazolidinones and phenicols would contain P-site tRNA attached to a long nascent polypeptide chain, a structure that is conceivably refractory to VmlR and MsrE binding and action. By contrast, ribosomes stalled by streptogramins of

group A, lincosamides, pleuromutilins during initiation would have an fMet-tRNA$_i^{Met}$ trying to accommodate at the P-site, and thereby represent an appropriate substrate for VmlR action. In this regard, the C-terminal extension (CTE) of VmlR may have a role in recognition of the initiation state, as the CTE reaches into the cavity on the 30S subunit where the Shine-Dalgarno-helix is located[35]. Indeed, the CTE is critical for resistance in VmlR[35] and is conserved in Vga-type proteins. However, potentially arguing against a specific role for the CTE during initiation is the fact that this region is absent in Lsa-type ARE ABC-F proteins that have the same antibiotic specificity as Vga and Vml proteins, but are present in OptrA, which mediates resistance to oxazolidinones and phenicols that stall ribosomes during elongation[32,37]. Lastly, it will also be interesting to understand how Optr and Poxt proteins manage to dislodge oxazolidinones and phenicols from the ribosome, as these proteins have a very short ARD that would not be expected to reach into the PTC[32].

[H1] FusB-type proteins

The antibiotic fusidic acid inhibits bacterial protein synthesis by binding to translation elongation factor EF-G on the ribosome and preventing disassembly of the post-translocation complex; the resultant steric occlusion of the A-site by EF-G blocks the delivery of incoming aminoacyl-tRNA species into the ribosome, causing cessation of protein synthesis[51-53]. Resistance to fusidic acid amongst clinical isolates of *Staphylococcus aureus* and other staphylococci has increased dramatically in recent years, and predominantly results from horizontal acquisition of determinants encoding FusB-type proteins[54-58]. This family, the best studied of which is FusB itself, comprises small (~25 kDa), two-domain metalloproteins that bind to the C-terminal domains of EF-G and rescue translation in the presence of the drug [59-61] (**Fig. 4a**). In contrast to the target protection mechanisms described above, these fusidic acid resistance proteins do not bind the target in close proximity to the drug; FusB recognises a region in EF-G that is entirely distinct from the fusidic acid-binding site, and indeed involves different domains of the protein (FusB makes contacts with domain IV and domain V of EF-G, whereas fusidic acid binds at a site located between domain II and domain III) [62,63] (**Fig. 4b,c**). FusB-type resistance does therefore not result from direct physical displacement of the antibiotic from the drug target, nor is there evidence to implicate an allosteric mechanism of drug removal. Instead, resistance is attributed to the ability of FusB-type proteins to modulate EF-G function in a manner that overcomes fusidic acid-mediated inhibition. In biochemical assays monitoring dissociation of EF-G•GDP•ribosome complexes, FusB mediates a dose-dependent increase in the rate at which EF-G leaves the ribosome, an effect that is observed even in the absence of fusidic acid[60]. By driving disassembly of the post-translocation complex, FusB effectively counters the opposing action of fusidic acid, thereby mitigating the inhibitory effect of the drug[60] (**Fig. 4a**).

Although a comprehensive description of the target protection mechanism of FusB-type proteins awaits additional molecular elucidation, our current understanding supports the following model. Substantial conformational rearrangement within EF-G is required to enable its dissociation from the post-translocation complex[64]. This rearrangement is driven by GTP hydrolysis within the N-terminal super-domain of the protein (domain I and domain II), with subsequent transmission to the C-terminal super-domain (domain III to domain V) to disrupt the contacts that domain IV makes with the 30S subunit[64,65]. By binding into a region that spans domain II and domain III, fusidic acid effectively tethers the two super-domains together and restricts this relay of conformational change, thereby inhibiting EF-G release[63]. Binding of FusB to EF-G has been shown to induce conformational change within domain IV and domain V and altered dynamics in domain III, changes that alone or together presumably drive EF-G dissociation from the ribosome[62]. By inducing these changes directly within the C-terminal super-domain of EF-G, the usual requirement for transmission of conformational change from the N terminus is lifted, thereby effectively nullifying the inhibitory action of fusidic acid. Although this target protection mechanism does not require or result from removal of the drug from the target, fusidic acid is likely to dissociate from EF-G once the latter has been dislodged from the ribosome, as it has only low affinity for free (non-ribosome bound) EF-G.

Thus, the third mechanistic type of target protection (type III target protection mechanism) does not involve protection of the target by reversal of antibiotic binding, but instead restores functioning of the target even with the antibiotic bound (**Fig. 1**).

[H1] Other examples of target protection.

The three target protection systems described above all have in common that there has been considerable recent progress in understanding the molecular mechanism underlying protection, which enables us to classify them into distinct types of target protection, and they are clinically significant causes of antibiotic resistance. The following paragraphs examine other antibiotic resistance proteins that, although failing to fulfil one or both of these criteria, nonetheless represent (or are likely to represent) examples of target protection, and further studies will provide important insights into the underlying mechanisms.

[H2] Target protection mediated by the quinolone resistance proteins. The quinolone resistance (Qnr) family of pentapeptide repeat proteins mediates reduced susceptibility to quinolones and fluoroquinolones in Gram-negative pathogens, such as the Enterobacteriaceae, by binding and

protecting the cellular targets (type II topoisomerases) from drug action[66-68]. Although the degree of protection provided by Qnr proteins is insufficient to render the bacteria that harbour them resistant according to clinical breakpoints, the *qnr* determinants are nonetheless of considerable importance because their presence both reduces the efficacy of fluoroquinolone treatment and facilitates the selection of higher-level ('true') fluoroquinolone resistance[69]. Qnr proteins adopt a right-handed β-helical fold that broadly mimics B-form DNA[70,71], a structure that could potentially enable them to bind into the central DNA-binding groove of type II topoisomerase enzymes[71]. Binding of Qnr to these enzymes is proposed to destabilize the complex that the drug forms with topoisomerase-bound cleavage sites on DNA, thereby enabling re-ligation of DNA and regeneration of the active enzyme[69-71]. It remains to be understood in detail how this protective effect is mediated, including whether Qnr-type proteins primarily drive the dissociation of the drug — either directly or indirectly (type I or type II target protection, respectively) — or whether they restore topoisomerase function despite the presence of the bound drug (type III target protection).

[H2] Target protection mediated by cis-acting peptides. It has long been known that certain short peptides can protect the ribosome translating them from the action of the related macrolide and ketolide antibiotic classes (reviewed in Ref. [72]). To explain this, a 'bottle-brush' model has been proposed[73] that effectively describes a type I target protection mechanism; the short peptide, as it is being translated, interacts with the antibiotic within the ribosomal tunnel, eventually dislodging it as the peptide is released from the P-tRNA during termination[74]. However, the biological relevance of this remains unclear. The majority of E-peptide and K-peptide sequences (named to indicate their ability to mediate resistance to erythromycin (representative macrolide) or ketolides, respectively) that have been studied derive from random peptide libraries[73,75,76]. Furthermore, although the original E-peptide (MRMLT) is encoded within the 23S rRNA of *Escherichia coli*[77], there is no evidence that it is expresses in native settings[72]. A recent study identified a novel 61 amino-acid long polyproline-containing peptide from a soil metagenome that shares sequence similarity with these short resistance peptides, and which when overexpressed in *E. coli* also confers resistance to macrolides and ketolides[78]. Specifically, the N-terminal sequence (MSWKL) of the peptide is reminiscent of E-peptides (**MS**L**K**V, **M**F**S**K**L**, **M**N**WKL**)[75] and K-peptides (**MSWK**I)[73], raising the possibility that it also confers resistance in *cis* by dislodging macrolides and ketolides from the ribosome as the peptide is being translated[78]. Although a compelling idea, it will need to be reconciled with the observation that 'classic' E-peptides are typically only functional in their short form, and extending them by removal of the stop codon or appending the E-peptide sequence to the C-terminus of a polypeptide abrogates their ability to confer resistance[77]. Further investigation will also be required to understand whether the central region of

the 61 amino-acid long peptide, which is extremely proline rich (25 proline residues within ten PPx motifs), has any role in drug displacement and antibiotic resistance.

[H2] Target protection mediated by HflX-type proteins. Treatment of *Listeria monocytogenes* with sub-inhibitory concentrations of lincosamides dramatically affects the gene expression program, including inducing transcription of Lmo0919 (an ARE ABC-F protein)[47] and Lmo0762[79]. The latter has been found to mediate modest levels of resistance to lincosamides and macrolides, an effect that is only apparent in a genetic background lacking Lmo0919[79]. Lmo0762 exhibits homology to HflX[79], a ribosome-splitting GTPase that rescues stalled ribosomes under stress conditions[80,81]. Reflecting this similarity and the fact that the protein mediates a degree of antibiotic resistance, it was subsequently termed HflXr, a descriptor that also distinguishes it from another listerial HflX protein (Lmo1296) that has no role in resistance[79]. Analogous to HflX, HflXr seems to induce dissociation of 70S ribosomes into their 30S and 50S subunits. However, it remains unclear whether HflXr is also directly responsible for antibiotic displacement or whether another factor is recruited to the antibiotic-bound 50S particles to fulfil this function[79]. We note that in the cryo-electron microscopy structure of the *E. coli* HflX•GDPNP•50S complex, the loop connecting two helices of subdomain II within the N-terminal domain of HflX is positioned at the PTC in close proximity to the lincosamide-binding site (**Fig. 5a**); as the HflXr loop is two residues longer than HflX and differs in sequence (**Fig. 5b**), this offers the possibility that a distinct conformation adopted by HflXr could reach towards the macrolide-binding site to mediate antibiotic displacement using a type I or type II target protection mechanism. Independently evolved insertions within the loop region have also arisen in the HflX proteins of some organisms that lack an HflXr protein, including *Streptomyces fradiae* and *Mycobacterium abscessus* (**Fig. 5b**), and it has recently been established that mycobacterial HflX proteins also mediate resistance to macrolides and lincosamides[82]. It would be interesting to examine whether HflX and HflXr proteins are associated with resistance to other PTC-binding antibiotics, such as pleuromutilins, oxazolidinones and streptogramins, which have overlapping binding sites with macrolides and lincosamides (**Fig. 5a**).

[H2] Target protection mediated by antimicrobial peptide 'transporters'. Until very recently, all confirmed or apparent examples of target protection occurred inside bacterial cells. A study [83] has now provided evidence that the BceAB system of *Bacillus subtilis* mediates target protection at the outer surface of the cytoplasmic membrane to resist bacitracin and other peptide antibiotics that inhibit cell-wall biogenesis through binding of lipid II cycle intermediates. The following model has been proposed to describe this target protection mechanism. BceAB spans the cytoplasmic membrane, with its extracellular portion presented at the cell surface where it can recognise complexes of the antibiotic

bound to the target (undecaprenyl pyrophosphate in the specific case of bacitracin). Subsequent ATP hydrolysis by the intracellular ATPase domains of BceAB provides the energy to catalyse splitting of these extracellular antibiotic-target complexes, a process that may constitute a type I target protection mechanism.

This model provides a compelling explanation for the long-standing conundrum as to how a protein complex that resembles a transporter can mediate resistance to an antibiotic that acts on the outer surface of the bacterium. It also describes a target protection mechanism that is potentially responsible for resistance to various antibacterial compounds acting outside the cell, and across a range of bacteria that includes important pathogens. For example, it seems a reasonable assumption that other BceAB-type systems (for example, the VraDE system in *Staphylococcus aureus*[84]) mediate resistance through this same mechanism. VraDE makes a substantial contribution to intrinsic resistance to clinically deployed antibiotics, including daptomycin and bacitracin[85], and upregulation of expression of this peptide detoxification module constitutes a key route by which staphylococci can evolve resistance to antimicrobial peptides such as nisin[86,87]. A considerable number of other transporter-like systems are known to mediate resistance to antimicrobial peptides in bacteria[88], and future work should seek to distinguish those truly functioning as transporters from those mediating resistance via target protection.

[H1] The origin of target protection mechanisms

Although some target proteins (for example, the TRPPs) have conceivably evolved as dedicated antibiotic resistance factors, in other cases resistance is likely to be coincidental to their native cellular role or roles. For example, the ARE ABC-F and FusB-type proteins seem to be accessory translation factors that have evolved to optimise functioning of the core protein synthesis machinery[31,32,59,60], but in modulating the conformational and functional properties of this machinery, resistance to antibiotics results as a by-product. Reinforcing the idea that the original raison d'etre for these proteins is probably not to provide resistance, they are encoded within the core genomes of organisms that are highly unlikely to encounter the corresponding antibiotics in their natural habitats[59,89].

The target protection proteins with the clearest evolutionary origins are the enzymatic factors that protect the ribosome (ARE ABC-Fs, HflXr and TRPPs). In all cases, these have evolved from duplication of a housekeeping factor; HflX in the case of HflXr, an EF-G-like or EF2-like elongation factor in the case of TRPPs (**Fig. 6**), and in the case of ARE ABC-Fs, translation factors of unknown function, but probably with a role involving PTC modulation for optimisation of translation, perhaps similar to ABC-F EttA[32-34]

(**Fig. 6**). Although ARE ABC-Fs and TRPPs have evolved by the same process of duplication, their phylogenetic trees look very different (**Fig. 6**). The TRPPs form one very distinct branch in the EF2 family, which indicates that they have a single point of origin that is likely to be extremely ancient (**Fig. 6a**). By contrast, known ARE ABC-Fs do not branch together, have probably evolved multiple times independently, and functional diversification into dedicated translation and resistance factors is likely to be an ongoing process (**Fig. 6b**).

As with the ABC-Fs, target protection proteins in the HflX family may constitute a mix of dedicated resistance factors (HflXr) and multifunctional translation and/or resistance factors. Phylogenetic analysis indicates that the *hflX* gene duplication is present in many firmicutes, such as *Bacillus cereus* and *Clostridium difficile*, but is also observed in other phyla, including alpha-, beta-, gamma- and deltaproteobacteria[79], which suggests that HflXr proteins capable of mediating antibiotic resistance may exist in many different bacterial species. As indicated above, HflX itself seems to be involved in resistance in some bacteria. Beyond the very recent demonstration that mycobacterial HflX mediates macrolide resistance[82], the *S. fradiae hflX* gene resides within the biosynthetic gene cluster of the macrolide, spiramycin[90], and functional metagenomic databases constructed from antibiotic-rich environments have identified *hflX* genes as putative resistance determinants in *Simkania negevensis* and *Emergencia timonensis*[78,91].

Evolutionary parallels can be drawn between target protection and target modification mechanisms of antibiotic resistance, with examples again coming from the ribosome. The Cfr and Erm resistance proteins evolved from the housekeeping rRNA methyltransferases RlmN and KsgA, respectively[92-94]. Thus, in addition to carrying out their primary roles, proteins that work with the core cellular machinery are an important reservoir from which resistance could evolve by virtue of their innate ability to interact with or functionally modulate the target of the antibiotic. As a consequence, and as with target protection mechanisms, the boundary is blurred between what is a housekeeping or resistance factor. The fact that target protection can be 'accidental' helps to explain the existence of mechanisms against wholly synthetic antibacterial agents unlike those that exist in nature (for example, OptrA and PoxtA as a mechanism of resistance to the oxazolidinones), and highlights the scope for resistance to future antibacterial drugs (including synthetic agents) to emerge through target protection.

[H1] Overcoming target protection.

A growing appreciation of the molecular detail of target protection could help to inform the rational development of therapeutic approaches for overcoming this class of resistance mechanism. There are

two basic strategies for mitigating resistance to a given antibacterial drug class, both of which have been successfully used clinically to restore the therapeutic utility of agents whose activity has become compromised by resistance. The first of these involves generating analogues of the drug scaffold, with a view to 'designing-out' the resistance liability; such an approach has breathed new life into multiple antibiotic classes that include the β-lactams and the tetracyclines. The second pairs the antibacterial drug in question with a small-molecule inhibitor of the resistance mechanism, an approach uniquely exemplified clinically by the use of β-lactamase inhibitors (for example, clavulanic acid) that spare β-lactams from hydrolytic destruction.

Proof-of-principle already exists that type I target protection can be overcome via the former approach; as described above, the C9-moiety of the third generation tetracyclines enables them to evade TRPP-mediated resistance. Whether type II or type III target protection can be similarly addressed by chemical modification of drug classes subject to these resistance mechanisms is unclear. As both types of target protection effectively proceed via an allosteric mechanism, chemical modification of an inhibitor to comprehensively evade resistance would probably need to fundamentally alter the nature of its interaction with the target, something drug analogues do not routinely achieve. Nevertheless, modification of an antibacterial drug scaffold to increase affinity and/or potency at the level of the target has been demonstrated to deliver some degree of improvement in antibacterial activity against bacteria expressing a type II target protection mechanism. Tedizolid, a newer-generation oxazolidinones, exhibits greater potency than the parent compound of the class (linezolid) against purified ribosomes in an *in vitro* translation assay, probably because the drug makes additional interactions with the 23S rRNA[95]. This effect on potency is associated with a 4-8 fold increase in antibacterial activity[95], an improvement that is retained against bacteria carrying the ARE ABC-F protein, OptrA[36]. This example implies that if an analogue can achieve a sufficiently dramatic improvement in potency against the target, the impact of a type II target protection mechanism could effectively be negated by reducing the level of reduced susceptibility it mediates below the threshold for true clinical resistance.

In principle, it should also be feasible to generate small-molecule inhibitors of the different types of target protection mechanisms. In this regard, a recent study used fragment-based screening to identify an inhibitor of OptrA that competes with ATP for binding, and which thereby effects a 30% reduction in the essential ATPase activity of the enzyme[96]. In practice, the potential therapeutic utility of inhibiting a specific target protection protein will need to be carefully assessed on a case-by-case basis; only for an antibacterial drug for which the target protection mechanism in question is the major —

preferably the sole — source of resistance encountered clinically will it likely prove worthwhile to generate a specific target protection inhibitor with a view to rejuvenating antibacterial activity.

[H1] Concluding remarks

Target protection can no longer be considered a rare or unusual antibiotic-resistance mechanism of limited clinical importance; it is in fact one of the predominant mechanisms by which bacterial pathogens resist a host of drug classes that include the fluoroquinolones and the overwhelming majority of protein synthesis inhibitors in clinical use.

Target protection does not proceed via a single, uniform mechanism, and three mechanistic types of target protection have now been defined (**Fig. 1**): direct antibiotic displacement (type I); allosteric antibiotic removal (type II); and restoration of target function to overcome antibiotic-mediated inhibition (type III). Despite detailed structural and functional analysis of target protection systems in recent years, and as discussed above, gaps in our understanding remain. Some of these gaps may prove challenging to fill given the inherent difficulty of dissecting the complex interplay between a resistance protein, an antibiotic target and an antibiotic molecule. Nevertheless, a more comprehensive understanding of target protection will be vital both to raise our fundamental knowledge to a level comparable to that already gained for other mechanistic classes of resistance, and to assist efforts already underway to devise approaches for overcoming target protection-mediated antibiotic resistance and restoring the therapeutic efficacy of a broad cross-section of antibacterial drugs.

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Author contributions

A.J.O. and D.N.W. led the drafting of the manuscript, with substantial input from the other authors.

Competing interests

The authors declare no competing interests.

Publisher's note

Figure 1. Overview of target protection types. Target protection proteins (TPPS) can mediate antibiotic resistance by (a) sterically removing the drug from the target (type I), (b) by inducing conformational changes within the target that allosterically dissociate the drug from the target (type II), or (c) by inducing conformational changes within the target that restore functionality despite the presence of the bound antibiotic (type III).

Figure 2. Protection of ribosomes from tetracyline by Tet(M). (a) Model for Tet(M)-mediated tetracycline (Tet) resistance via ribosome binding and release of tetracycline (Tet). (b) Structure of Tet(M) on the 70S ribosome[24]. (c-d) Relative binding position of loop III of domain IV of Tet(M) relative to tetracycline (c) and tigecycline (d) [10,24]. Part a modified from Ref.[22].

Figure 3. Ribosomal protection against antibiotics mediated by the ARE ABC-F proteins. (a) Model for ARE ABC-F-type antibiotic resistance, using VmlR as example. (b) Overview of VmlR and P/V-tRNA on the ribosome with transverse section of the 50S subunit revealing the nascent polypeptide exit tunnel. (c) (b) VmlR superimposed with the group A streptogramin A, virginiamycin M (VgM, PDB ID 1YIT)[97], the lincomycin (Lnc, PDB ID 5HKV) and the pleuromutilin, tiamulin (Tia, PDB ID 1XBP). (d) Comparison of the binding-site of MsrE (PDB ID 5ZLU), the group B streptogramin virginiamycin S (VgS, PDB ID 1YIT)[97] and the macrolide, erythromycin (Ery, PDB ID 4V7U)[98].

Figure 4. Target protection mediated by FusB-type proteins. (a) FusB-type fusidic acid (Fus) resistance results from the ability of the resistance protein to bind elongation factor G EF-G and drive its dissociation from the ribosome even in the presence of fusidic acid. Although not central to the protection mechanism, fusidic acid probably dissociates from EF-G once the elongation factor has left the ribosome, as it has only low affinity for free EF-G. (b) Structure of EF-G stalled by fusidic acid on the 70S ribosome[63]. (c) Model for the interaction of FusB with domain IV of EF-G (blue). Part c modified from*Ref* [62].

Figure 5. Proposed mechanism of target protection by HflXr proteins. (a) Overview of HflX on the 50S subunit with transverse section revealing the nascent polypeptide exit tunnel (NPETZoom of the loop within the N-terminal domain of HflX superimposed with lincomycin (Lnc, , PDB ID 5HKV, left panel)[99] and erythromycin (Ery, PDB ID 4V7U, left panel)[98], and with virginiamycin M (VgM, PDB ID 1YIT, right panel)[97], tiamulin (Tia, PDB ID 1XBP, right panel)[100], linezolid (Lnz, PDB ID 3DLL, right panel)[101] and virginiamycin S (VgS, PDB ID 1YIT, right panel)[97]. (b) Sequence alignment of the resistance-associated

loop region within the N-terminal domain of selected HflX and HflXr representatives, showing independently evolved insertions in HflXr and HflX.

Figure 6. The evolution of target protections proteins within the elongation factor 2 and ABC-F families of translation factors. (a) Tetracycline ribosomal protection proteins (TRPP), translation elongation factor-G (EF-G), eukaryotic elongation factor 2 (eEF2) and archaeal EF2 (aEF2) sequences were selected from the translational GTPase database from Ref. [102]. (b) The ABC-F sequences and classifications are taken from a previous analysis[32]. The bacterial branches (in black) are members of multiple subfamilies, some of which may be uncharacterised resistance factors, but some are almost certainly specialised translation factors such as the indicated EttA clade[33,34]. The eukaryotic group (in green) contains three known translation factors (ABC50/ABCF1[103], Arb1/ABCF2[104] and Gcn20/ABCF3[105]). Trees shown are maximum likelihood protein phylogenies generated using RaxML[106], using the LG model, 100 bootstrap replicates and alignments trimmed to remove columns with >50% gap characters. Archaeal and eukaryotic proteins are shown with green and purple respectively; all other sequences are bacterial, with clades containing known TRPPs highlighted in red.

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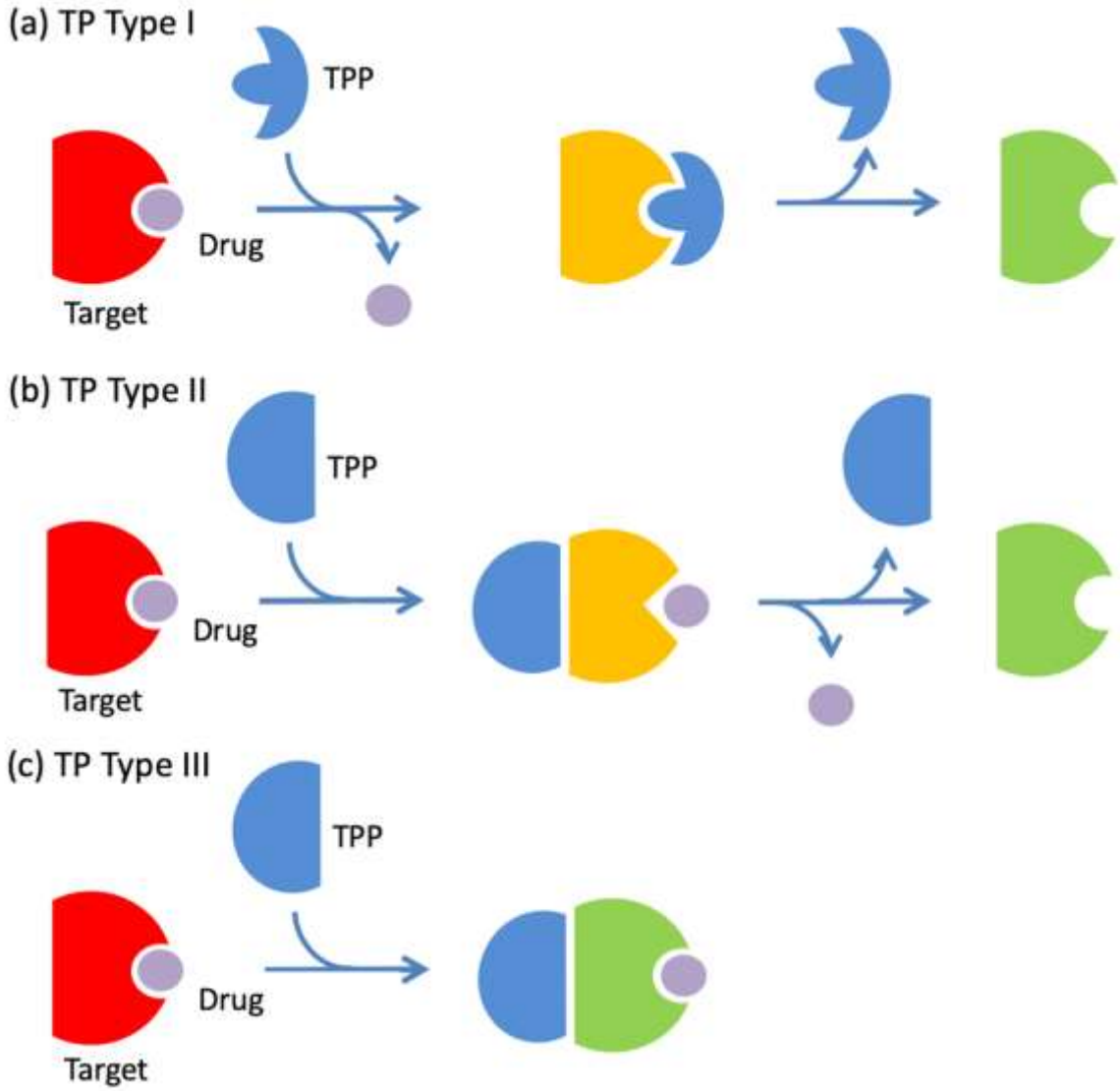

Figure 1. Overview of target protection (TP) Types I-III. Target protection proteins can mediate antibiotic resistance by (a) sterically removing the drug from the target (Type I TP), (b) inducing conformational changes within the target that allosterically dissociate the drug from the target (Type II TP), or (c) inducing conformational changes within the target that preserve functionality despite the presence of the bound antibiotic (Type III TP).

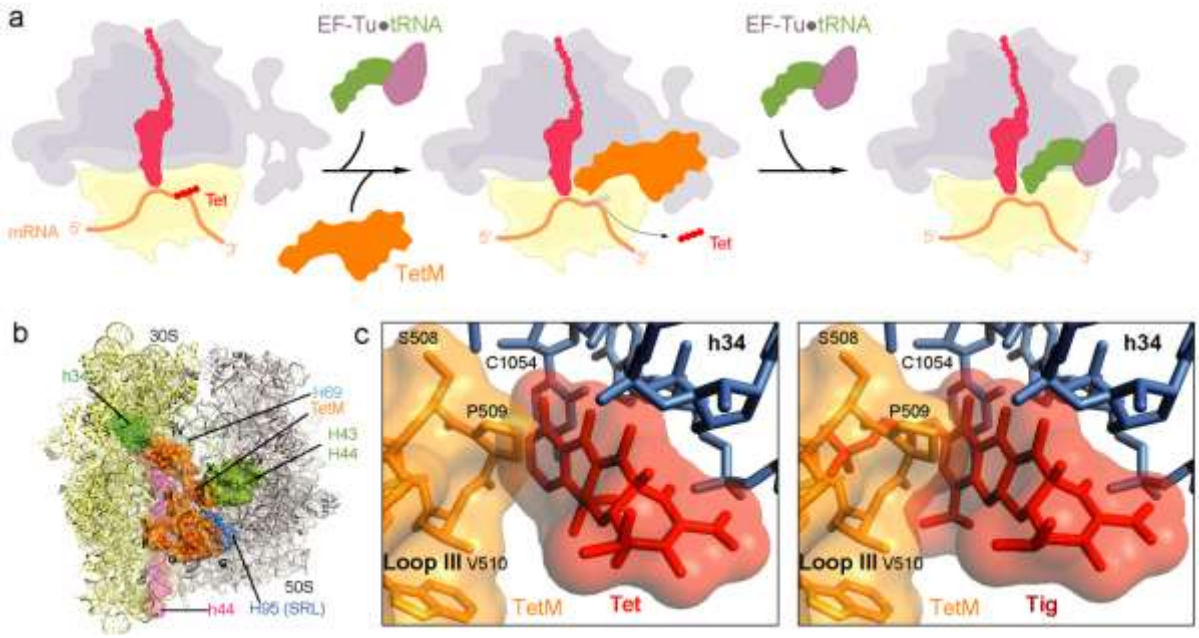

Figure 2. Protection of ribosomes from tetracyline by Tet(M). (a) Model for Tet(M)-mediated tetracycline resistance via ribosome binding and release of tetracycline (Tet) (modified from Dönhöfer *et al.*[22]). (b) Structure of Tet(M) on the 70S ribosome[24]. (c-d) Relative binding position of loop III of domain IV of Tet(M) relative to (c) tetracycline and (d) tigecycline[10,24].

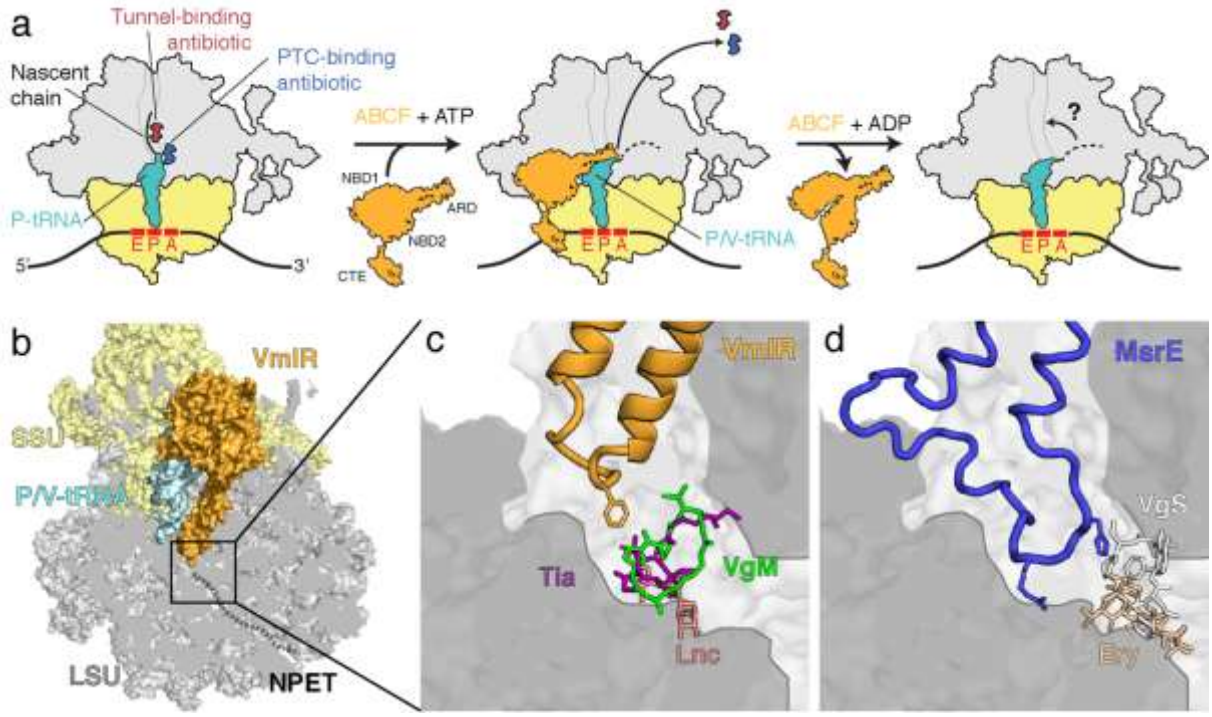

Figure 3. Ribosomal protection against antibiotics mediated by the ARE ABC-F proteins. (a) Model for ARE ABC-F-type antibiotic resistance (a). (b) Overview of VmlR (orange) and P/V-tRNA (green) on the ribosome (30S shown in yellow, 50S in grey) with transverse section of the 50S subunit revealing the nascent polypeptide exit tunnel[33]. (c) Zoom of (b) with VmlR (orange) superimposed with the STG_A, virginiamycin M (VgM, green, PDB ID 1YIT)[94], the LIN, lincomycin (Lnc, salmon, PDB ID 5HKV) and the PLM, tiamulin (Tia, purple, PDB ID 1XBP). (d) same view as (c) but comparing the binding site of MsrE (blue, PDB ID 5ZLU), the STG_B virginiamycin S (VgS, white, PDB ID 1YIT)[94] and the MAC, erythromycin (Ery, tan, PDB ID 4V7U)[95].

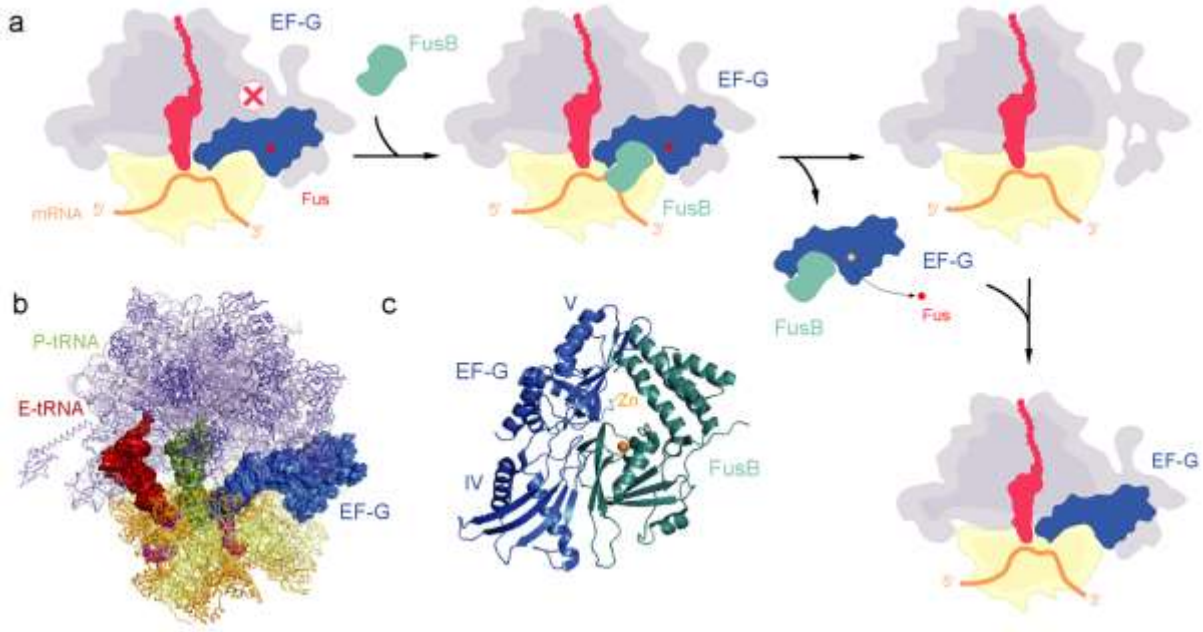

Figure 4. Target protection mediated by FusB-type proteins. (a) FusB-type fusidic acid (FA) resistance results from the ability of the resistance protein to bind EF-G and drive its dissociation from the ribosome even in the presence of FA. Although not central to the protection mechanism, FA likely dissociates from EF-G once the latter has left the ribosome, since it has only low affinity for free EF-G. (b) Structure of EF-G stalled by fusidic acid on the 70S ribosome[57]. (c) Model for the interaction of FusB (teal) with domain IV of EF-G (blue) [based on Tomlinson *et al.*[56]].

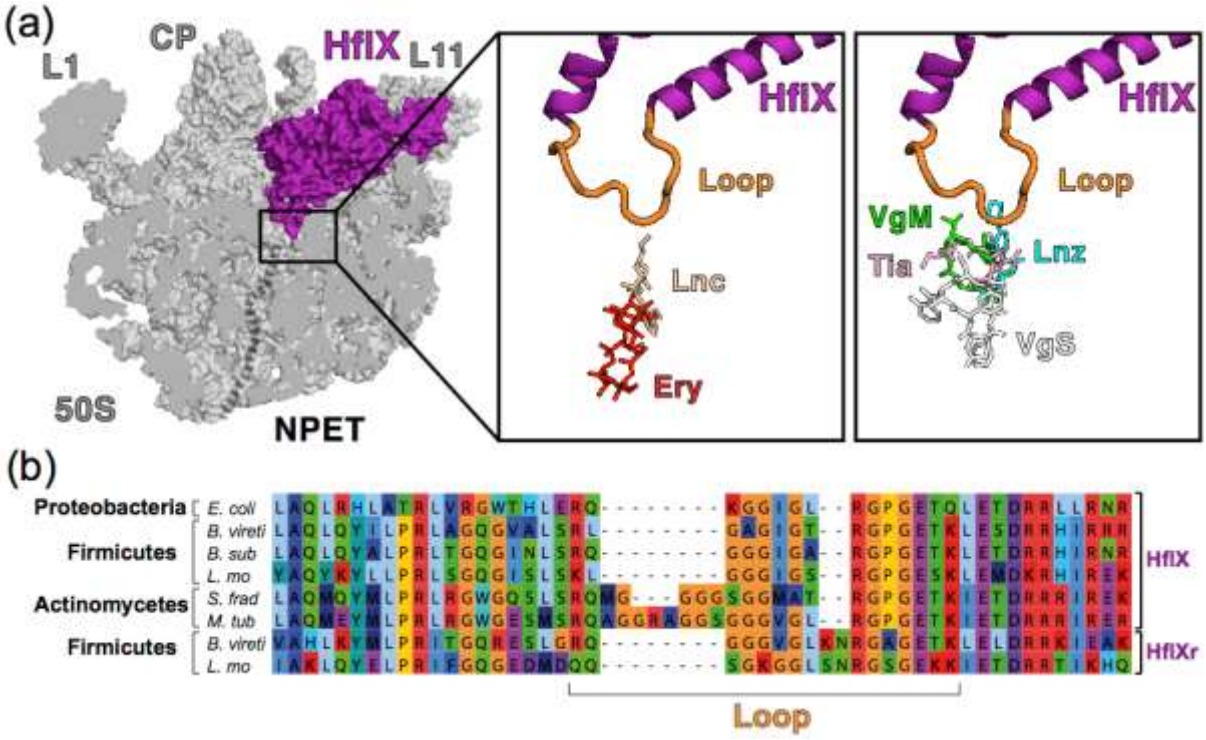

Figure 5. Proposed mechanism of target protection by HflXr proteins. (a) Overview of HflX (purple) on the 50S subunit (grey) with transverse section revealing the nascent polypeptide exit tunnel (NPET). Zoom of the loop (orange) within the N-terminal domain of HflX (purple) superimposed with (left) lincomycin (Lnc, salmon, PDB ID 5HKV)[96] and erythromycin (Ery, tan, PDB ID 4V7U)[95], and (right) with virginiamycin M (VgM, green, PDB ID 1YIT)[94], tiamulin (Tia, purple, PDB ID 1XBP)[97], linezolid (Lnz, blue, PDB ID 3DLL)[98] and virginiamycin S (VgS, white, PDB ID 1YIT)[94]. (b) Sequence alignment of the resistance-associated loop region (shaded) within the N-terminal domain of selected HflX and HflXr representatives, showing independently-evolved insertions in HflXr and HflX.

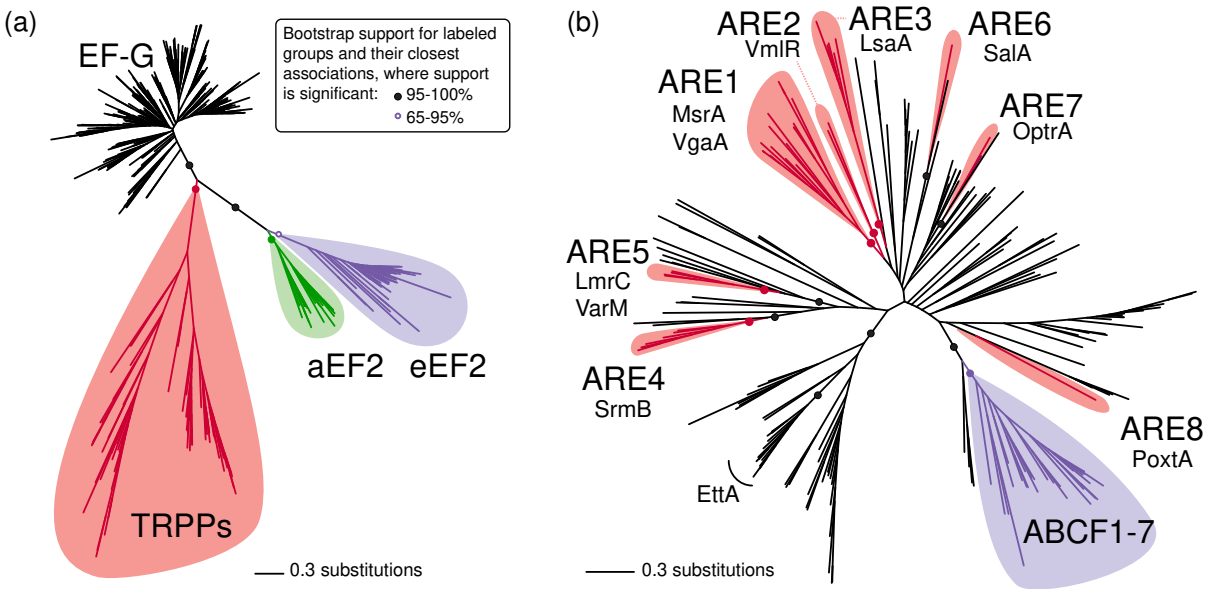

Figure 6. The evolution of TP proteins within the EF2 and ABC-F families of translation factors. (a) TRPP, EF-G, eEF2 and aEF2 sequences were selected from our translational GTPase database[99]. (b) The ABC-F sequences and classifications are from our previous analysis[32]. The bacterial branches (in black) are members of multiple subfamilies, some of which may be uncharacterised resistance factors, but some are almost certainly specialised translation factors such as the indicated EttA clade[85,86]. The eukaryotic group (in green) contains three known translation factors (ABC50/ABCF1[100], Arb1/ABCF2[101] and Gcn20/ABCF3[102]). Trees shown are maximum likelihood protein phylogenies made with RaxML[103], using the LG model, 100 bootstrap replicates and alignments trimmed to remove columns with >50% gap characters. Archaeal and eukaryotic proteins are shown with green and purple highlighting respectively; all other sequences are bacterial, with clades containing known TRPPs highlighted in red.