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**Target protection as a key antibiotic resistance mechanism**

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19 **Abstract**

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

## 29 [H1] Introduction

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

40

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

54

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

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

67

## 68 **[H1] Tetracycline ribosomal protection proteins**

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

82

83 Tet(M) and Tet(O) are closely related GTPases with sequence homology (<25% identity) to translation  
84 elongation factor-G (EF-G), and thus seem to represent EF-G paralogs that have evolved the specialized  
85 ability to rescue translation in the presence of tetracyclines<sup>7</sup>. Indeed, structural studies have shown  
86 that Tet(O) and Tet(M) bind to the ribosome in manner analogous to EF-G<sup>21-24</sup>. However, whereas EF-  
87 G recognises the ribosome in a pre-translocational state (that is, the A-site and P-site are occupied by  
88 tRNAs), Tet(O) and Tet(M) bind to a post-translocational state (that is, the P-site and E-site are  
89 occupied, with the A-site being free owing to the presence of tetracycline)<sup>21-24</sup>. The first, low-resolution  
90 (16 Å), cryo-electron microscopy structure of Tet(O) bound to the ribosome led to the suggestion that  
91 this TRPP indirectly drives the removal of tetracycline from the target by inducing a local disturbance  
92 in helix 34 (Ref. <sup>21</sup>) However, subsequent higher-resolution (3.9 Å to 9.6 Å) structures have established  
93 that both Tet(O) and Tet(M) directly overlap the tetracycline binding site on the ribosome<sup>22-24</sup> (**Fig. 2**),  
94 thereby mediating resistance through direct physical displacement of the drug. In all of these structural  
95 studies, the TRPP was trapped on the ribosome using non-hydrolysable GTP analogs, consistent with  
96 earlier biochemical studies indicating that GTP hydrolysis is required for factor dissociation rather than

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

108

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

118

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

121

### 122 **[H1] Antibiotic resistance ABC-F proteins.**

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

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

133

134 ABC-F proteins lack the transmembrane portions found in most other members of the ATP-binding  
135 cassette (ABC) superfamily, and instead comprise two ABC domains that are separated by a linker  
136 region<sup>30-32</sup>. This linker has been designated the P-site tRNA-interaction motif (PtIM)<sup>33,34</sup>, although  
137 amongst the ARE ABC-F proteins, it is also referred to as the antibiotic resistance domain (ARD)<sup>35</sup>. Three  
138 major categories of ARE ABC-F proteins can be differentiated on the basis of their antibiotic specificity:  
139 Vga, Lsa, Sal and Vml belong to the first category, and they mediate resistance to streptogramins of  
140 group A, lincosamides and sometimes pleuromutilins; Msr-type proteins mediate resistance to  
141 streptogramins of group B and macrolide antibiotics (and sometimes ketolides); and Optr and Poxr  
142 mediate resistance to oxazolidinones and phenicols<sup>31,32,36-38</sup>. Structures of representatives from the  
143 first two groups (VmlR from *Bacillus subtilis* and MsrE from *Pseudomonas aeruginosa*) in complex with  
144 the ribosome have revealed that these proteins bind into the E-site, with their ARDs reaching into the  
145 peptidyltransferase centre (PTC)<sup>35,39,40</sup> (**Fig. 3**). To access the PTC, these proteins induce a  
146 conformational change within the P-site tRNA, shifting the acceptor arm out of the PTC and towards  
147 the ribosomal A-site<sup>35,39</sup>. The loop located at the tip of the ARD varies considerably in length and  
148 sequence between ARE ABC-F proteins, and mutations within this region can affect antibiotic  
149 specificity<sup>31,35,39,41,42</sup>. When bound to the ribosome, a residue (Phe<sub>237</sub>) within the ARD loop of VmlR  
150 sterically overlaps the binding site of PTC-inhibiting antibiotics, such as lincosamides, streptogramins  
151 of group A and pleuromutilins<sup>35</sup>, consistent with the reported resistance spectrum of this protein (**Fig.**  
152 **3**)<sup>35,43</sup>. However, an engineered VmlR variant in which Phe<sub>237</sub> was replaced with Ala — a substitution  
153 that would be predicted to remove the overlap — still conferred resistance to lincosamides and  
154 pleuromutilins<sup>35</sup>, which suggests that the steric overlap is not critical for VmlR-mediated removal of  
155 these antibiotics from the ribosome. By contrast, this same VmlR variant lost the ability to mediate  
156 resistance to virginiamycin M<sup>35</sup>, which implies that an important steric component exists for removal  
157 of streptogramins of group A. In the case of MsrE, the ARD loop is longer and reaches deeper into the  
158 ribosomal exit tunnel, where Leu<sub>242</sub> of MsrE overlaps with the binding site of macrolides and  
159 streptogramins of group B<sup>39</sup> (**Fig. 3**). Substitution of Leu<sub>242</sub> to Ala leads to near-complete loss of ability  
160 to mediate azithromycin resistance, which suggests a strong steric component to the mechanism of  
161 macrolide removal from the ribosome<sup>39</sup>. Whether MsrE functions sterically or allosterically on  
162 streptogramins of group B remains to be determined. Taken together, these observations imply that  
163 the precise nature of the target protection mechanism of ARE ABC-F proteins depends not only on the  
164 target protection protein itself, but can also vary amongst the targeted antibiotic class. Thus, in some

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

170

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

186

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

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

210

### 211 **[H1] FusB-type proteins**

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

233

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

250

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

254

### 255 **[H1] Other examples of target protection.**

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

263

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

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

280

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

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

303

304 [H2] Target protection mediated by HflX-type proteins. Treatment of *Listeria monocytogenes* with sub-  
305 inhibitory concentrations of lincosamides dramatically affects the gene expression program, including  
306 inducing transcription of Lmo0919 (an ARE ABC-F protein)<sup>47</sup> and Lmo0762<sup>79</sup>. The latter has been found  
307 to mediate modest levels of resistance to lincosamides and macrolides, an effect that is only apparent  
308 in a genetic background lacking Lmo0919<sup>79</sup>. Lmo0762 exhibits homology to HflX<sup>79</sup>, a ribosome-splitting  
309 GTPase that rescues stalled ribosomes under stress conditions<sup>80,81</sup>. Reflecting this similarity and the  
310 fact that the protein mediates a degree of antibiotic resistance, it was subsequently termed HflXr, a  
311 descriptor that also distinguishes it from another listerial HflX protein (Lmo1296) that has no role in  
312 resistance<sup>79</sup>. Analogous to HflX, HflXr seems to induce dissociation of 70S ribosomes into their 30S and  
313 50S subunits. However, it remains unclear whether HflXr is also directly responsible for antibiotic  
314 displacement or whether another factor is recruited to the antibiotic-bound 50S particles to fulfil this  
315 function<sup>79</sup>. We note that in the cryo-electron microscopy structure of the *E. coli* HflX•GDPNP•50S  
316 complex, the loop connecting two helices of subdomain II within the N-terminal domain of HflX is  
317 positioned at the PTC in close proximity to the lincosamide-binding site (**Fig. 5a**); as the HflXr loop is  
318 two residues longer than HflX and differs in sequence (**Fig. 5b**), this offers the possibility that a distinct  
319 conformation adopted by HflXr could reach towards the macrolide-binding site to mediate antibiotic  
320 displacement using a type I or type II target protection mechanism. Independently evolved insertions  
321 within the loop region have also arisen in the HflX proteins of some organisms that lack an HflXr protein,  
322 including *Streptomyces fradiae* and *Mycobacterium abscessus* (**Fig. 5b**), and it has recently been  
323 established that mycobacterial HflX proteins also mediate resistance to macrolides and lincosamides<sup>82</sup>.  
324 It would be interesting to examine whether HflX and HflXr proteins are associated with resistance to  
325 other PTC-binding antibiotics, such as pleuromutilins, oxazolidinones and streptogramins, which have  
326 overlapping binding sites with macrolides and lincosamides (**Fig. 5a**).

327

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

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

339

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

353

### 354 **[H1] The origin of target protection mechanisms**

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

363

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

369 (Fig. 6). Although ARE ABC-Fs and TRPPs have evolved by the same process of duplication, their  
370 phylogenetic trees look very different (Fig. 6). The TRPPs form one very distinct branch in the EF2  
371 family, which indicates that they have a single point of origin that is likely to be extremely ancient (Fig.  
372 6a). By contrast, known ARE ABC-Fs do not branch together, have probably evolved multiple times  
373 independently, and functional diversification into dedicated translation and resistance factors is likely  
374 to be an ongoing process (Fig. 6b).

375

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

387

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

399

#### 400 [H1] Overcoming target protection.

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

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

411

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

430

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

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

439

#### 440 **[H1] Concluding remarks**

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

445

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

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

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

490

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

495

496 **Figure 3. Ribosomal protection against antibiotics mediated by the ARE ABC-F proteins.** (a) Model for  
497 ARE ABC-F-type antibiotic resistance, using VmlR as example. (b) Overview of VmlR and P/V-tRNA on  
498 the ribosome with transverse section of the 50S subunit revealing the nascent polypeptide exit tunnel.  
499 (c) (b) VmlR superimposed with the group A streptogramin A, virginiamycin M (VgM, PDB ID 1YIT)<sup>97</sup>,  
500 the lincomycin (Lnc, PDB ID 5HKV) and the pleuromutilin, tiamulin (Tia, PDB ID 1XBP). (d) Comparison  
501 of the binding-site of MsrE (PDB ID 5ZLU), the group B streptogramin virginiamycin S (VgS, PDB ID  
502 1YIT)<sup>97</sup> and the macrolide, erythromycin (Ery, PDB ID 4V7U)<sup>98</sup>.

503

504 **Figure 4. Target protection mediated by FusB-type proteins.** (a) FusB-type fusidic acid (Fus) resistance  
505 results from the ability of the resistance protein to bind elongation factor G EF-G and drive its  
506 dissociation from the ribosome even in the presence of fusidic acid. Although not central to the  
507 protection mechanism, fusidic acid probably dissociates from EF-G once the elongation factor has left  
508 the ribosome, as it has only low affinity for free EF-G. (b) Structure of EF-G stalled by fusidic acid on the  
509 70S ribosome<sup>63</sup>. (c) Model for the interaction of FusB with domain IV of EF-G (blue). Part c modified  
510 from *Ref*<sup>62</sup>.

511

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

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

520

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

533

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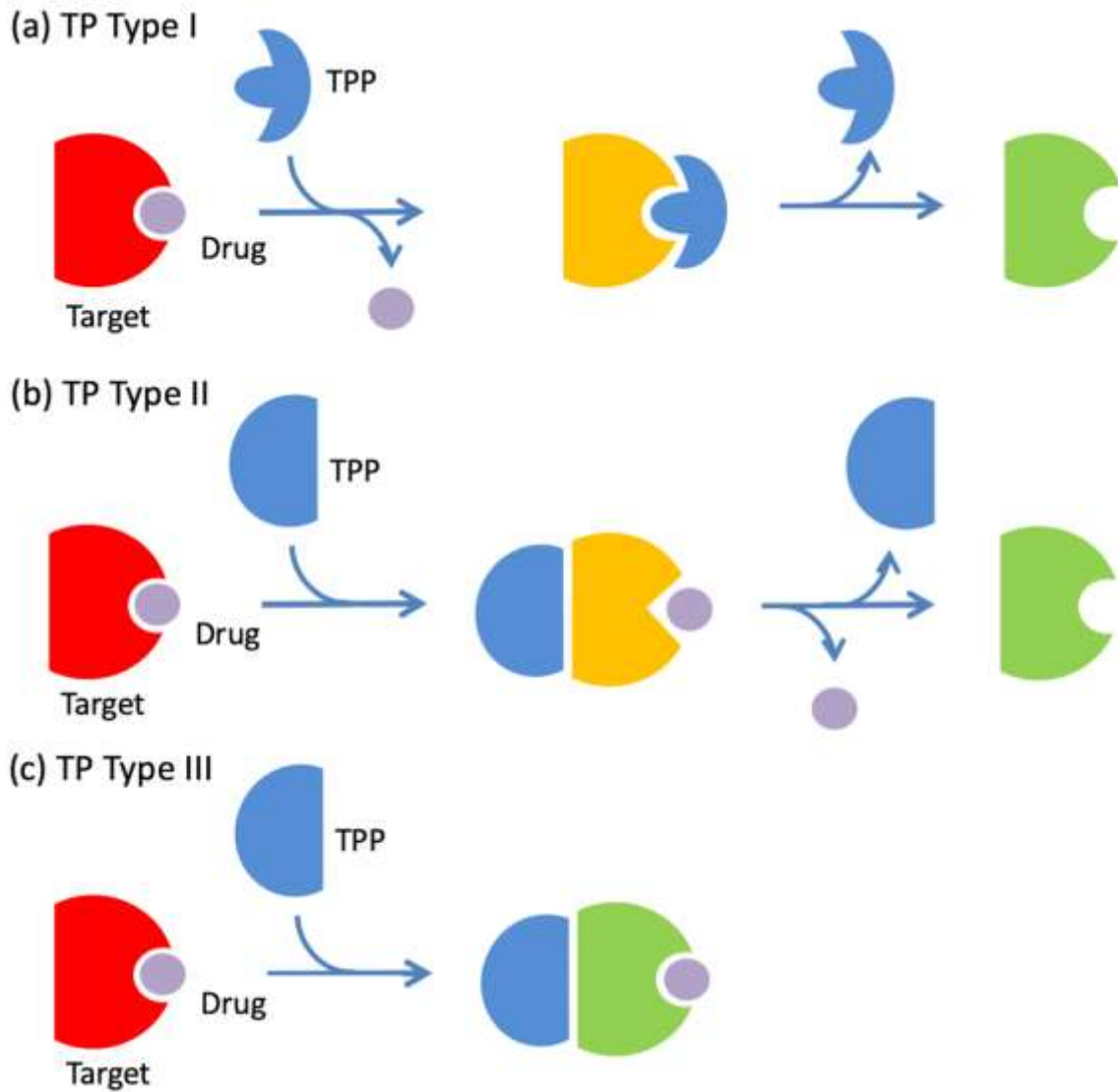
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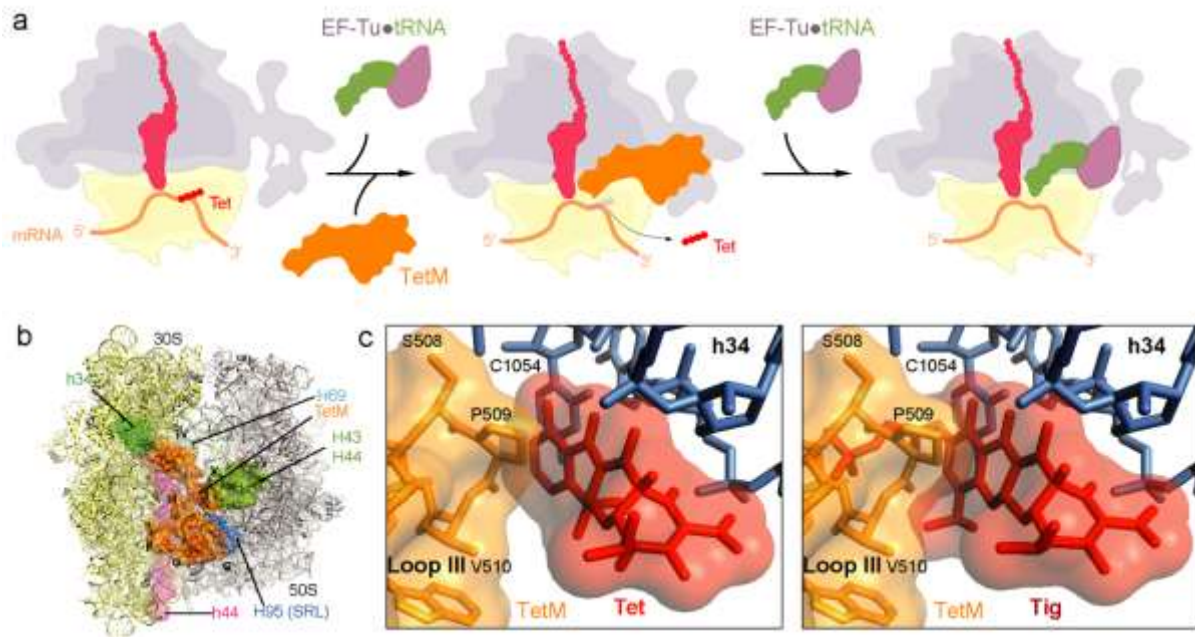
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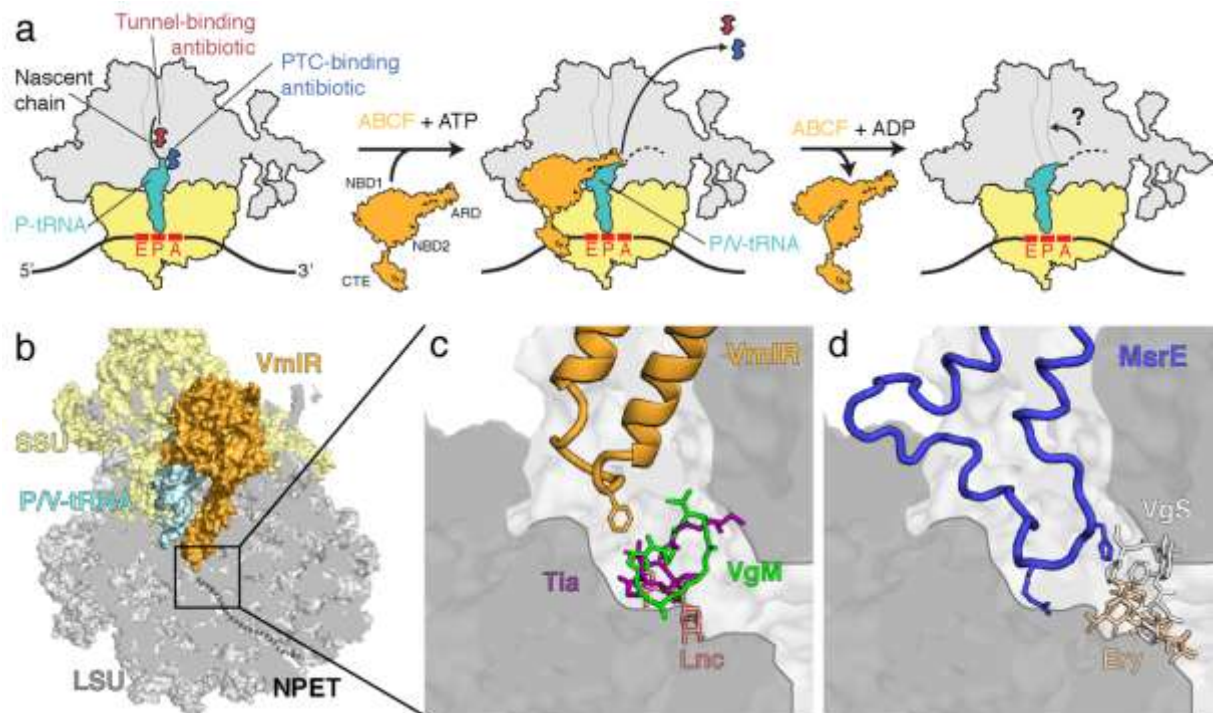
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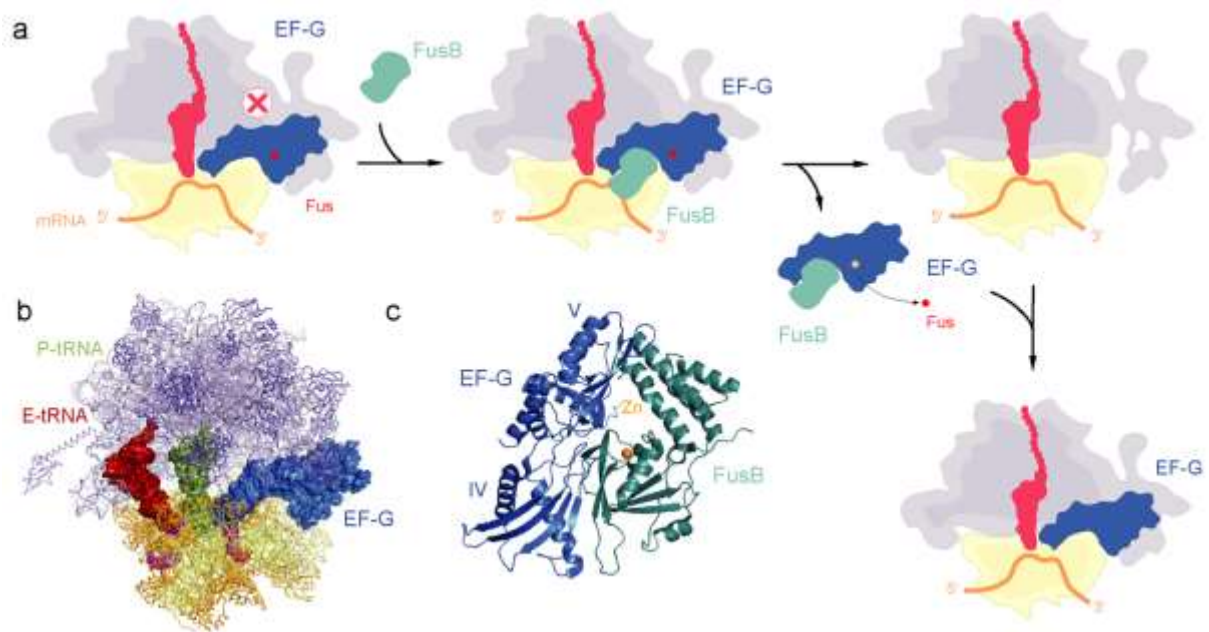
**Figure 1. Overview of target protection (TP) Types I-III.** Target protection proteins can mediate antibiotic resistance by (a) sterically removing the drug from the target (Type I TP), (b) inducing conformational changes within the target that allosterically dissociate the drug from the target (Type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



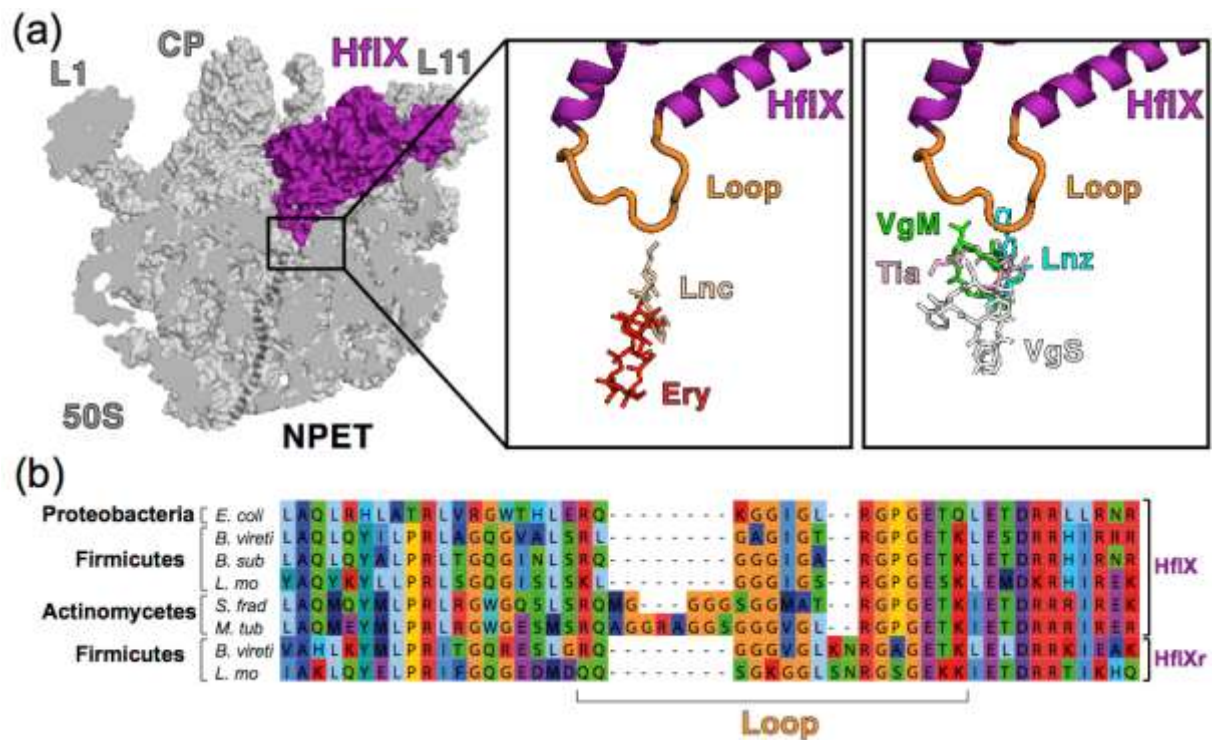
**Figure 2. Protection of ribosomes from tetracycline by Tet(M).** (a) Model for Tet(M)-mediated tetracycline resistance via ribosome binding and release of tetracycline (Tet) (modified from Dönhöfer *et al.*<sup>22</sup>). (b) Structure of Tet(M) on the 70S ribosome<sup>24</sup>. (c-d) Relative binding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



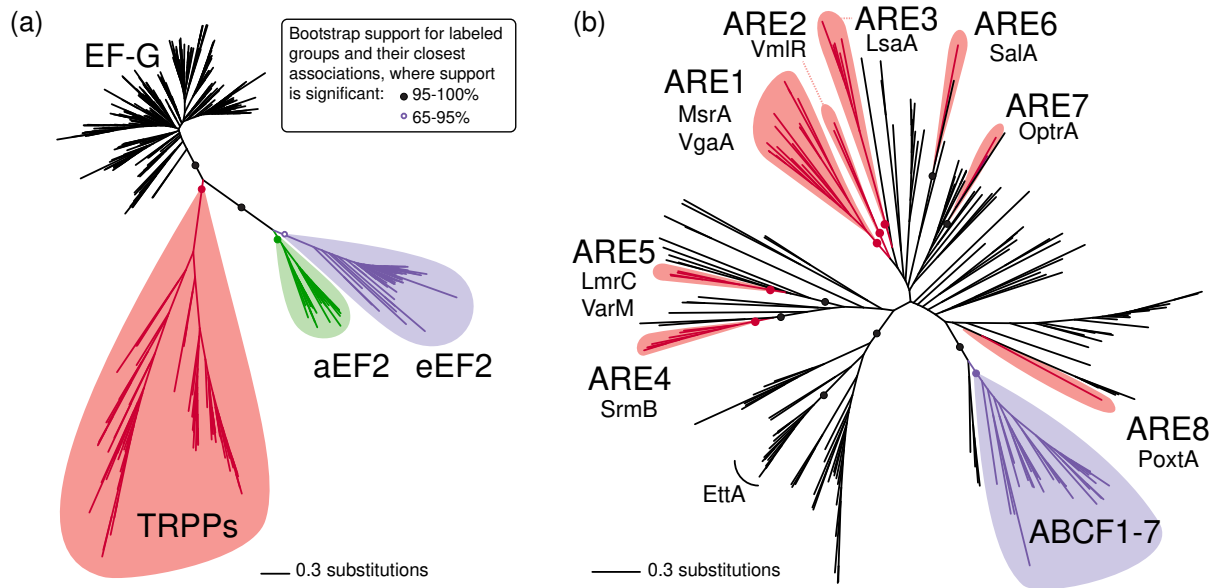
**Figure 3. Ribosomal protection against antibiotics mediated by the ARE ABC-F proteins.** (a) Model for ARE ABC-F-type antibiotic resistance (a). (b) Overview of VmlR (orange) and P/V-tRNA (green) on the ribosome (30S shown in yellow, 50S in grey) with transverse section of the 50S subunit revealing the nascent polypeptide exit tunnel<sup>33</sup>. (c) Zoom of (b) with VmlR (orange) superimposed with the STG<sub>A</sub>, virginiamycin M (VgM, green, PDB ID 1YIT)<sup>94</sup>, 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<sub>B</sub> virginiamycin S (VgS, white, PDB ID 1YIT)<sup>94</sup> and the MAC, erythromycin (Ery, tan, PDB ID 4V7U)<sup>95</sup>.



**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<sup>57</sup>. (c) Model for the interaction of FusB (teal) with domain IV of EF-G (blue) [based on Tomlinson *et al.*<sup>56</sup>].



**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)<sup>96</sup> and erythromycin (Ery, tan, PDB ID 4V7U)<sup>95</sup>, and (right) with virginiamycin M (VgM, green, PDB ID 1YIT)<sup>94</sup>, tiamulin (Tia, purple, PDB ID 1XBP)<sup>97</sup>, linezolid (Lnz, blue, PDB ID 3DLL)<sup>98</sup> and virginiamycin S (VgS, white, PDB ID 1YIT)<sup>94</sup>. (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<sup>99</sup>. (b) The ABC-F sequences and classifications are from our previous analysis<sup>32</sup>. 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<sup>85,86</sup>. The eukaryotic group (in green) contains three known translation factors (ABC50/ABCF1<sup>100</sup>, Arb1/ABCF2<sup>101</sup> and Gcn20/ABCF3<sup>102</sup>). Trees shown are maximum likelihood protein phylogenies made with RaxML<sup>103</sup>, 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.