Structural basis for TetM-mediated tetracycline resistance

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Ribosome protection proteins (RPPs) confer tetracycline resistance by binding to the ribosome and chasing the drug from its binding site. The current model for the mechanism of action of RPPs proposes that drug release is indirect and achieved via conformational changes within the drug-binding site induced upon binding of the RPP to the ribosome. Here we report a cryo-EM structure of the RPP TetM in complex with the 70S ribosome at 7.2-Å resolution. The structure reveals the contacts of TetM with the ribosome, including interaction between the conserved and functionally critical C-terminal extension of TetM and the decoding center of the small subunit. Moreover, we observe direct interaction between domain IV of TetM and the tetracycline binding site and identify residues critical for conferring tetracycline resistance. A model is presented whereby TetM directly dislodges tetracycline to confer resistance.

Tetracyclines are broad-spectrum antibiotic agents that bind to the translational apparatus to inhibit protein synthesis. The third generation of tetracycline derivatives, such as tigecycline, display enhanced antimicrobial activity, overcoming resistance mechanisms, for example, by stimulating delivery of the ternary complex Tgc, the TetM tetrameric complex formed in the presence of the nonhydrolyzable GTP analogue GDPNP (500 μM). Pelleting assays confirmed that binding of TetM to the 70S ribosome was observed in the presence of Tgc, suggesting that analysis of this complex by cryo-EM may enable TetM and Tgc to be visualized simultaneously on the same ribosome. To analyze the interplay between TetM and Tgc, the TetM•70S complex formed in the presence of Tgc was selected for analysis by cryo-EM and single particle reconstruction. From a total of 406,687 particles, in silico sorting yielded three main subpopulations of 70S ribosomal particles (SI Appendix, Fig. S2A): rotated (40%) and nonrotated (33%) 70S ribosomes without density for TetM and a TetM-bound 70S ribosome (27%) exhibiting a nonrotated conformation. Further refinement of the TetM•70S complex resulted in a final density map (Fig. 1A) with a resolution of 7.2 Å (EMD-2183), as determined by using the Fourier shell correlation cutoff value of 0.5 (SI Appendix, Fig. S2B). The absence of a subpopulation of TetM bound to rotated 70S ribosomes suggests that TetM binds preferentially to the nonrotated state, i.e., analogous to the C1054 within h34 from chemical modification, whereas the reactivity of A1408 in h44 is enhanced (15). As TetO is not observed to directly interact with C1054 or A1408 (14), TetO was suggested to chase tetracycline from the ribosome indirectly via inducing local disturbances within h34 (9, 14, 15). Moreover, the conformational changes were proposed to persist after TetO has dissociated from the ribosome, preventing rebinding of tetracycline as well as stimulating delivery of the ternary complex (9, 16).

To gain further structural insights into the interaction of RPPs with the ribosome and the mechanism of RPP-mediated tetracycline release, we have determined a cryo-EM structure of the RPP TetM bound to the 70S ribosome at 7.2-Å resolution. The improved quality of the map allows us to present the first molecular model for TetM as well as a detailed account of TetM interactions with the ribosome. Surprisingly, the higher resolution enables us to observe density for a loop in domain IV of TetM that interacts directly with the tetracycline binding site, indicating that RPP action uses a direct mechanism of action to dislodge and release tetracycline from the ribosome.

Results and Discussion

Cryo-EM Structure of a TetM•70S Complex. Escherichia coli 70S ribosomes (0.4 μM) were mixed together with Tgc (10 μM), and subsequently incubated at 37 °C for 20 min in the presence of purified recombinant Enterococcus faecalis TetM protein (4 μM) and the nonhydrolysable GTP analogue GDPNP (500 μM). Pelleting assays confirmed that binding of TetM to the 70S ribosome was observed in the presence of Tgc, suggesting that analysis of this complex by cryo-EM may enable TetM and Tgc to be visualized simultaneously on the same ribosome. To analyze the interplay between TetM and Tgc, the TetM•70S complex formed in the presence of Tgc was selected for analysis by cryo-EM and single particle reconstruction. From a total of 406,687 particles, in silico sorting yielded three main subpopulations of 70S ribosomal particles (SI Appendix, Fig. S2A): rotated (40%) and nonrotated (33%) 70S ribosomes without density for TetM and a TetM-bound 70S ribosome (27%) exhibiting a nonrotated conformation. Further refinement of the TetM•70S complex resulted in a final density map (Fig. 1A) with a resolution of 7.2 Å (EMD-2183), as determined by using the Fourier shell correlation cutoff value of 0.5 (SI Appendix, Fig. S2B). The absence of a subpopulation of TetM bound to rotated 70S ribosomes suggests that TetM binds preferentially to the nonrotated state, i.e., analogous to the
expected (posttranslational) substrate resulting from tetra-
cycline inhibition.

Molecular Model for the TetM•70S Complex. The quality and resolu-
tion of the density map for the TetM•70S complex allowed an
unambiguous fit of molecular models for the E. coli 30S and 50S
subunits (17–19) (SI Appendix, Fig. S3). A large additional density
within the subunit interface was attributed to TetM (Fig. 1A), as
expected from the similarity in location of TetO (14) and EF-G
(20–22) on the ribosome. In the absence of a crystal structure of
any RPP, a homology model for TetM was built on the basis of
the TetO map was not deposited in a public database. TetM
significantly overlaps with the anticodon stem-loop of the A-
tRNA (Fig. 1C), as observed for EF-G bound to a post-
translocation state ribosome (22). However, the binding position of
TetM does not overlap in position with the mRNA, and, unlike
EF-G, TetM does not appear to encroach on the P-site (SI Ap-
pendix, Fig. S5). Moreover, whereas the overall orientation of
TetM on the ribosome is similar to that of EF-G (22), EF-G is shifted in
position relative to TetM, being located closer to the 30S subunit
and further away from the stalk base of the 50S subunit (Fig. 1D
and SI Appendix, Fig. S6). The overall orientation of TetM appears
rather to structurally mimic more closely the position of EF-
Tu•mRNA bound to the 70S ribosome (SI Appendix, Fig. S6). Based
on the fit of the molecular model of TetM and the 70S ribosome
to the cryo-EM density, a list of interactions was compiled (SI Ap-
pendix, Table S1 and Fig. S7). At 7.2-Å resolution, the inter-
actions can only be approximated, and thus the closest residues
between TetM and the ribosome at the site of density fusion are
listed. In general, the contacts are similar to those reported pre-
viously for other translational GTPases, such as EF-G (21, 22),
LepA (23), and, at the domain level, TetO (14), and are discussed in
more detail in the SI Appendix.

CTE of TetM Interacts with the Ribosomal Decoding Site. The
homology model for TetM based on the EF-G template encompasses
residues 1 to 610, leaving 29 C-terminal residues that are not in-
cluded in the initial TetM model. However, fitting of the initial
TetM homology model to the TetM•70S map revealed additional
unsigned density that initiates at the C terminus of domain V,
traverses domain IV, and, eventually forms a large rod-like density
adjacent to the tip of domain IV (Fig. 2A). This rod-like density
remains present at high thresholds and exhibits the characteristics
of an α-helix (SI Appendix, Fig. S4). Therefore, the CTE of TetM
was modeled as a short 11-aa α-helix (residues 627–637) connected
to domain V by a flexible unstructured loop (Fig. 2B), in accor-
dance with secondary structure predictions (Fig. 2C).

The CTE interacts with a loop region at the tip of domain IV
of TetM, but also with H69 of the 23S rRNA (Fig. 2D). Addi-
tionally, density is seen connecting the distal end of helix-αA of
the TetM-CTE with helix 44 of the 16S rRNA, in close proximity
to A1492 and A1493 (Fig. 2E). As these bases are known to be
flexible and can flip out of helix 44 during mRNA-tRNA decoding
(24), the flipped-out conformation of these bases was also fitted
(Fig. 2D and E). We believe this flipped-out conformation
not only correlates with the fused electron density between h44
and the CTE (Fig. 2D and E), but also explains the hole in the
density of helix 44 caused by the absence of A1492 and A1493
stacking within the helix (Fig. 2E). Binding of TetO to the ri-
bose leads to an enhancement in the chemical reactivity of
A1408 of the 16S rRNA to DMS modification (15). Consistently,
the stacked conformation of A1493 would protect A1408 from
modification (SI Appendix, Fig. S7 C and D), whereas the flipped-
out conformation would expose A1408, allowing easier access for
DMS modification (Fig. 2E). Collectively, these results suggest
that binding of both TetM (and TetO) to the ribosome leads to
the flipping out of A1492 and A1493—a conformation that is
stabilized via interaction with the CTE of TetM. The enhance-
ment of A1408 is also observed when TetO is bound with GTP
rather than GDPNP (15), suggesting that the flipped-out con-
formation of A1492 and A1493 remains after the RPP has left the
ribosome.

Domain IV of TetM Directly Encroaches Upon the Tetracycline Binding Site. Domain IV of TetM interacts with the cleft between the head and body of the small subunit (Fig. 3A). Sequence align-
ments (SI Appendix, Fig. S1) and homology modeling suggest that
domain IV of TetM is structurally analogous to EF-G, containing
a four-stranded β-sheet and two α-helices, with an overall ββββ-β
(topology (Fig. 3B). Three loops protrude from one end of domain

Fig. 1. Cryo-EM reconstruction of a TetM•70S complex. (A) Final map of the
TetM•70S complex with TetM (orange), 30S (yellow), and 50S (gray). (b, body; CP, central protuberance; h, head; sp, spur). (S) Schematic color code of the
domain structure of EF-G and TetM (domain I, G′ subdomain, II, III, IV, V, and CTE are shown in green, blue, red, yellow, pink, pale blue, and orange,
respectively), with fit of the homology model for TetM into the extracted cryo-EM density (gray mesh). (C) Relative binding position of domain
IV of TetM (orange) compared with mRNA (tan), A-tRNA (green), and P-tRNA (blue). (D) Relative positions of domain V of TetM (orange) and EF-G
(22) (blue) with their respective stalk base regions (H43/H44 and L11-NTD)
colored pale blue and yellow, respectively. Arrows in D indicate the shift in the
position of the stalk base between TetM and EF-G.
IV of TetM, hereafter referred to as loops I, II, and III (Fig. 3B). The proline-rich loop I, located between β2α and β3α, is bent significantly to allow interaction with the RPP-specific C-terminal helix αCTE (SI Appendix, Fig. S8A), whereas, in contrast, loop I of EF-G is longer and adopts an extended conformation on the ribosome that establishes interaction with the P-tRNA (22) (SI Appendix, Fig. S8B). Loop II between β4α and α3α interacts with the proximal end of h34 of the 16S rRNA, with residues Ser465–Leu466–Gly467 (465SLG467) coming into close proximity with the backbone of C1209 and the nucleobase of C1214 (Fig. 3C). Consistently, binding of TetO to the ribosome protects C1214 from DMS modification (15, 16).

Loop III of TetM linking β5α to helix αB is less well-resolved than loops I and II, yet additional electron density is clearly observable within the TetM–70S map (Fig. 3C), which, in contrast, is absent in other cryo-EM maps lacking A-site ligands, such as the SecM-stalled ribosome nascent chain complex (26) (Fig. 3D). This additional density in the TetM–70S map fuses directly with 30S showing lack of density for Tgc in comparison with (F) the nonrotated 70S map without TetM from sorting (SI Appendix, Fig. S2) that reveals density for Tgc.

### Interplay of TetM and Tgc on the Ribosome.

Although the TetM–70S complex was formed in the presence of 10 μM Tgc, no density for the drug is observable in the TetM–70S map (Fig. 3E). As we observe the presence of a small population of 70S ribosome containing TetM (27%) (SI Appendix, Fig. S2A), we conclude that TetM can bind to the Tgc–70S complex and recycle Tgc; however, it does so too inefficiently (compared with tetracycline) as to allow translation levels necessary for viability. Density for Tgc is, however, clearly present in the nonrotated (Fig. 3F) and rotated (SI Appendix, Fig. S10A–C) 70S ribosomes where TetM is not bound. Because there is no structure reported for Tgc–70S, the observed binding position of Tgc was modeled into the primary tetracycline binding site (2, 3) based on similarity of the chemical structures of the two drugs (SI Appendix, Fig. S10D–H). In contrast to tetracycline, which has multiple binding sites on the ribosome (2, 3), a careful examination of the TetM–70S map reveals no additional density for Tgc within any of the secondary tetracycline binding sites (SI Appendix, Fig. S10I–M). Given that the TetM–70S complex was formed with 10 μM Tgc, which is ~100 times its IC50 for in vitro translation (8), and that

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**Fig. 2.** Localization and interaction of the CTE of TetM. (A) Density (gray mesh) of domain IV of TetM fitted with homology model (colors are as in Fig. 1B). The arrow indicates the site where the homology with EF-G ends, yet additional density is observed extending from domain V toward domain IV (asterisk). (B) Same as A but with extended CTE (orange), modeled based on (C) PISCRED secondary structure prediction, with sequence (Seq.), prediction (Pred.), and probability (Prob.) as indicated. Underlined residues were deleted to create TetM–ΔCTE. (D and E) Interaction of the CTE of TetM (orange) with H69 (blue) of the 23S rRNA and h44 of the 16S rRNA (pale blue), modeled with A1492 and A1493 flipped out of h44 [Protein Data Bank ID 2XQD (25)].
Tgc has a ~10-fold higher affinity for the ribosome than tetracycline (7, 8, 27), the absence of secondary binding sites illustrates the increased specificity of Tgc compared with tetracycline.

Interplay of TetM and Tetracycline on the Ribosome. Compared with Tgc, tetracycline that lacks the C9-glycyl side chain (SI Appendix, Fig. S10) exhibits significantly less overlap with the TetM density and would still permit interaction between the side chains within loop III and C1054 in h34 of the 16S rRNA (Fig. 4A). In contrast, the attached C9-glycyl side chain of Tgc would prevent access of the residues of loop III of TetM (Fig. 3E and F), leading us to suggest that this steric hindrance contributes, together with the increased affinity of Tgc (7, 8, 27), to explaining how Tgc overcomes TetM-mediated resistance whereas tetracycline cannot (8). Curiously, loop III of EF-G contains a highly conserved histidine residue (HS83; SI Appendix, Fig. S8B), which has been shown to be critical for the translocation activity of EF-G (28). The equivalent residue to HS83 of E. coli EF-G in TetM is tyrosine 507 (Y507; SI Appendix, Fig. S1), which, in the TetM·70S model, comes into close proximity of C1054 (Fig. 4A). Moreover, loop III contains a number of residues that are highly conserved in RPPs, in particular the s98SPV/S11 motif that directly follows Y507 (SI Appendix, Fig. S1). Thus, to investigate whether residues located within loop III of TetM are important for conferring tetracycline resistance, alanine-scanning mutagenesis was used to generate TetM variants with single-residue mutations Y506A, Y507A, S508A, P509A, V510A, and S511A. In addition, we introduced a premature stop codon at residue 623, thus truncating the last 17 aa (Fig. 2C, underlined) and generating a TetM variant lacking the CTE α-helix (∆CTE).

The growth of WT E. coli strain BL21 (~TetM) in the presence of increasing concentrations of tetracycline (0–128 μg/mL) was compared with the same strain bearing a plasmid overexpressing E. faecalis TetM (+TetM) or one of the TetM variants (Fig. 4B).

In the absence of TetM protein, the WT E. coli strain (Fig. 4B, black squares) is sensitive to tetracycline with minimal inhibition concentration (MIC50) of ~0.6 μg/mL, whereas, as expected, overexpression of E. faecalis TetM (Fig. 4B, red circles) raises the MIC50 by 14-fold to ~10 μg/mL (Fig. 4B). Surprisingly, no single alanine substitution within loop III of TetM exhibited a significant effect on the ability of TetM to confer tetracycline resistance (shown for Y506A and Y507A in Fig. 4B), whereas, in contrast, deletion of the CTE of TetM extensively inhibited resistance activity, decreasing the MIC50 value (~0.4 μg/mL; Fig. 4B, blue triangles) to that observed in the absence of TetM overexpression. Subsequent analysis of double and triple mutations within loop III of TetM led to the identification of the Y506A–Y507A (YY/AA) and Y507A–S508A–P509A (YP/AA) TetM variants as inactive, whereas the S508A–P509A–V510A (SP/AAA) triple mutant retained activity (Fig. 4B).

Conclusion

The cryo-EM structure of the TetM-GDPNP·70S complex reveals that loop III of domain IV directly interacts with the tetracycline binding site in proximity to C1054, but does not appear to overlap dramatically with the binding site of tetracycline. This suggests that TetM dislodges tetracycline from its binding site on the ribosome by disrupting the reported stacking interaction between the aromatic ring D of tetracycline and the nucleobase of C1054 (2), in agreement with a previous proposal (15). In the model of the TetM·70S complex, the best fit for EM density in terms of C1054 and the neighboring U1196 was using the 30S subunit from the EF-Tu·70S structure (25) (Fig. 4C, blue). In contrast, the position of C1054 and U1196 when tetracycline is bound does not fit the density as well (Fig. 4C, pink), supporting the suggestion that TetM alters the conformation of these nucleotides to chase tetracycline from the ribosome (15).
Additionally, the mutagenesis data (Fig. 4B) suggest that residues especially Y506 and Y507 within loop III of TetM are likely to be important for inducing this conformational change.

Resistance to tetracycline by TetM, however, not only requires that TetM chase the drug from its binding site, but that it also prevent immediate rebinding of tetracycline to the ribosome (9). This has been proposed to be brought about via TetM-induced conformational changes within the ribosome that prevent tetracycline rebinding, and yet promote binding of the ternary complex EF-TuGTPaa-tRNA (9, 15, 16). Indeed, our findings provide structural support for this model, as the conformation of C1054 and U1196 in the TetM70S structure appears to be most compatible with binding of EF-TuGTPaa-tRNA (Fig. 4D) and would disfavor rebinding of tetracycline. Moreover, binding of TetM to the ribosome induces A1492 and A1493 to adopt a flipped-out conformation (Fig. 2C and D), analogous to that observed during decoding of the mRNA by EF-TuGTPaa-tRNA (25) (Fig. 4D). Footprinting experiments suggest that the flipped-out conformation of A1492 and A1493 persists upon dissociation of TetM from the ribosome (16), which promote binding of the ternary complex. Finally, we observe that TetM also induces a conformational change in the stalk base, analogous to that observed in the EF-Tu70S structure (25). Collectively, we believe these structural features imparted by TetM on the ribosome would contribute to the synergistic effect that TetM has been proposed to have on EF-Tu binding to the ribosome (16). In conclusion, our structure suggests that TetM confers resistance to tetracycline using a direct mechanism (Fig. 5). (i) TetM employs residues including Y506 and Y507 within loop III of domain IV to directly interact and alter the conformation of nucleotide C1054 within h34 of the 16S rRNA that comprises part of the tetracycline binding site (Fig. 5A). (ii) The altered conformation of C1054 perturbs stacking interaction with tetracycline, leading to its dissociation from the ribosome and prevents rebinding (Fig. 5B). (iii) The altered conformation of C1054, together with the flipped-out conformation of A1492 and A1493 induced by interaction with the CTE of TetM, as well as the closed conformation of the stalk base, promote rapid binding of the ternary complex EF-TuGTPaa-tRNA (Fig. 5C).

Materials and Methods

*E. faecalis* TetM and *E. coli* ribosomes were purified as described previously (29), and ribosome binding was verified by pelleting assays as described for EF-G (30). Cryo-EM data collection on a Titan Krios transmission electron microscope (FEI Company) and processing using the SPIDER software package (31) was as described previously (32). The protein homology model of *E. faecalis* TetM was generated by using HHPred (33) and MODELLER (34). The TetM homology model and ribosome crystal structures (18, 19, 35, 36) were fitted as rigid bodies to the cryo-EM density by using Coot (37) and Chimera (38). The QuikChange mutagenesis kit (Qiagen) was used to introduce site-specific mutations into the tetM gene according to the manufacturer's instructions, and minimal inhibitory concentrations were determined as described previously (8). Detailed materials and methods can be found in the SI Appendix, Materials and Methods.

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Supporting Information

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SI Text

Interaction of TetM with the ribosome. The major sites of contact involve domains III and IV of TetM with the small subunit and domains I and V of TetM with the large subunit (SI Appendix, Fig. S7). Like EF-G and LepA, domain III of TetM interacts with ribosomal protein S12 on the small subunit (SI Appendix, Fig. S7A), however, unlike EF-G and LepA, no interaction was observed between domain II and helix 5 (h5) or h15 of the 16S rRNA (SI Appendix, Fig. S7B). This is likely due to the aforementioned shift in orientation of EF-G towards the small subunit compared to TetM (SI Appendix, Fig. S6). Domain IV of TetM interacts with the cleft between the head and body of the small subunit (Fig. 3A), with loop II between β4 and αA4 interacting with the proximal end of helix 34 of the 16S rRNA, such that residues Ser465-Leu466-Gly467 (465SLG467) come into close proximity with the backbone of C1209 and the nucleobase of C1214 (Fig. 3C). Loop III of TetM linking β5 to helix αB4 fuses directly with C1054 of the 16S rRNA, a component of the primary tetracycline binding site (Fig. 3C). The CTE interacts with loop I of domain IV of TetM, but also with H69 of the 23S rRNA as well as h44 of the 16S rRNA (Fig. 2D,E and SI Appendix, Fig. S7C,D). We believe that interaction of the CTE with h44 stabilizes the flipped-out conformation of A1492 and A1493 (seen in Fig. 2D,E), since this conformation not only correlates with the electron density of the fused density between h44 and the CTE (Figure 3F), but also explains better the hole in the density of helix 44 compared with conformation when A1492 and A1493 are stacked within h44 (SI Appendix, Fig. S7C,D).

On the large subunit, the bulk of the interactions of TetM are with ribosomal proteins L6, L7 and L11 as well as helices H43/H44, H89 and H95 of the 23S rRNA (SI Appendix, Fig. S7E-H). The C-terminus of L6, which contains two terminal lysine residues (K175 and K176), extends towards the distal end of αB5 of domain V of TetM, as was reported for EF-G (1) (SI Appendix, Fig. S7E). Density for the neighboring loop of TetM connecting αB5 with β45 fuses with nucleotide A2660 located at the tip of the sarcin-ricin loop (SRL, H95) (SI Appendix, Fig. S7E). Cleavage, depurination or mutation at this position in the SRL leads to defects in EF-
G GTPase activity (2, 3). In the TetM●70S map, additional density is observed adjacent to L11-NTD in the position where the C-terminal domain of L7 (L7-CTD) has been previously observed to interact with EF-G (1, 4, 5) (SI Appendix, Fig. S7F).

Domain V of TetM also forms a large network of interactions that encompass the stalk base and H89 (SI Appendix, Fig. S7G): The proximal portion of helix αA5 of TetM contacts nucleotide U2473 at the tip of H89 whereas the mid to distal portions of α-helix A5 appear to fuse with the nucleotides A1067 and A1095 located at the tips of H43 and H44, respectively (SI Appendix, Fig. S7G). This region encompasses the binding site of the thiopeptide antibiotics thiostrepton and micrococcin, which inhibit TetM and TetO GTPase activity (6-8).

The network of interactions of domain V of TetM with the stalk base also encompasses interaction between the distal end of β-strand 25 and the proline-rich 3₁₀ helix within the N-terminal domain (NTD) of L11 (SI Appendix, Fig. S7G). The stalk base is flexible and is found in a different position in TetM●70S compared to EF-G●70S (1, 9). Moreover, in such EF-G●70S complexes, the L11-NTD is observed in an open conformation, shifted away from H43/H44 (Fig. 1D and SI Appendix, Fig. S6). In contrast, in TetM●70S, the L11-NTD adopts a more closed conformation (SI Appendix, Fig. S7G), with density connecting H43/44 with L11-NTD observable. This difference in the conformation of the stalk base in TetM●70S and EF-G●70S is in agreement with the distinct footprinting patterns observed in this region upon binding of TetO and EF-G to the ribosome (7). The conformation and position of the stalk base in TetM●70S is rather more similar to that observed when EF-Tu●tRNA is bound to the 70S ribosome (10, 11) (SI Appendix, Fig. S6). TetO binding has been proposed to invoke a conformational change in the ribosome that persists after it has left the ribosome, explaining how TetO can enhance the ribosome-dependent GTPase activity of EF-Tu (7). A specific conformation and optimal positioning of the stalk base by TetM that enhances EF-Tu activity may thus contribute to the observed synergy between these two factors.

The sarcin-ricin loop (SRL, H95) also establishes a number of interactions with the G domain (domain I) of TetM (SI Appendix, Fig. S7E, H and Table S1). In the TetM●70S map, the G domain of TetM is fairly well ordered, with density observable for the GDPNP nucleotide as well as for most of the nucleotide binding motif containing loops (SI Appendix, Fig. S7H and Fig. S11). The exception is the density for the switch 1 (G2 motif) and switch 2 (G3 motif) loops that are better
visible at lower thresholds (SI Appendix, Fig. S7H and Fig. S11): The switch 1 loop contains the putative catalytic histidine (H78), the density of which fuses at lower thresholds with the backbone of the SRL near to G2661 (SI Appendix, Fig. S7H). Density for the switch 2 loop of TetM suggests that the conformation of this loop is similar to that observed for EF-Tu (11), EF-G (9), EF-2 (12) (eukaryotic EF-G homologue) and LepA (13) bound to the ribosome in the presence of non-hydrolysable GTP analogues (SI Appendix, Fig. S11), whereas this loop is disordered in the ribosome-bound GDP conformations of EF-Tu (10, 14), EF-2 (12) and EF-G (1, 15) structures. However, unlike in the EF-G●GDPNP●70S (9) and LepA●GDPNP●70S (13), we observe no interaction between switch 1 and domain III of TetM, even at very low thresholds.

SI Materials and methods
Preparation of the TetM●70S complex. Enterococcus faecalis TetM from transposon TnFO1 (Q47810) was purified using the N-terminally encoded 6x histidine tag and Ni-NTA chromatography as described previously (8). The TetM●70S complex was formed mixing E. coli 70S ribosomes (0.4 µM) with tigecycline (10 µM) and then incubating at 37°C for 20 min with recombinant TetM (4 µM) in the presence of 500 µM GDPNP (Roche) in a buffer containing 20mM Hepes-KOH pH 7.8, 30 mM NH₄Cl and 10 mM MgCl₂. Binding of TetM to the ribosome was verified using pelleting assays, as described previously for EF-G (16).

Cryo-electron microscopy and image processing. Freshly prepared TetM●70S sample was applied to 2 nm pre-coated Quantifoil R3/3 holey carbon supported grids and vitrified using a Vitrobot Mark IV (FEI Company) and visualized on a Titan Krios TEM (FEI Company) under low-dose conditions (about 20 e− per Å²) at a nominal magnification of 75,000× with a nominal defocus between −1 µm and −3.5 µm. Data was collected at 200 keV at a magnification of ×148,721 at the plane of CCD using a TemCam-F416 CMOS camera (TVIPS GmbH, 4,096 × 4,096 pixel, 15.6 µm pixel, 1 s/full frame), resulting in an image pixel size of 1.049 Å (object scale). Data collection was facilitated by the semi-automated software EM-TOOLS (TVIPS GmbH), allowing manual selection of appropriate grid meshes and holes in the holey carbon film.
Data processing was performed using the SPIDER software package (17) using an automated workflow including import of the original .tif files, automated conversion into SPIDER and MRC format, CTF determination using the SPIDER TFD command and automated particle selection based on the program Signature (18). After initial particle selection, a second selection of the dataset was performed using MAPPOS (http://arxiv.org/abs/1112.3173v2), a newly developed machine-learning algorithm that detects wrongly selected particles (‘non-ribosome particles’) such as contaminations, noise, carbon edges etc. that were then omitted from the data set. Two separate datasets were collected on the same sample, with the general workflow described here for dataset 2 (similar procedure applied to dataset 1). Initially, TetM dataset 2 was comprised of 261412 particles, which were sorted into 70S ribosomes (56.2%), 50S subunits (28%) and noise-derived particles (15.8%) (SI Appendix, Fig. S2A). The 70S ribosomes could be further sorted into rotated (40%) and non-rotated (33%) 70S ribosomes that lacked density for TetM as well as non-rotated 70S ribosomes with TetM bound (27%). A second data set of 145275 was collected and sorted as above. The 40776 particles from dataset 1 were combined with the 39996 particles from dataset 2, resulting in a final dataset with 52701 particles (after further refinements) with a final resolution of 7.2 Å using the Fourier shell correlation (FSC) cut-off value of 0.5 (SI Appendix, Fig. S2B).

Molecular modeling and map-docking procedures. The protein homology model of E. faecalis TetM was generated using HHPred (19) and Modeller (20). The crystal structure of Thermus thermophilus EF-G●GDP trapped using the antibiotic fusidic acid in the post-translocational state (PDB2WRI) (1) served as the model template. The individual domains of the TetM homology model were then fitted as rigid bodies using Coot (21) and Chimera (22) with the clearly resolved secondary structure elements, in particularly the α-helices (SI Appendix, Fig. S5), serving as constraints. In addition, density for the Switch 2 loop and GDPNP molecule, which was disordered in EF-G●GDP structure (PDB2WRI) (1), was modeled in part on the basis of the structure of T. thermophilus EF-Tu●GDPCP●tRNA bound to ribosome (11) (SI Appendix, Fig. S8). The model was then refined in DireX (23) and manually fine-tuned using Coot (21).

The models for the 30S subunit of the TetM●70S and rotated/non-rotated 70S complexes were generated by fitting the body (1-921), head (922-1396) and helix
44/45 (1397-1530) of crystal structures (PDB and references given in the legends) as rigid bodies to the EM density using Chimera (fit in map function) (see SI Appendix, Fig. S4). The core model for the 50S subunits was generated from PDB2WWQ (24), which in turn was based on large subunit from the crystal structure of the E. coli 70S ribosome (25, 26). Three exceptions are that (i) H43/44 and a homology model for E. coli L11 generated by SWISS MODEL (27) was derived from the crystal structure of L11-RNA complex (PDB1MMS) (28) and (ii) E. coli L31 was based on a SWISS MODEL (27) derived a homology model using T. thermophilus L31 (PDB3I8I) (29) as a template (SI Appendix, Fig. S4).

**Mutagenesis and tetracycline resistance determination.** The Quick-change mutagenesis kit (Qiagen) was used to introduce site-specific mutations into the tetM gene on the pET-TetM (8), according to the manufacturers instructions. The TetMΔCTE construct was generated by introducing a stop codon directly after domain V (Δ623-639). The primers for mutagenesis are listed in SI Appendix, Table S2. All mutants were confirmed by sequencing and transformed into E. coli BL21 strain. Overnight cultures were grown in LB at 37°C in the presence of 100µg/ml ampicillin and then diluted into fresh LB containing 1 mM IPTG and increasing concentrations of tetracycline (ranging from 0-128 µg/ml). Growth at 37°C was monitored over a period of 0-16h by measuring the optical density at 600nm with a Tecan-Infinite M1000 microplate reader. The expression of all TetM mutants was confirmed to be equivalent to that observed for the wildtype TetM using Western blotting against the 6xHis-tag.

**Coordinates, alignments and figures.** Alignment of all PDBs and generation of structure figures was performed using Chimera (22) and PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC.). Secondary structure predictions were performed using PSIPRED (30, 31). The Cryo-EM map of the TetM●70S complex is deposited in the EMDatabank under accession number EMD-2183. The model for TetM is deposited in PDB under accession number PDB ID 3J25.
SI Text

SI References
Figure S1 Alignment of RPP sequences and EF-G. ClustalW2 alignment of RPP sequences from *Enterococcus faecalis* (E.faTetM), *Lactococcus lactis* subsp. *lactis* (L.lac_TetS), *Campylobacter jejuni* (C.jej_TetO), *Bifidobacterium bifidum* (B.bif_TetW), *Bacteroides thetaiotaomicron* (B.the TetQ), *Streptococcus pyogenes* (St.pyo_TetT), *Clostridium perfringens* (Cl.per_TetP), *Streptomyces rimosus* (S.rim_OtrA) and *Escherichia coli* and *Thermus thermophilus* EF-G (E.col EF_G and T.the EF-G). Domain boundaries are shown for domains I (G domain, green and G’ subdomain, blue), II (red), III (yellow), IV (pink), V (pale blue) and C-terminal extension (CTE, orange) with secondary structure assignments and nomenclature for the *E. faecalis* TetM homology model. The G1-G5 nucleotide binding motifs, switch 1 and 2 loops as well as the TetM domain IV loops I-III are also indicated.
**Figure S2 In silico sorting and resolution of the TetM•70S complex.** (A) The dataset was sorted into sub-datasets containing healthy ribosomal particles, noisy/edged particles and 50S subunits. Subsequently, ribosome particles were sorted into non-rotated and rotated ribosomes without TetM and TetM-bound ribosomes. TetM•70S particles from dataset 1 were then joined with this dataset and after improvement, a final map could be visualized showing TetM (highlighted in orange) bound to the ribosome with (B) a resolution of 7.2 Å using the Fourier shell correlation (FSC) cut-off value of 0.5.
Figure S3 Electron density and fit of a molecular model for the TetM•70S complex. (A-C) Cryo-EM density (grey mesh) with fitted molecular model for the *E. coli* 30S (yellow, PDB2AVY)(25, 26) and 50S (blue, PDB2WWQ(24)/1MMS(28)/1CTF(32)) subunit, as well as TetM (orange), viewed from (A) factor binding site, (B) 30S solvent side and (C) birds-eye view onto top of ribosome. (D) Molecular model for *E. coli* ribosomal protein L31 (orange) based on the *T. thermophilus* L31 from PDB3I8I(29) fitted into the remaining cryo-EM density after fitting of the crystal structures of the *E. coli* 30S and 50S subunits.
Figure S4 Electron density and model for TetM at different thresholds. (A-C)
Two views of the isolated cryo-EM density for TetM from the TetM•70S complex, shown at increasing thresholds ranging from (A) 2 $\sigma$, (B) 3 $\sigma$ to (C) 4 $\sigma$. The domains are labeled I-V as well as the G’ subdomain and C-terminal extension (CTE). Note the persistence of cylindrical rods for $\alpha$-helices at higher thresholds (C), such as the terminal helix in the CTE.
Figure S5 Relative position of TetM, EF-G and EF-Tu compared to tRNA and mRNA. (A-I) Relative binding position of (A-C) TetM, (D-F) EF-G (PDB2WRI)(1) and (G-I) EF-Tu●tRNA (PDB2WRQ)(10) on the 30S subunit (yellow) compared to messenger RNA (mRNA, tan) and A- (green), P- (blue) and E-site (red) tRNAs (taken from PDB3I8H)(29). Note that TetM overlaps significantly with the position of the A-tRNA but does not approach the P-tRNA.
Figure S6 Comparison of TetM on the ribosome relative to EF-G and EF-Tu. (A-G) Comparison of ribosome binding positions and interactions of TetM (orange) with (A,B,E,F) EF-G (blue, PDB2WRI)(1) and (C,D,G) EF-Tu●tRNA (PDB2WRQ)(10), aligned relative to the (A,C) 16S rRNA of the 30S subunit (yellow) and (B,D-G) 23S rRNA of the 50S subunit. In (B,D-G), the stalk base (H43-H44 and L11) and C-terminal domain (CTD) of L7 of the 50S subunit when TetM bound is shown in yellow. Arrows in (A,B) indicate the shift in EF-G (relative to TetM) closer to the 30S subunit and away from the 50S subunit. Arrows in (E) indicate the shifted position of the stalk base and NTD of L11 in the EF-G●70S (blue) compared to the TetM●70S (yellow) complex, whereas the respective conformations for EF-Tu are similar to that observed for TetM (G).
**Fig. S7 Interaction of TetM with the 70S ribosome.** Predicted contact sites (spheres in model taken from Table S1) between density (grey mesh) of TetM and the (A-D) 30S and (E-H) 50S subunit. (A) Helix αA3 of domain III of TetM (orange) interacts with S12 (green), whereas (B) no interaction is observed between TetM-domain II (orange) and 16S rRNA helix 5 (h5, blue). (C,D) The CTE of TetM interacts with h44 (the flipped-in conformation of A1492/3 is shown). Large subunit contacts with TetM include (E) domain V of TetM (orange) with A2660 of the SRL (H95) (blue) and the CTE of L6 (purple), (G) domain V of TetM (orange) with H89, the tips of H43 and H44 as well as with L11 (green), (F) helices αA6 and αB6 of the G’ subdomain of TetM (orange) with the C-terminal domain of L7/L12 (blue), and (H) interaction between domain I (G domain) of TetM (orange with switch 2 loop in pink/purple) and the SRL (blue).
Figure S8 Comparison of the orientation of domain IV of TetM and EF-G on the ribosome. (A-B) Comparison of the binding position of (A) TetM (orange) and (B) EF-G (PDB2WRI)(1) relative to mRNA (green) and P-tRNA (red) (taken from PDB3I8H)(29), with zoom showing domain IV of (A) TetM and (B) EF-G and the respective orientations of Loops I-III. Histidine 583 (H583) in Loop III of EF-G and the equivalent tyrosine (Y507) in Loop III of TetM are shown as sticks. (C) Superimposition of (A) and (B).
Figure S9 Filtering of the TetM•70S complex to lower resolutions. (A) Electron density map of the TetM•70S complex at 7.2 Å compared with the same map filtered at (B) 15 Å and (C) 20 Å. The upper panel shows an overview with TetM (orange), 30S (yellow) and 50S (grey), while the lower panel shows zoomed view focused on domain IV of TetM with PDB model (orange) and map (grey surface), compared with the binding position of tetracycline (Tet). Note the loss of density for loop III of domain IV of TetM at lower resolutions.
Figure S10 Binding sites of tigecycline and tetracycline on the ribosome. (A-C) Electron density map (grey mesh) of the (A) rotated 70S map without TetM from sorting (Fig. S2) that reveals density for tigecycline, compared with (B) SecM-RNC (EMD-1829)(33) and (C) TetM•70S. In (B,C), the rotated 70S map is shown as a grey mesh and the SecM-RNC and TetM maps as opaque grey surfaces. (G,H) Chemical structures for tigecycline and tetracycline, with differences highlighted in red. (I-M) Overview of tetracycline binding sites of the 30S subunit(34, 35) with enlargements showing lack of density in the TetM•70S map (grey mesh) for secondary binding sites, Tet1 and Tet2 (red)(34) and Tet1-Tet6 (gold)(35).
Figure S11 Comparison of G domains of TetM, EFG, EF-Tu and LepA. (A) Ribbon representation of the G domain of TetM (orange) with nucleotide binding motifs G1 (yellow), G2 (purple), G3 (brown), G4 (green), G5 (cyan) and switch 1 (pink) and switch 2 (light brown) highlighted. (B,C) Electron density of the G domain of TetM shown at (B) high and (C) low thresholds. Note that at low threshold additional density appears that would correspond with the conformation of the switch 1 as observed previously for EF-Tu•tRNA trapped on the ribosome with GDPCP (PDB2XQD)(11). Note that lack of density for part of the switch 2 in the region of the catalytic histidine 78 (H78). (D-F) Ribbon representation of the G domain with same view as (A) but for (D) EF-G•FA•GDP•70S complex (blue, PDB2WRI) with disordered switch 1, (E) EF-Tu•tRNA•GDPCP•70S complex (teal, PDB2XQD)(11) and (F) LepA•GDPCP•70S complex (grey, PDB3DEG). (G-I) Superimposition of (A) with (D)-(F). Note the similarity in the conformation of the switch 1 region of TetM with (H) EF-Tu and (I) LepA.
### Table S1 Contacts between TetM and the ribosome

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* shaded yellow = 30S components and shaded blue = 50S components
### Table S2 Primers for site-directed mutagenesis

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