Directed evolution of the rRNA methylating enzyme Cfr reveals molecular basis of antibiotic resistance

Short title: Directed evolution of the Cfr resistance enzyme

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1 ABSTRACT

2 Alteration of antibiotic binding sites through modification of ribosomal RNA (rRNA) is a common form
 3 of resistance to ribosome-targeting antibiotics. The rRNA-modifying enzyme Cfr methylates an
 4 adenosine nucleotide within the peptidyl transferase center, resulting in the C-8 methylation of A2503
 5 (m⁸A2503). Acquisition of *cfr* results in resistance to eight classes of ribosome-targeting antibiotics.
 6 Despite the prevalence of this resistance mechanism, it is poorly understood whether and how bacteria
 7 modulate Cfr methylation to adapt to antibiotic pressure. Moreover, direct evidence for how m⁸A2503
 8 alters antibiotic binding sites within the ribosome is lacking. In this study, we performed directed
 9 evolution of Cfr under antibiotic selection to generate Cfr variants that confer increased resistance by
 10 enhancing methylation of A2503 in cells. Increased rRNA methylation is achieved by improved
 11 expression and stability of Cfr through transcriptional and post-transcriptional mechanisms, which may
 12 be exploited by pathogens under antibiotic stress as suggested by natural isolates. Using a variant which
 13 achieves near-stoichiometric methylation of rRNA, we determined a 2.2 Å cryo-EM structure of the
 14 Cfr-modified ribosome. Our structure reveals the molecular basis for broad resistance to antibiotics and
 15 will inform the design of new antibiotics that overcome resistance mediated by Cfr.

16 **KEY WORDS:** Cfr; directed evolution; antibiotic resistance; RNA modification; 23S rRNA; peptidyl
 17 transferase center; cryo-EM structure

1 INTRODUCTION

2 A large portion of clinically-relevant antibiotics halt bacterial growth by binding to the ribosome and
 3 inhibiting protein synthesis (Arenz and Wilson, 2016; Tenson and Mankin, 2006; Wilson, 2009). Since
 4 antibiotic binding sites are primarily composed of ribosomal RNA (rRNA), rRNA-modifying enzymes
 5 that alter antibiotic binding pockets are central to evolved resistance (Vester and Long, 2013; Wilson,
 6 2014). The rRNA-methylating enzyme Cfr modifies an adenosine nucleotide located within the peptidyl
 7 transferase center (PTC), a region of the ribosome essential for catalyzing peptide bond formation and
 8 consequently, a common target for antibiotics (Kehrenberg et al., 2005; Schwarz et al., 2000). Cfr is a
 9 radical SAM enzyme that methylates the C8 carbon of adenosine at position 2503 (m⁸A2503, *E. coli*
 10 numbering) (Giessing et al., 2009; Grove et al., 2011b; Kaminska et al., 2010; Yan et al., 2010; Yan and
 11 Fujimori, 2011). Due to the proximal location of A2503 to many antibiotic binding sites, introduction of
 12 a single methyl group is sufficient to cause resistance to eight classes of antibiotics simultaneously:
 13 **phenicols**, **lincosamides**, **oxazolidinones**, **pleuromutilins**, **streptogramin A** (PhLOPS_A), in addition to
 14 nucleoside analog A201A, hygromycin A, and 16-membered macrolides (Long et al., 2006; Polikanov
 15 et al., 2015; Smith and Mankin, 2008). Among rRNA modifying enzymes, this extensive
 16 cross-resistance phenotype is unique to Cfr and presents a major clinical problem.

17 Cfr emergence in human pathogens appears to be a recent event, with the first case reported in 2007
 18 from a patient-derived *Staphylococcus aureus* isolate (Arias et al., 2008; Toh et al., 2007). Since then,
 19 the *cfr* gene has been identified across the globe in both gram-positive and gram-negative bacteria (Shen
 20 et al., 2013; Vester, 2018), and has been associated with several clinical resistance outbreaks to the
 21 oxazolidinone antibiotic, linezolid (Bonilla et al., 2010; Cai et al., 2015; Dortet et al., 2018; Layer et al.,
 22 2018; Lazaris et al., 2017; Locke et al., 2010; Morales et al., 2010; Weßels et al., 2018). The vast spread
 23 of Cfr is attributed to its association with mobile genetic elements and relatively low impact on bacterial
 24 fitness, suggesting that *cfr* can be rapidly disseminated within bacterial populations (LaMarre et al.,
 25 2011; Schwarz et al., 2016).

26 Due to the ability of Cfr to confer resistance to several antibiotics simultaneously, it is critical to
 27 understand how bacteria may adapt under antibiotic pressure to enhance Cfr activity and bolster
 28 protection against ribosome-targeting molecules. Identification of Cfr mutations that improve resistance
 29 will also be critical for informing clinical surveillance and designing strategies to counteract resistance.
 30 A major limitation in our current understanding of Cfr-mediated resistance is the lack of structural

1 insight into changes in the ribosome as a result of Cfr modification. Steric occlusion of antibiotic
2 binding has been proposed as a model to rationalize altered antibiotic susceptibility (Polikanov et al.,
3 2015). Additionally, the observation that A2503 can adopt both *syn* and *anti*-conformations in
4 previously reported ribosome structures suggests that methylation may regulate conformation of the
5 base, as previously proposed (Schlünzen et al., 2001; Stojković et al., 2020; Toh et al., 2008; Tu et al.,
6 2005). However, direct evidence for how m⁸A2503 alters antibiotic binding sites to inform the design of
7 next-generation molecules that can overcome Cfr resistance is lacking.

8 In this study, we identified mechanisms that enhance antibiotic resistance by performing directed
9 evolution of a *cfr* found in a clinical MRSA isolate under antibiotic selection (Barlow and Hall, 2003).
10 The obtained highly resistant Cfr variants show increased rRNA methylation, driven primarily by robust
11 improvements in Cfr cellular levels, achieved either by higher transcription or increased translation and
12 improved cellular stability. In particular, mutation of the second Cfr amino acid to lysine strongly
13 enhances translation and resistance. Lastly, we used an evolved variant which achieves
14 near-stoichiometric rRNA methylation to generate a high-resolution cryo-EM structure of the
15 Cfr-modified *E. coli* ribosome. The obtained structural insights provide a rationale for how m⁸A2503
16 causes resistance to ribosome antibiotics.

17 RESULTS

18 *Evolved Cfr variants confer enhanced antibiotic resistance*

19 To perform directed evolution of Cfr, we used error-prone PCR (EP-PCR) to randomly introduce 1-3
20 mutations into the *cfr* gene obtained from a clinical MRSA isolate (Toh et al., 2007), herein referred to
21 as CfrWT (**Fig. 1a**). Mutagenized *cfr* sequences were then cloned into a pZA vector where Cfr was
22 expressed under tetracycline-inducible promoter P_{tet} introduced to enable precise control of Cfr
23 expression (Wellner et al., 2013). The resulting library of ~10⁷ *E. coli* transformants was selected for
24 growth in the presence of increasing amounts of tiamulin, a pleuromutilin antibiotic to which Cfr
25 confers resistance. During each round, a subset of the surviving colonies was sequenced to identify new
26 mutations. After two rounds of evolution, *wild-type* Cfr was no longer detected, indicating that the
27 introduced mutations provide enhanced survivability in the presence of tiamulin. After five rounds of

1 mutation and selection, we performed two rounds of selection without mutagenesis, and with high
2 tiamulin concentrations, thus leading to fixation of mutations that provide robust resistance.

3 Analysis of surviving *cfr* sequences from the final rounds of selection revealed notable trends
4 (**Supplementary Table 2**). Three positions were primarily mutated: N2, I26, and S39. By homology
5 modeling, these mutational hotspots appear distal from the enzyme active site (>12 Å; **Fig. 1b**).
6 Secondly, ~28% of sequences contained alterations to the promoter. These alterations consist of either
7 P_{tet} duplication, or insertion of a partial P_{tet} sequence (**Supplementary Table 3**).

8 We selected 7 evolved Cfr variants, referred herein as CfrV1-V7, as representative mutational
9 combinations for further characterization (**Fig. 1c**). All selected Cfr variants contain mutations in the *cfr*
10 open reading frame while CfrV6 and CfrV7 also harbor P_{tet} alterations (**Fig. 1d**). Compared to CfrWT,
11 these variants confer ~2 to ~16-fold enhanced resistance to PhLOPS_A antibiotics, yet with no changes in
12 susceptibility to trimethoprim, an antibiotic that does not inhibit the ribosome (**Fig. 1e, Supplementary**
13 **Table 4**). Interestingly, the promoter alterations enable CfrV7 to be expressed and confer resistance to
14 tiamulin in the absence of inducer (**Supplementary Fig. 1**). The robustness of resistance, and the
15 absence of active-site mutations, suggests Cfr variants do not act as dominant-negative enzymes that
16 inhibit C-2 methylation of A2503, as observed in a previous directed evolution experiment (Stojković et
17 al., 2016). Furthermore, the specificity of resistance to PhLOPS_A antibiotics suggests that these Cfr
18 variants elicit their effects through PTC modification rather than triggering a stress response that confers
19 global resistance.

Fig. 1. Evolved variants of Cfr exhibit improved resistance to PhLOPS_A ribosome antibiotics. (a) Evolution of Cfr under selection by the PTC-targeting antibiotic tiamulin. (b) Cfr homology model based on RlmN generated by I-TASSER server (Yang and Zhang, 2015) with mutagenic hotspots in red. Active site denoted by S-adenosylmethionine (grey) and [4Fe-4S] cluster (orange). (c) Evolved variants containing Cfr mutations selected for further study. Ptet* indicates alterations to promoter sequence. (d) Promoter architecture of CfrV6 and CfrV7 where pPtet designates a partial Ptet promoter sequence and Ins designates a variable insertion sequence. (e) Fold improvement in MIC resistance value for PhLOPS_A antibiotics and trimethoprim compared to empty pZA vector control determined from three biological replicates. Trimethoprim is a negative control antibiotic that does not target the ribosome. Numerical MIC values displayed in **Supplementary Table 4**.

1 Variants exhibit increased rRNA methylation and Cfr protein levels

2 To test the hypothesis that Cfr variants mediate higher resistance by increasing the fraction of ribosomes
 3 with m⁸A2503, we evaluated the methylation status of A2503 by mass spectrometry. Specifically, we
 4 expressed Cfr in *E. coli* and used oligonucleotide protection to isolate a 40-nt fragment of 23S rRNA
 5 containing A2503. The isolated fragment was then enzymatically digested and analyzed by
 6 MALDI-TOF mass spectrometry (**Fig. 2a, Supplementary Fig. 2**). As expected, an empty vector
 7 produces a 1013 m/z fragment corresponding to the mono-methylated m²A2503, modification installed
 8 by the endogenous enzyme RlmN. Upon expression of Cfr, we observe a reduction in the 1013 m/z peak
 9 and the emergence of a new peak at 1027 m/z, corresponding to m²A2503 conversion into
 10 hypermethylated m²m⁸A2503. CfrWT is able to convert less than ~40% of m²A2503 into the
 11 hypermethylated m²m⁸A2503 product. In contrast, the evolved variants achieve ~50-90% methylation of
 12 A2503, indicating that variants are more active than CfrWT *in vivo*.

13 The ability of evolved Cfr variants to achieve enhanced ribosome methylation *in vivo* could be attributed
 14 to enhanced enzymatic activity and/or higher levels of functional enzyme. To test the hypothesis that Cfr
 15 variants achieve higher turnover number, we anaerobically purified and reconstituted CfrWT and a
 16 representative evolved variant, CfrV4. We then evaluated the ability of CfrWT and CfrV4 to methylate a
 17 23S rRNA fragment (2447-2625) *in vitro* by monitoring the incorporation of radioactivity from
 18 [³H-methyl] S-adenosylmethionine (SAM) into RNA substrate under saturating conditions (Bauerle et
 19 al., 2018). However, no significant difference in k_{cat} between CfrWT ($3.45 \times 10^{-2} \pm 3.2 \times 10^{-3} \text{ min}^{-1}$) and
 20 CfrV4 ($2.25 \times 10^{-2} \pm 1.3 \times 10^{-3} \text{ min}^{-1}$) was observed (**Supplementary Fig. 3**).

21 Given these findings, we hypothesized that the variants might alter protein levels. To monitor Cfr
 22 protein levels, we inserted a flexible linker followed by a C-terminal FLAG tag, which does not alter
 23 resistance (**Supplementary Table 5**). Interestingly, immunoblotting against FLAG revealed that in
 24 addition to full-length Cfr, N-terminally truncated Cfr proteins are also produced (**Fig. 2b**). The
 25 truncations result from translation initiation at internal methionines but do not contribute to resistance
 26 (**Supplementary Fig. 4**), indicating that they are non-functional enzymes unable to methylate A2503.
 27 Interestingly the larger molecular weight truncation is present only in CfrV1/V4/V6 and is generated by
 28 the I26M mutation introduced during directed evolution. Quantification of resistance-causative,
 29 full-length Cfr proteins alone revealed that the evolved variants achieve ~20-100-fold higher
 30 steady-state protein levels than CfrWT (**Fig. 2b**).

1 We measured transcript levels for all variants to assess the contribution of altered transcription to
2 increased protein levels. For Cfr variants with promoter alterations, enhanced production of the Cfr
3 transcript is a large contributor to Cfr protein expression, as CfrV6 and CfrV7 exhibit ~6 and ~10-fold
4 enhancement in Cfr mRNA levels compared to CfrWT, respectively (**Fig. 2c**). However, the ~2 to 3-fold
5 increase in mRNA levels for CfrV1-5 cannot explain the multi-fold improvement in protein expression
6 and indicates that these variants also boost protein production through a post-transcriptional process.
7 This is further supported by the expression profiles for CfrV1-5, which are dominated by the full-length
8 protein (**Fig. 2d**). Interestingly, enhanced production of Cfr protein correlates with larger fitness defects
9 in *E. coli*, with an increase in doubling time of ~4 min for CfrV7 compared to empty vector in the
10 absence of antibiotics (**Fig. 2e**).

Fig. 2. Cfr variants cause increased methylation of 23S rRNA at A2503, correlating with enhanced production of Cfr protein. (a) Endogenously modified (m²A2503) and Cfr-hypermodified (m²m⁸A2503) rRNA fragments correspond to m/z values of 1013 and 1027, respectively. MALDI-TOF mass spectra of 23S rRNA fragments isolated from *E. coli* expressing CfrWT, and evolved Cfr variants V2, V4, and V7. Ψ is pseudouridine, m²A is 2-methyladenosine, is m²m⁸A is 2,8-dimethyladenosine. (b) Relative protein expression of full-length Cfr variants compared to full-length CfrWT detected by immunoblotting against a C-terminal FLAG tag and quantification of top Cfr bands. Signal was normalized to housekeeping protein RNA polymerase β-subunit. Data is presented as the average of four biological replicates with standard deviation on a log₂ axis. Asterisks denote N-terminally truncated versions of Cfr that do not contribute to resistance. Em = empty vector control. (c) Relative transcript levels for variants compared to CfrWT determined from three biological replicates with standard deviation. (d) Percentage of total Cfr expression attributed to production of full-length Cfr protein, presented as the average of four biological replicates with standard deviation. (e) Doubling times for *E. coli* expressing empty plasmid, CfrWT, or Cfr variants determined from three biological replicates with standard error.

Promoter and second position mutations drive Cfr resistance

Given that the evolved variants achieve robust enhancement in Cfr expression we sought to elucidate the mechanism(s) by which this occurs. To evaluate the importance of promoter alterations, we generated a construct where the P_{tet} * promoter sequence from CfrV6 was inserted upstream of CfrWT open reading frame, herein referred to as P_{tet} *V6-CfrWT. The insertion of P_{tet} * alone was sufficient to elicit improvement in Cfr expression (**Fig. 3a**). Furthermore, *E. coli* expressing P_{tet} *V6-CfrWT resembled CfrV6 in its ability to survive in the presence of chloramphenicol (**Fig. 3b**). Together, these results suggest the altered promoter drives expression and resistance for CfrV6.

To investigate the contributions of mutations within the Cfr protein, we generated constructs containing Cfr mutations N2K/I, I26M, and S39G in isolation. Interestingly, we observe that mutations at the second position, N2K and N2I, display the largest enhancements in expression, ~27-fold and ~12-fold respectively (**Fig. 3a**). The dominance of the second position mutants is further manifested in *E. coli* expressing CfrN2K, but not I26M or S39G, exhibiting similar survival in the presence of chloramphenicol to that of the triple mutant, CfrV3 (**Fig. 3c**). Similarly, *E. coli* expressing CfrN2I also exhibits increased resistance to chloramphenicol when compared to the corresponding directed evolution variant, CfrV5, albeit weaker than CfrN2K (**Supplementary Fig. 5a**). Together, these results suggest that the second position mutations drive the robust expression and resistance observed for CfrV1-5. Of note, ribosome methylation by the produced Cfr does not impact the translation of CfrN2K, as this mutant and its corresponding catalytically inactive double mutant protein CfrN2K_C338A are similarly highly expressed (**Supplementary Fig. 5b-c**).

Fig. 3. Mutations to the second amino acid and promoter are the largest contributors to Cfr expression and resistance. (a) Effect of Cfr mutations and promoter alteration on relative Cfr protein expression was assessed by immunoblotting against a C-terminal FLAG tag. Quantification was performed for full-length Cfr protein normalized to housekeeping protein RNA polymerase β -subunit. Data is presented as the average of four biological replicates with standard deviation on a $\log_2$ axis. Asterisks denote N-terminally truncated Cfr protein products that do not contribute to resistance and were not included in quantification. Em = empty vector control. (b) and (c) Dose-dependent growth inhibition of *E. coli* expressing pZA-encoded CfrWT, CfrV6 (panel b), CfrV3 (panel c) and individual mutants that comprise these variants towards chloramphenicol (CHL) presented as an average of three biological replicates with standard error.

1 *Mutations impact Cfr translation and degradation*

2 The Cfr coding mutations drive enhanced steady-state protein levels of Cfr protein through a
3 post-transcriptional process. However, because levels at steady-state reflect the net effect of protein
4 synthesis and degradation, we sought to evaluate how Cfr mutations impact both processes, especially
5 since the nature of N-terminal amino acids and codons can greatly influence both translation and
6 degradation in bacteria (Bentele et al., 2013; Bhattacharyya et al., 2018; Boël et al., 2016; Goodman et
7 al., 2013; Gottesman, 2003; Looman et al., 1987; Sato et al., 2001; Stenström et al., 2001a, 2001b;
8 Stenström and Isaksson, 2002; Tuller et al., 2010a; Verma et al., 2019).

9 To test the hypothesis that second position mutations enhance translation of mutants, we used polysome
10 profiling to evaluate the relative abundance of Cfr mRNA in polysome fractions. Polysome profiles
11 derived from 10-55% sucrose gradients appear similar across biological conditions, suggesting
12 expression of CfrWT and its evolved mutants do not affect global translation (**Fig. 4a-b**). CfrWT
13 transcripts migrate with low polysomes (fractions 10, 11) (**Fig. 4c**). In contrast, CfrV4 transcripts are
14 strongly shifted toward high polysomes (fractions 16, 17), which indicate that CfrV4 mRNA is
15 associated with a large quantity of ribosomes and is better translated than CfrWT (**Fig. 4d**). Further
16 support that CfrV4 is well-translated is the observation that CfrV4 mRNA co-migrates with mRNA of
17 the well-translated housekeeping gene, *recA* (Li et al., 2014) (**Supplementary Fig. 6a-c**). At least in
18 part, this is due to the N2K mutation which shifts transcripts to higher polysomes fractions (fractions 12,
19 13) (**Fig. 4c**). The *recA* control mRNA shows excellent reproducibility across biological samples,
20 indicating that the observed shift of mutant Cfr transcripts towards higher polysomes is due to
21 introduced mutations (**Fig. 4b**). Taken together, these results suggest that enhanced translation is a
22 cumulative effect of N2K and other ORF mutations obtained by directed evolution.

23 To further interrogate the role of second position mutations in Cfr translation, we determined the second
24 codon identity for all sequenced variants from the final rounds of evolution (**Supplementary Table 2**).
25 Interestingly, all N2K mutations were encoded by an AAA codon, while AUU encoded all N2I
26 mutations. In *E. coli*, the tRNA molecules that decode K(AAA) and I(AUU) are slightly more abundant
27 than the wild-type N(AAU), accounting for 3.0% and 5.4% of the tRNA pool compared to 1.9%,
28 respectively (Dong et al., 1996). To test if tRNA abundance and codon sequence contribute to enhanced
29 translation, we evaluated the impact of synonymous codons on protein expression. Lysine codons AAA
30 and AAG are decoded by the same tRNA^{Lys} in *E. coli*. Interestingly, mutating CfrN2K from AAA to

1 AAG, which increases G/C content, did not significantly impact expression (**Supplementary Fig. 6d**).
2 The isoleucine AUA codon is decoded by the low-abundant tRNA^{Ile2} (Del Tito et al., 1995; Nakamura et
3 al., 2000). Mutation of N2I from AUU to the AUA rare codon resulted in a ~2-fold decrease in Cfr
4 expression, supporting tRNA abundance as a contributing factor (**Supplementary Fig. 6d**).

5 To evaluate impact of mutations introduced during directed evolution on protein half-life, we monitored
6 changes in protein abundance over time after halting expression with rifampicin (**Fig. 4e**,
7 **Supplementary Fig. 7**). While CfrWT is rapidly degraded with a half-life of ~20 min, CfrN2K/I exhibit
8 increased half-lives of ~60 min. These results suggest that mutation of the second amino acid to lysine
9 or isoleucine contribute to improved steady-state expression both by enhancing translation and stability
10 of Cfr in the cell. CfrS39G also exhibits an increased half-life of ~60 min. The half-life increase is the
11 most pronounced for the I26M single point mutant and similar to that of the triple-mutant, CfrV3 (>100
12 min for both proteins). Together, these results suggest that evolved variants achieve higher expression
13 through mutations that both enhance translation and decrease degradation of mutant Cfr proteins.

Fig. 4. Directed evolution mutations impact Cfr translation and degradation. (a) Sucrose gradient fractionation of polysomes from *E. coli* expressing empty vector or CfrWT/N2K/V4 denoting fractions corresponding to low- and high-density polysomes. (b) mRNA distribution of well-translated, housekeeping gene *recA* across polysome profiles. (c) mRNA distribution of Cfr transcripts expressing CfrWT or CfrN2K. (d) mRNA distribution of Cfr transcripts expressing CfrWT or CfrV4. For B-D, transcript levels for each fraction were determined by RT-qPCR and normalized by a luciferase mRNA control spike-in. Values presented as the average of three biological replicates with standard error. (e) Protein degradation kinetics of CfrWT, single mutations CfrN2K/N2I/S39G/I26M, and evolved variant CfrV3 in *E. coli* after halting expression by rifampicin treatment. Percentage of Cfr protein remaining over time was determined by immunoblotting against C-terminal FLAG tag and presented as the average of three biological replicates with standard error.

1 *Evolved Cfr enables understanding of the structural basis of resistance*

2 Molecular understanding of Cfr-mediated resistance to antibiotics necessitates structural insights into
3 methylated ribosomes. However, obtaining the structure of Cfr-modified ribosome has been so far
4 hampered by moderate methylation efficiency of *S. aureus* Cfr, a challenge that can be addressed by the
5 improved methylation ability of directed evolution variants. Of all characterized evolved variants, CfrV7
6 achieves the highest levels of antibiotic resistance and methylation of rRNA, providing a unique tool for
7 structural determination. Relative peak quantification of the MALDI spectra revealed that CfrV7
8 achieved near-stoichiometric (~90%) m⁸A2503 methylation (**Supplementary Fig. 2**).

9 Ribosomes were purified from *E. coli* expressing CfrV7 to obtain a 2.2 Å cryo-EM structure of the
10 Cfr-modified 50S ribosomal subunit (**Fig. 5a, Supplementary Fig. 8, Supplementary Table 6**). The
11 high resolution cryo-EM density map enabled modeling all known modified nucleotides including the
12 novel C8 methylation of A2503 (**Fig. 5b**). Furthermore, comparison of the Cfr-modified ribosome with
13 the high resolution cryo-EM structure of unmodified, *wild-type* ribosome we published previously
14 (Stojković et al., 2020) allowed us to identify with high confidence any structural changes due to the
15 presence of m⁸A2503. Importantly, modification of A2503 by Cfr does not affect the conformation or
16 position of the A2503 nucleotide. The adenine ring remains in the *syn*-conformation and places the
17 newly installed C8-methyl group directly into the PTC to sterically obstruct antibiotic binding (**Fig.**
18 **5c-d**).

19 Strikingly, beyond the addition of a single methyl group to the substrate nucleotide, presence of
20 m⁸A2503 does not result in any additional structural changes to the PTC region of the ribosome (**Fig.**
21 **5c**). Furthermore, the increased resistance provided by CfrV7 appears to be mediated specifically by
22 improved methylation of A2503. No off-target activity of the evolved variant was observed as manual
23 inspection did not reveal density that could correspond to additional C8-methyl adenosines within the
24 high-resolution regions of the 50S ribosomal subunit. This result was cross-validated using our qPTxM
25 tool (Stojković et al., 2020), which identified only A2503 and A556 as possible C8-methyl adenosines.
26 Closer examination of A556 reveals it registered as a false positive (**Supplementary Fig. 9a-d**).

27 Contrary to previous reports, we do not observe changes to methylation of C2498, a distal PTC
28 nucleotide whose endogenous 2'-O-ribose modification has previously been reported to be suppressed
29 by Cfr methylation of A2503 and hypothesized to alter the PTC through long-range effects (Giessing et

1 al., 2009; Kehrenberg et al., 2005; Purta et al., 2009). Although it is unclear what percentage of C2498
2 retains the native modification in our structure, we observe clear density for the methyl group and the
3 nucleotide conformation is unaltered. The density for the methyl group is slightly off of the rotameric
4 position, but the dropoff in density along the methyl bond matches the expected shape (**Supplementary**
5 **Fig. 9e-g**). Together, the results do not indicate that conformational changes to C2498 are involved in
6 Cfr-mediated resistance.

7 Structural superposition of the Cfr-modified ribosome with ribosomes in complex with PhLOPS_A
8 antibiotics enables direct identification of chemical moieties responsible for steric collision with
9 m⁸A2503 for these five antibiotic drug classes (**Supplementary Fig. 10**). For example, overlay of a
10 bacterial ribosome in complex with the pleuromutilin derivative tiamulin, the selection antibiotic used
11 during directed evolution, reveals steric clashes between the C10 and C11 substituents of the antibiotic
12 with the Cfr-introduced methyl group (**Fig. 5d**). The pleuromutilin class of antibiotics have recently
13 regained interest for their applications as antimicrobial agents in humans but existing molecules remain
14 ineffective against pathogens with Cfr (Goethe et al., 2019). Given recent synthetic advances that enable
15 more extensive modification of the pleuromutilin scaffold (Farney et al., 2018; Murphy et al., 2017), the
16 structural insights we obtained will inform the design of next-generation antibiotics that can overcome
17 Cfr-mediated resistance.

Fig. 5. Near-stoichiometric ribosome methylation by CfrV7 enables structural understanding of Cfr-mediated resistance to antibiotics. (a) Cfr-modified 50S ribosomal subunit highlighting adenosine 2503 (A2503) within 23S rRNA and the binding site of PTC-targeting antibiotics. Cfr methylates A2503 at the C8 carbon to produce m²m⁸A2503. (b) Cryo-EM density maps of adenosine 2503 in 23S rRNA contoured to 3σ. Cfr-modified (m²m⁸A2503) in cyan. Wild-type (m²A2503) in orange; PDB 6PJ6. (c) Close up view of 23S rRNA nucleotides in the 50S ribosomal subunit. Cfr-modified ribosome in cyan. Wild-type ribosome in orange; PDB 6PJ6. (d) Structural overlay of Cfr-modified ribosome (cyan) and *H. marismortui* 50S ribosome in complex with pleuromutilin antibiotic tiamulin (purple, PDB 3G4S) highlighting steric clashes between m⁸A2503 and the antibiotic.

1 DISCUSSION

2 By relying on directed evolution under antibiotic selection, we identified strategies that increase the
3 ability of a multi-antibiotic resistance determinant Cfr to cause resistance. Enhanced resistance is
4 associated with improved *in vivo* methylation of rRNA at C8 position of A2503. The positive correlation
5 between extent of rRNA modification and resistance aligns with previous studies that investigated
6 linezolid resistance caused by mutation of rRNA, where the severity of linezolid resistance was
7 proportional to the number of 23S rRNA alleles harboring the resistance mutation (Besier et al., 2008;
8 Ebihara et al., 2014; Lobritz et al., 2003). While alteration of the antibiotic binding site through
9 mutations and enzymatic modification of 23S rRNA are functionally distinct, dependence on the extent
10 of rRNA modification provides parallels between the two mechanisms. Although Cfr-mediated
11 methylation is an enzymatic process, the ability of Cfr to confer resistance is restricted by ribosome
12 assembly. Since the A2503 is only accessible to Cfr prior to incorporation of 23S rRNA into the large
13 ribosomal subunit (Yan et al., 2010), the extent of resistance correlates with the ability of the enzyme to
14 methylate 23S rRNA prior to its incorporation into the 50S subunit. The results of our evolution
15 experiment indicate that increasing the intracellular concentrations of Cfr, rather than improving
16 catalysis of an enzyme with a complex radical mechanism (Bauerle et al., 2018; Grove et al., 2011a;
17 McCusker et al., 2012; Yan and Fujimori, 2011) is the preferred strategy to increase the proportion of
18 ribosomes with the protective m⁸A2503 modification.

19 The evolved Cfr variants improve expression using two mechanisms. Improved Cfr expression for
20 CfrV6/7 is driven by increased transcription due to alterations to the P_{tet} promoter likely introduced by
21 primer slippage during the error-prone PCR step of directed evolution. CfrV6 contains a full duplication
22 of P_{tet}, providing two sites for transcription initiation, likely responsible for enhanced *cfr* transcript
23 levels. Interestingly, this result parallels a clinical instance of high Cfr resistance discovered in a *S.*
24 *epidermidis* isolate where transcription of *cfr* was driven by two promoters (LaMarre et al., 2013) and
25 highlights transcriptional regulation as an important mechanism for modulating the *in vivo* activity of
26 Cfr.

27 Improved expression for evolved variants CfrV1-5 is mediated by mutations that improve both
28 translational efficiency and protein stability *in vivo*. Of the tested mutations, I26M provides the largest
29 improvement in stability. Of note, the N-terminally truncated Cfr derived from translation initiation at
30 I26M is rapidly degraded, as no detectable protein is observed after 60 min (**Fig. 4e**). However, these

1 results indicate that the costly production and clearance of this nonfunctional protein is offset by the
2 improved cellular stability of the full-length Cfr carrying the I26M mutation.

3 Of the mutations investigated, N2K is the largest contributor to enhanced Cfr expression and resistance.
4 Although N2K contributes to cellular stability, our results suggest that improved Cfr translation is the
5 dominant role of this mutation. Our results indicate that the effect of N2K on translation may be
6 mediated, at least in part, by tRNA abundance. The influence of N-terminal residues on early stages of
7 translation has been well documented, with several proposed models to explain how tRNA abundance,
8 in addition to local mRNA sequence and structure, amino acid composition of the nascent chain, and
9 interaction between the mRNA or the nascent chain and the ribosome itself influence translation
10 initiation and elongation (Bentele et al., 2013; Bhattacharyya et al., 2018; Boël et al., 2016; Cambray et
11 al., 2018; Goodman et al., 2013; Gorochoowski et al., 2015; Kudla et al., 2009; Riba et al., 2019; Tuller et
12 al., 2010a, 2010b; Verma et al., 2019). Although the mechanism is poorly understood, the presence of an
13 AAA lysine codon after the start codon can be associated with improved translation efficiency (Brock et
14 al., 2007; Looman et al., 1987; Sato et al., 2001; Stenström et al., 2001a, 2001b; Stenström and Isaksson,
15 2002; Zalucki et al., 2007). (Brock et al., 2007; Looman et al., 1987; Sato et al., 2001; Stenström et al.,
16 2001a, 2001b; Stenström and Isaksson, 2002; Zalucki et al., 2007). Interestingly, the observed internal
17 translation start sites (I26M, M95) that are responsible for producing Cfr truncations (**Fig. 2B**,
18 **Supplementary Fig. 4**) contain a lysine immediately after methionine, further highlighting the putative
19 role for lysine codons in early steps of translation.

20 To date, only a few *S. aureus* Cfr variants have been reported and no mutations matching those obtained
21 from directed evolution have been found in clinical isolates. However, enhanced expression through
22 positioning of Lys as the second amino acid of Cfr can be recapitulated by accessing an upstream
23 translational start site found in a native sequence context of *cfr* (**Supplementary Fig. 11**). In the
24 specific case of the pSCFS1 resistance plasmid, the sequence upstream of the annotated start codon,
25 which we validated as the start site under the experimental conditions tested (**Supplementary Fig. 12**),
26 contains regulatory elements that have been proposed to modulate Cfr expression (Kehrenberg et al.,
27 2007; Schwarz et al., 2000). It is plausible that in response to antibiotics, the upstream start codon is
28 used to add three amino acids (MKE) to the N-terminus of Cfr and thus placement of a lysine (K) at
29 position two of the newly expressed protein, analogous to the N2K mutation. Although start codon
30 selection requires further investigation, N-terminal addition of MKE to Cfr expressed under non-native

1 P_{tet} promoter phenocopies the N2K directed evolution mutation, resulting in increased expression and
2 resistance compared to CfrWT (**Supplementary Fig. 11**). Since our assessment of the evolved variants
3 indicates that an increase in Cfr expression is accompanied by a decrease in fitness (**Fig. 2e**), start site
4 selection in response to antibiotic pressure would mitigate detrimental impact on fitness while enabling
5 higher resistance when acutely needed.

6 In addition to identifying mechanisms that increase Cfr-mediated resistance, directed evolution of Cfr
7 also provided an indispensable reagent that enabled structural determination of the Cfr-modified
8 ribosome. The high-resolution cryo-EM structure revealed that broad resistance is due to steric effects of
9 the judiciously positioned methyl group within the shared binding site of PTC-targeting antibiotics. Lack
10 of notable changes in position or orientation of A2503 or surrounding PTC nucleotides upon Cfr
11 methylation suggests that the resulting modification does not obstruct the translation capabilities of the
12 ribosome. This absence of PTC disruption is consistent with the observation that the fitness cost of Cfr
13 acquisition is not due to ribosome modification, but rather results from expression of the exogenous
14 protein (LaMarre et al., 2011). Importantly, overlay with existing structures containing PTC-targeting
15 antibiotics provides direct visualization of chemical moieties that are sterically impacted by m⁸A2503
16 and will inform design of antibiotic derivatives that can overcome resistance mediated by Cfr.

1 MATERIALS AND METHODS

2 *E. coli* strains and plasmids

3 *E. coli* ER2267 expressing Cfr from a pZA vector (Stojković et al., 2016; Wellner et al., 2013) was used
4 in directed evolution experiments. Antibiotic resistance, fitness, *in vivo* RNA methylation, and
5 protein/transcript expression, polysome analysis, and protein degradation experiments were conducted
6 with *E. coli* BW25113 expressing Cfr protein from a pZA vector under the Ptet promoter (or Pcfr
7 promoter where noted). *E. coli* BW25113 *acrB::kan*, where the efflux pump *acrB* was replaced with a
8 kanamycin cassette, was used for antibiotic susceptibility testing of the oxazolidinone antibiotic,
9 linezolid. For experiments for which tagless versions of evolved Cfr variants were used, comparisons
10 were made to the *wildtype* Cfr protein to which the original C-terminal His tag had been removed. *E.*
11 *coli* Rosetta2 BL21(DE3) pLysS was used for overexpression of N-His₆-SUMO-tagged Cfrs from a
12 pET28a vector. *E. coli* MRE600 was used for preparation of Cfr-modified ribosomes for structural
13 studies.

14 Cfr mutagenesis and selection scheme

15 The wild-type *cfr* gene (accession: EF450709.1) with a C-terminal His₆-tag, or pooled *cfr* genes from
16 the previous round of evolution, were randomly mutagenized by error-prone polymerase chain reaction
17 as described previously (Stojković et al., 2016). The mutagenized *cfr* gene pool was then recloned into a
18 pZA vector and transformed into *E. coli* ER2267. The frequency of mutations was determined by
19 sequencing randomly selected library variants and was on average 1-3 mutations per gene. *E. coli*
20 transformants were then subjected to selection by plating cells on LB agar containing tiamulin (Wako
21 Chemicals USA), in addition to 100 µg/mL ampicillin for plasmid maintenance and 20 ng/mL
22 anhydrotetracycline (AHT, Sigma) for induction of Cfr expression. For each round of evolution, the *E.*
23 *coli* transformants were divided equally and plated on 4-5 plates of LB agar containing different
24 concentrations of tiamulin and grown at 37°C for up to 48 h. The tiamulin concentration was increased
25 in 50-100 µg/ml increments. For example, in the first round of evolution the transformation was plated
26 on the 150, 200, 250 and 300 µg/ml tiamulin plates, in the last round we selected on 250, 350, 450 and
27 550 µg/ml tiamulin plates. Two microliters were plated on tiamulin deficient plates in order to determine
28 transformation efficiency. In general, colonies isolated from tiamulin plates in which the ≤10% of the
29 transformants grew were taken for the next round. After 5 rounds of mutagenesis and selection, 2 rounds
30 of enrichment (selection without mutagenesis) using high tiamulin concentrations (400-1500 µg/mL)
31 was conducted. After each round of selection or enrichment, 5-10 randomly selected colonies were
32 sequenced from each plate.

33 Determination of antibiotic resistance

34 Antibiotic resistance experiments by broth microdilution followed established protocols (Wiegand et al.,
35 2008). In brief, 2 mL of LB media with selection antibiotic was inoculated with a freshly transformed
36 colony containing either empty plasmid, CfrWT, or Cfr mutants. Cultures were grown at 37°C with
37 shaking for approximately 2.5 h. After measuring the OD₆₀₀ value, cultures were diluted to 10⁶ cells and
38 50µL of this dilution was dispensed into 96-well plates containing 50 µL of LB media with antibiotic of

1 interest, ampicillin (100 µg/mL), and AHT (30 ng/mL). Antibiotic resistance of evolved Cfr variants
2 were evaluated using 2-fold serial dilution of antibiotic with the following concentration ranges:
3 tiamulin (50-6400 µg/mL, TCI America); clindamycin (50-6400 µg/mL, Cayman Chemical),
4 chloramphenicol (0.5-64 µg/mL, Acros), linezolid (1-256 µg/mL, Acros), and trimethoprim (0.125 – 0.2
5 µg/mL, Sigma). Chloramphenicol resistance of single Cfr mutations were evaluated using
6 concentrations of 1, 2-12 µg/mL (in 2 µg/mL-step increments), followed by 16-64 µg/mL (2-fold
7 dilution). The minimum inhibitory concentration (MIC) required to inhibit visible bacterial growth was
8 determined after incubating plates at 37°C with shaking for 18 h. Plate OD₆₀₀ values were also recorded
9 with a microtiter plate reader (SpectraMax M5, Molecular Devices). Antibiotic resistance determination
10 on LB agar plates was conducted as described previously (Stojković et al., 2016; Wiegand et al., 2008).
11 In brief, 3 µL of 10⁸, 10⁶, and 10⁴ dilutions *E. coli* harboring Cfr were spotted on LB agar plates
12 containing various concentrations of tiamulin. LB agar plates also contained ampicillin (100 µg/mL) and
13 AHT (30 ng/mL). LB agar plates were incubated at 37°C for 24–48 h.

14 **Oligo-protection of rRNA and MALDI-TOF analysis**

15 *E. coli* expressing empty plasmid or Cfr were grown at 37°C to an OD₆₀₀ of 0.4-0.6 with shaking by
16 diluting an overnight culture 1:100 into LB media containing ampicillin (100 µg/mL) and AHT inducer
17 (30 ng/mL). Total RNA purification, oligo-protection of the 23S rRNA fragment C2480-C2520, and
18 RNaseT1 digestion was performed as described previously (Andersen et al., 2004; Stojković and
19 Fujimori, 2015). Mass spectra were acquired in positive ion, reflectron mode on an AXIMA
20 Performance MALDI TOF/TOF Mass Spectrometer (Shimadzu). Relative peak intensity values were
21 calculated using the Shimadzu Biotech MALDI-MS software.

22 **Expression and purification of Cfr**

23 CfrWT and CfrV4 were expressed, purified, and reconstituted using modified published protocols
24 (Stojković and Fujimori, 2015; Yan et al., 2010). In brief, N-His₆-SUMO-tagged CfrWT/V4 were
25 overexpressed in minimal media conditions with 800 µM IPTG and 1,10-phenanthroline to obtain
26 enzyme lacking a [4Fe-4S] iron-sulfur cluster. Minimal media also contained selection antibiotics
27 kanamycin (50 µg/mL) and chloramphenicol (34 µg/mL). All purification steps were performed in a
28 glovebox (MBraun, oxygen content below 1.8 ppm) that was cooled to 10°C. Cfr was purified by Talon
29 chromatography (Clontech) from clarified lysates. Following chemical reconstitution of the [4Fe-4S],
30 the N-His₆-SUMO-tag was cleaved by incubating the fusion protein with SenP1 protease (prepared
31 in-house, 1 mg SenP1:100 mg Cfr) for 1 h at 10°C in buffer containing 50 mM EPPS (pH 8.5), 300 mM
32 KCl 15% glycerol, and 5 mM DTT. The cleaved protein was purified away from SenP1 and the
33 N-His₆-SUMO-tag by FPLC on a Mono Q 10/100 GL anion exchange column (GE Healthcare Life
34 Sciences) using buffers containing 50 mM EPPS (pH 8.5), 50 mM or 1M KCl (low-salt or high-salt),
35 15% glycerol, and 5 mM DTT. Protein was eluted using a linear gradient of 100% low-salt to 100%
36 high-salt buffer over 8 column volumes. Fractions containing apo-reconstituted, tag-less Cfr were
37 combined, concentrated using a concentrator cell (Amicon Ultra- 0.5 mL, 30 MWCO), and stored at
38 -80°C. Protein concentration was determined by Bradford assay (Bio-Rad).

1 Preparation of rRNA substrate

2 The *E. coli* 23S rRNA fragment 2447-2624 used for *in vitro* experiments was prepared using modified
3 published protocols (Stojković and Fujimori, 2015). The desired DNA fragment was amplified from
4 plasmid pKK3535 using previously established primers (Yan et al., 2010) and used as the template in the
5 *in vitro* transcription reaction. Following DNase treatment and purification, RNA was precipitated
6 overnight at -20°C by addition of 1/10th volume of 3 M NaOAc, pH 5.5 and 3 volumes of ethanol
7 (EtOH). The RNA was then pelleted and washed with 70% aqueous EtOH, dried, and resuspended in
8 nuclease-free water to obtain a final concentration of ~ 6 mg/mL. The rRNA fragment was refolded and
9 purified by size exclusion chromatography. To refold the RNA, the sample was heated at 95°C for 2 min
10 and then cooled to 65°C over 5 min. MgCl_2 was subsequently added to a final concentration of 10 mM
11 prior to a final cooling step at room temperature for at least 30 min. After removing insoluble debris,
12 RNA was purified by FPLC on a HiLoad 26/60 Superdex 200 column (GE Healthcare Life Sciences)
13 using buffer containing 50 mM HEPES (pH 7.5), 10 mM MgCl_2 , and 50 mM KCl. Fractions containing
14 the desired rRNA product were combined and precipitated overnight at -20°C by addition of 1/10th
15 volume of 3 M NaOAc, pH 5.5 and 3 volumes of EtOH. The RNA was then pelleted and washed with
16 ice-cold 80% aqueous EtOH, dried, and resuspended in nuclease-free water. After confirming RNA
17 purity on a denaturing 5% TBE, 7M Urea-PAGE gel, the RNA sample was concentrated to ~ 450 mM
18 using a SpeedVac Vacuum Concentrator prior to storage at -80°C .

19 Cfr Kinetic Assay

20 Methylation activity of CfrWT and CfrV4 were assessed by monitoring radioactivity incorporation into
21 RNA. Flavodoxin and flavodoxin reductase enzymes were prepared as described previously (McCusker
22 et al., 2012). Prior to assembling reaction components, the RNA substrate was refolded as described
23 above. Reactions were conducted in 52 μL volumes in an anaerobic chamber (MBraun, oxygen levels
24 less than 1.8 ppm) under the following conditions: 100 mM HEPES (pH 8.0), 100 mM KCl, 10 mM
25 MgCl_2 , 2 mM DTT, 50 μM Flavodoxin, 25 μM Flavodoxin reductase, 100 μM rRNA substrate, 2 mM
26 [^3H -methyl] *S*-adenosylmethionine (175.8 dpm/pmol), and 5 μM apo-reconstituted Cfr. Reactions were
27 equilibrated at 37°C for 5 min and subsequently initiated by addition of NADPH (Sigma, final
28 concentration 2 mM). The reaction was allowed to proceed at 37°C and timepoints at 0, 2, 4, 6, and 8
29 min of 10 μL volume were quenched by the addition of H_2SO_4 (50 mM final concentration). For each
30 timepoint, the RNA volume was brought up to 100 μL with nuclease-free water and was purified away
31 from other reaction components by an RNA Clean & Concentrator kit (Zymo Research) by following
32 the manufacturer's instructions. Purified RNA eluate was added to Ultima Gold scintillation fluid, and
33 the total amount of radioactivity incorporated in the product was detected using a Beckman-Coulter
34 LS6500 scintillation counter. Amount of product generated at each time point was calculated by
35 subtracting background radioactivity ($t=0$ min) and taking into account that 2 of the 3 tritium atoms
36 from [^3H -methyl] *S*-adenosylmethionine would be incorporated into the final methylated RNA product
37 (Bauerle et al., 2018; Yan and Fujimori, 2011).

1 Evaluation of Cfr protein expression by quantitative western blot

2 *E. coli* expressing empty plasmid, CfrWT, or Cfr mutants were grown at 37°C to an OD₆₀₀ of ~0.4 with
 3 shaking by diluting an overnight culture 1:100 into 10 mL LB media containing ampicillin (100 µg/mL)
 4 and AHT inducer (30 ng/mL). Cells were harvested by centrifugation. Cell pellets were lysed for 15
 5 min using B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) containing DNase I (New
 6 England Biolabs) and 1X cOmplete, EDTA-free protease inhibitor cocktail (Roche). Whole cell lysate
 7 samples containing 4 µg of protein were fractionated using a 4–20% SDS-PAGE gel (Bio-Rad). Proteins
 8 were transferred to a 0.2 µm nitrocellulose membrane using a Trans-Blot Turbo transfer system
 9 (Bio-Rad) with a 7 min, mixed MW protocol. Membranes were incubated with TBST-Blotto buffer (50
 10 mM Tris-pH 7.5, 150 mM NaCl, 0.1% Tween-20, 5% w/v Bio-Rad Blotting Grade Blocker) for 1 h at
 11 room temperature, followed by TBST-Blotto containing two primary antibodies: monoclonal mouse
 12 anti-FLAG M2 (1:2,000 dilution, Sigma) and monoclonal rabbit anti-RNA polymerase beta (1:2,000
 13 dilution, Abcam) for 1 h at room temperature. After washing 3 x 5 min with TBST, membranes were
 14 then incubated overnight at 4°C with TBST-Blotto containing two secondary antibodies: goat anti-rabbit
 15 IgG cross-absorbed DyLight 680 (1:10,000 dilution, Thermo) and goat anti-mouse IgG cross-absorbed
 16 IRDye 800CW (1:10,000 dilution, Abcam). Membranes were rinsed 3 x 5 min with TBST and allowed
 17 to dry completely prior imaging using a Bio-Rad ChemiDoc Molecular Imager. Quantification was
 18 performed using Image Lab Software (Bio-Rad) within the linear range of detection. The house-keeping
 19 protein RNA polymerase beta, which was stably expressed in all experimental conditions, was used as
 20 an internal loading control.

21 Determination of *E. coli* growth rate

22 *E. coli* expressing empty plasmid, CfrWT, or Cfr variants were grown at 37°C with shaking by diluting
 23 an 50 µL of an overnight culture into 10 mL of LB media containing ampicillin (100 µg/mL) and AHT
 24 inducer (30 ng/mL). OD₆₀₀ values were recorded every 20 min with a microtiter plate reader
 25 (SpectraMax M5, Molecular Devices).

26 qPCR Primer Design and Validation

27 qPCR primer sequences for *cfr*, *recA*, and *luc* were designed using NCBI Primer Blast. Template
 28 accession numbers, amplicon length, and primer sequences are described in **Supplementary Table 1**.
 29 Primer sequences for *rrsA* were used as published previously (Zhou et al., 2011). For each primer pair
 30 primer, qPCR was performed on a 10-fold dilution series of desired samples. Amplification efficiency
 31 was calculated from the slope of the graph of Cq values plotted against log₁₀ of the at least four template
 32 concentrations. Primers for *recA*: Y = -3.238*X + 38.46, R² = 0.9992, PCR efficiency = 103.6%. Primers for
 33 *luc*: Y = -3.316*X + 34.52, R² = 0.9967, PCR efficiency = 100.2%. Primers for *cfr*: Y = -3.254*X + 37.52, R² =
 34 0.9960, PCR efficiency = 102.9%. Primers for *rrsA*: Y = -3.629*X + 32.24, R² = 0.9965, PCR efficiency =
 35 90.0%.

36 Determination of Cfr mRNA expression by RT-qPCR

37 Bacterial growth. *E. coli* expressing empty plasmid control, CfrWT, or Cfr variants were grown at 37°C
 38 with shaking by diluting an overnight culture 1:100 into 5 mL of LB media containing ampicillin (100
 39 µg/mL) and AHT inducer (30 ng/mL). When cells reached an OD₆₀₀ of ~ 0.4, RNAProtect Bacteria

1 Reagent (Qiagen) was added to the culture following manufacturer's instructions. Cells were then
2 harvested by centrifugation for 10 min at 5000 x g at 4°C and frozen on dry ice.
3 Total RNA isolation and DNase treatment. Pellets were then thawed and resuspended in 200 µL of lysis
4 buffer containing 30 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 15 mg/mL lysozyme, and Proteinase K
5 (New England Biolabs). Following lysis for 10 min at room temperature, total RNA was isolated using a
6 RNeasy mini kit (QIAGEN) following the manufacturer's instructions. Yield and purity of isolated RNA
7 was assessed by NanoDrop UV spectrophotometer (Thermo). RNA integrity was assessed by
8 performing 1% TBE agarose gel electrophoresis with samples that had been boiled for 95°C for 5 min in
9 RNA loading dye (New England Biolabs). Genomic DNA was eliminated by incubating 2 µg of RNA
10 with 2 U of RQ1 RNase-free DNase I (Promega) for 30 min at 30°C. The DNase reaction was halted by
11 the addition of RQ1 Stop Solution (Promega) and incubation for 10 min at 65° C.
12 cDNA synthesis. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad)
13 following the manufacturer's instructions with 10-fold diluted DNase-treated RNA. In brief, reactions of
14 20 µL volume were prepared by combining 4 µL 5X iScript buffer, 1 µL iScript RNase H⁺ MMLV
15 reverse transcriptase, 11 µL nuclease-free water, and 4 µL of RNA. Reactions were incubated for 5 min
16 at 25 °C, followed by 20 min at 42 °C and 1 min at 95 °C. If not used immediately, cDNA was stored at
17 -20°C.
18 RT-qPCR. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used for 10 µL qPCR
19 reactions. Each reaction contained 5 µL of 2X Supermix, 0.3 µM of each forward and reverse primer,
20 and 4 µL of diluted cDNA. The cDNA was diluted 40-fold for reactions with *cfr* primers and 4,000-fold
21 for reactions *rrsA* primers. Reactions were prepared in a 96-well PCR Plate (Bio-Rad, MLL9601) and
22 run on a Bio-Rad CFX qPCR Machine. The thermal cycling conditions were as follows: 98°C for 30 s,
23 followed by 35 cycles of 98°C for 10 s and 60°C for 45 s with plate read, ending with melt curve
24 analysis using 5s, 0.5 °C increment steps from 65 °C to 95 °C. A no template control and no reverse
25 transcription control were included on each plate for each primer pair. Cq values were determined using
26 CFX Maestro Software using a single threshold method. For each sample, the average of three triplicate
27 Cq values was used for further analysis. Relative transcript expression was calculated using the Pfaffl
28 method (Pfaffl, 2001). Expression was normalized to *rrsA* transcripts which is stably expressed in *E. coli*
29 BW25113 (Zhou et al., 2011) and across our experimental conditions.

30 **Polysome analysis**

31 Lysate preparation and sucrose gradient fractionation were adapted from previously published protocols
32 with modification (Li et al., 2014; Mohammad and Buskirk, 2019).
33 Lysate preparation. *E. coli* expressing empty plasmid control, CfrWT, or Cfr mutants were grown at
34 37°C with shaking by diluting an overnight culture 1:100 into 400 mL of LB media containing
35 ampicillin (100 µg/mL) and AHT inducer (30 ng/mL). Cells were harvested at an OD₆₀₀ ~0.4-0.5 in 200
36 mL batches by rapid filtration at 37°C followed by flash freezing in liquid nitrogen as described
37 previously (Li et al., 2014). Each frozen cell pellet was combined with 650 µL lysis buffer as frozen
38 droplets containing 20 mM Tris (pH 8.0), 10 mM MgCl₂, 100 mM NH₄Cl, 5 mM CaCl₂, 0.4% Triton
39 X-100, 0.1% NP-40, 100 U/ml RNase-free DNase I (Roche), and 10 U/mL SUPERase-In (Invitrogen).
40 Cells with lysis buffer were pulverized in a 10 mL jar containing a 12 mm grinding ball using a

1 TissueLyser II (QIAGEN) by performing 5 rounds of 3 min at 15 Hz. Canisters were pre-chilled by
2 submersion in liquid nitrogen for at least 1 min prior to each round of pulverization. Lysates were
3 recovered from the frozen jars using a spatula pre-chilled in liquid nitrogen and stored at -80°C until
4 further use.

5 Sucrose Gradient Fractionation. Pulverized lysates were thawed at 30°C for 2 min followed by an
6 ice-water bath for 20 min. Lysates were clarified by centrifugation at 20,000 x g for 10 min at 4°C. The
7 RNA concentration of the clarified lysate was measured by NanoDrop UV spectrophotometer (Thermo)
8 and diluted to 2.5 mg/mL with lysis buffer. Ribosome and mRNA components were separated on a
9 linear, 12 mL, 10-55% (w/v) sucrose gradient containing 20 mM Tris (pH 8.0), 10 mM MgCl₂, 100 mM
10 NH₄Cl, 2 mM DTT, and 10 U/mL SUPERase-In. Sucrose gradients were generated using a Bio-Comp
11 Gradient Master with the following program: Time = 1:58 s; Angle = 81.5°, Speed = 16 rpm. For each
12 biological sample, 190 µL (~0.5 mg RNA) of clarified lysate was loaded onto sucrose gradients in
13 duplicate. Ultracentrifugation was performed using a SW Ti41 rotor (Beckman Coulter) for 201,000 x g
14 for 2.5 h at 4°C. Gradients were fractionated using a Bio-Comp Fractionator in 20 fractions at a speed of
15 0.25 mm/sec where absorbance at 260 nm was continuously monitored.

16 RNA Extraction and DNase Treatment. Fractions 1+2, 3+4, 16+17, and 18+19 were combined. RNA
17 was extracted from uniform volumes of each fraction or combination of fractions. RNA extraction was
18 performed by adding one volume of TRIzol reagent (Invitrogen), mixing until homogeneous, and
19 incubating at room temperature for 5 min. Samples were then incubated at room temperature for another
20 5 min following the addition of 0.4 volumes of chloroform. After centrifugation for 15 min at 12,000 x g
21 at 4°C, the aqueous supernatant was transferred to a new tube to which 250 pg of a luciferase control
22 RNA spike-in (luc, Promega). RNA was precipitated overnight at -20°C by the addition of 1 volume of
23 isopropanol and 2 µL of GlycoBlue (15 mg/mL, Invitrogen). RNA was pelleted by centrifugation,
24 washed twice with 75% ice-cold, aqueous ethanol, and allowed to dry at room temperature for ~30 min.
25 The RNA was then resuspended in 20 µL of nuclease-free water. RNA quality and concentration were
26 assessed by a NanoDrop UV spectrophotometer (Thermo). Genomic DNA was eliminated by incubating
27 10 µL of isolated RNA with 1 U of RQ1 RNase-free DNase I (Promega) for 30 min at 30°C. The DNase
28 reaction was halted by the addition of RQ1 Stop Solution (Promega) and incubation for 10 min at 65°C.

29 cDNA synthesis and RT-qPCR. Reverse transcription was performed using the iScript cDNA Synthesis
30 Kit (Bio-Rad) following the manufacturer's instructions. In brief, reactions of 20 µL volume were
31 prepared by combining 4 µL 5X iScript buffer, 1 µL iScript RNase H⁺ MMLV reverse transcriptase, 5
32 µL nuclease-free water, and 10 µL of DNase-treated RNA. Reactions were incubated for 5 min at 25 °C,
33 followed by 20 min at 42 °C and 1 min at 95 °C. SsoAdvanced Universal SYBR Green Supermix
34 (Bio-Rad) was used for 10 µL qPCR reactions in a 96-well plate as described above. Each reaction
35 contained 5 µL of 2X Supermix, 0.3 µM of each forward and reverse primer, and 4 µL of 10-fold diluted
36 cDNA. Reactions containing cfr, recA, and luc primers (**Supplementary Table 1**) were performed for
37 each fraction, including a no template control and no reverse transcription control for each primer set on
38 each plate. The average of three triplicate C_q values was used for further analysis.

39 Data Analysis. Normalized mRNA distribution profiles for the target mRNAs were calculated as
40 described previously (Pringle et al., 2019). In brief, the relative abundance of each target mRNA

1 normalized to luciferase RNA spike-in. The percentage of target mRNA found across gradient fractions
2 was calculated by dividing the amount of target mRNA detected in one fraction by the sum of the target
3 mRNA detected in all fractions.

4 **Protein Degradation Assay**

5 Bacterial growth and rifampicin treatment. *E. coli* expressing CfrWT or Cfr mutants were grown at 37°C
6 with shaking by diluting an overnight culture 1:100 into 25 mL of LB media containing ampicillin (100
7 µg/mL) and AHT inducer (30 ng/mL). When cells reached an OD₆₀₀ ~0.4-0.5, rifampicin (Sigma) was
8 subsequently added to a final concentration of 100 µg/mL, and cultures were allowed continued
9 incubation at 37°C with shaking. Timepoints at 0, 20, 40, 60, 80, 100 min were harvested by
10 centrifuging 3 mL of the culture at 8,000 rpm at 4°C for 10 min, decanting the supernatant, and
11 immediately flash-freezing the pellet in liquid nitrogen. Cell pellets for each time point were lysed using
12 B-PER Bacterial Protein Extraction Reagent as described above.

13 Western blot. Whole cell lysate samples containing 5 µg of protein were fractionated on a 4-20%
14 SDS-PAGE gel and transferred onto a 0.2 µm nitrocellulose membrane as described above. Membranes
15 were stained with Ponceau S stain (0.1% w/v Ponceau S, 5% v/v acetic acid) and imaged using a
16 Bio-Rad ChemiDoc Molecular Imager. After blocking in TBST-Blotto buffer for 1 h at room
17 temperature, membranes were incubated with TBST-Blotto containing primary monoclonal mouse
18 anti-FLAG M2 antibody (1:2,000 dilution, Sigma) or monoclonal mouse anti-GAPDH antibody
19 (1:2,000 dilution, Abcam) for 1 h at room temperature. After washing 3 x 5 min with TBST, membranes
20 were incubated overnight at 4°C with TBST-Blotto containing a secondary antibody, goat anti-mouse
21 cross-absorbed IRDye 800CW (1:10,000 dilution, Abcam). Membranes were rinsed and imaged as
22 described above.

23 Data Analysis. Quantification was performed using Image Lab Software (Bio-Rad) within the linear
24 range of detection. The Ponceau S total protein stain was used to normalize for differential sample
25 loading. Percentage (%) of initial Cfr protein remaining was calculated by dividing the amount of Cfr
26 protein at a given time point after rifampicin treatment by the amount of Cfr protein prior to rifampicin
27 treatment (t = 0 min).

28 **Purification of Cfr-modified *E. coli* ribosome**

29 Cfr-modified, 70S ribosomal subunit was purified from *E. coli* MRE600 expressing CfrV7 variant using
30 previously published protocol with modification (Mehta et al., 2012; Stojković et al., 2020). In short, *E.*
31 *coli* transformed with pZA-encoded CfrV7 were grown to an OD₆₀₀ of 0.5 in LB media containing
32 ampicillin (100 µg/mL) and AHT inducer (30 ng/mL) at 37°C with shaking. Cells were harvested by
33 centrifugation, washed, and lysed by using a microfluidizer. The lysate was clarified by
34 ultracentrifugation at 30,000 x g 30 min at 4°C using a Ti45 rotor (Beckman Coulter) twice. The
35 recovered supernatant was applied to a 32 % w/v sucrose cushion in buffer containing 20 mM
36 Hepes-KOH (pH 7.5), 500 mM NH₄Cl, 20 mM Mg(OAc)₂, 0.5 mM EDTA, 6 mM β-mercaptoethanol,
37 10 U/mL SuperASE-In and was ultracentrifuged at 100,000 x g for 16 h at 4 °C in a SW Ti41 rotor
38 (Beckman Coulter). After removing the supernatant, the pellet was resuspended slowly at 4°C over 1 h
39 in Buffer A containing 20 mM Hepes-KOH (pH 7.5), 200 mM NH₄Cl, 20 mM Mg(OAc)₂, 0.1 mM

1 EDTA, 6 mM β -mercaptoethanol, 10 U/mL SuperASE-In. Particulates that were not resuspended were
2 removed by centrifugation at 10,000 rpm for 10 min at 4°C. Sample concentration was determined by
3 NanoDrop UV spectrophotometer (Thermo), where $A_{260}=1$ corresponds to 24 pmol of 70S ribosome.
4 Tight-coupled 70S ribosomes were purified as described previously (Khusainov et al., 2017). In brief,
5 70S ribosomes were purified on a 15-30% w/v sucrose gradient in Buffer A. Sucrose gradients were
6 generated using a Bio-Comp Gradient Master. 300-400 pmol of 70S ribosomes were loaded on each
7 sucrose gradient. Ultracentrifugation was performed using a SW Ti41 rotor (Beckman Coulter) for
8 75,416 x g for 16 h at 4°C. Gradients were fractionated using a Bio-Comp Fractionator in 20 fractions at
9 a speed of 0.25 mm/sec where absorbance at 260 nm was continuously monitored. Fractions
10 corresponding to 70S ribosomes were combined and precipitated by slow addition at 4°C of PEG 20,000
11 in Buffer A to a final concentration of 9% w/v. Ribosomes were isolated by centrifugation for 10 min at
12 17,500 x g. After removing the supernatant, ribosomes were slowly resuspended overnight at 4°C in
13 buffer containing 50 mM Hepes-KOH (pH 7.5), 150 mM KOAc, 20 mM Mg(OAc)₂, 7 mM
14 β -mercaptoethanol, 20 U/mL SuperASE-In.

15 **Cryo-EM analysis**

16 Purified 70S ribosomal subunits were diluted from 2 to 0.5 mg/ml in Buffer A, applied to 300-mesh
17 carbon coated (2nm thickness) holey carbon Quantifoil 2/2 grids (Quantifoil Micro Tools) and
18 flash-frozen as described in (Khatter et al., 2015). Data were collected using serialEM on the in-house
19 Titan Krios X-FEG instrument (Thermo Fisher Scientific) operating at an acceleration voltage of 300 kV
20 and a nominal underfocus of $\Delta z = 0.2$ to 1.5 μ m at a nominal magnification of 29 000 (calibrated
21 physical pixel size of 0.822 Å). We recorded 2055 movies using a K2 direct electron detector camera in
22 super-resolution mode with dose fractionation (80 individual frames were collected, starting from the
23 first one). Total exposure time was 8 s, with the total dose of 80 e⁻ (or 1 e⁻/Å²/frame). Images in the
24 stack were aligned using the whole-image motion correction and patch motion correction (5 × 5 patches)
25 methods in MotionCor2 (Zheng et al., 2017). Before image processing, all micrographs were checked
26 for quality and 1531 best were selected for the next step of image processing. The contrast transfer
27 function of each image was determined using GCTF (Zhang, 2016) as a standalone program. For
28 particle selection we have used Relion 3.0 autopicking procedure (Scheres, 2012). For the first steps of
29 image processing we used data binned by a factor of 8 (C8 images). During the first round of 2D
30 classification we removed only images with ice or other contaminants. Subsequently, the initial structure
31 was generated using the *ab initio* procedure in CryoSPARC v2.0. Following this step, we performed
32 Relion 3D classification with bin by four data (C4) in order to exclude bad particles. The resulting 141
33 549 particle images of ribosomes were used for subsequent classification and refinement procedures.
34 For the initial refinement we used a spherical mask, which was followed by further refinement using a
35 mask around the stable part of 50S (excluding L1 stalk, L7/L12 region). A further improved cryo-EM
36 map was obtained by using CTF-refinement procedure from Relion 3.0. The post-processing procedure
37 implemented in Relion 3.0 (Scheres, 2012) was applied to the final maps with appropriate masking,
38 B-factor sharpening (automatic B-factor estimation was -55.86) and resolution estimation to avoid
39 over-fitting (final resolution after post-processing with 50S mask applied was 2.7 Å). Subsequently the
40 stack of CTF-refined particles was processed in a new version of CryoSPARC v2.0 (Punjani et al.,

1 2017). After homogeneous refinement the same stack of particles was additionally refined in cisTEM
2 (Grant et al., 2018). After Auto-Refine (with automasking within cisTEM) we performed local
3 refinement using 50S mask (the same one used for refinement in Relion) and also applied per particle
4 CTF refinement as implemented in cisTEM software. After such refinement the resolution was
5 improved to 2.2 Å (**Extended Data Fig. 6**). This map after Sharpen3D (Grant et al., 2018) was used for
6 model building and map interpretation.

7 **Atomic model building and refinement**

8 The final model of the 50S subunit was generated by multiple rounds of model building in Coot (Emsley
9 et al., 2010) and subsequent refinement in PHENIX (Adams et al., 2010). The restraints for the novel
10 m²m⁸A nucleotide for the atomic model fitting and refinements were generated using eLBOW (Moriarty
11 et al., 2009). The atomic model of the 50S subunit from the *E. coli* ribosome structure (PDB 6PJ6)
12 (Stojković et al., 2020) was used as a starting point and refined against the experimental cryo-EM map
13 by iterative manual model building and restrained parameter-refinement protocol (real-space refinement,
14 positional refinement, and simulated annealing). Final atomic model comprised of ~92 736 atoms
15 (excluding hydrogens) across the 3015 nucleotides and 3222 amino acids of 28 ribosomal proteins.
16 Proteins L7, L10, L11 and L31 were not modelled in. In addition, 179 Mg²⁺, 2716 water molecules, one
17 Zn²⁺ and one Na⁺ were included in the final model. Prior to running MolProbity (Chen et al., 2010)
18 analysis, nucleotides 878–898, 1052–1110, 2101–2189 of 23S rRNA, and ribosomal protein L9 were
19 removed, due to their high degree of disorder. Overall, protein residues and nucleotides show
20 well-refined geometrical parameters (**Supplementary Table 6**). Figures were prepared using Pymol
21 Molecular Graphics System, Version 2.4.1 unless otherwise noted.

22 **qPTxM analysis of post-transcriptional modifications**

23 The final model and map were run through qPTxM (Stojković et al., 2020) with default parameters
24 except for d_min=2 and cc_threshold=0.5 to search for evidence of posttranscriptional modifications. Of
25 a total of 39 sites with density suggesting possible modifications, two were C8-methyl adenosines, A556
26 and A2503. None of the identified sites were 2'O-methyl cytosines. To calculate expected density
27 dropoff curves for methylated and unmethylated nucleotides, the *phenix.fmodel* (Adams et al., 2010)
28 tool was used to generate noise-free maps from models of a single nucleotide in each state, and scripts
29 modified from qPTxM were used to collect measurements of the density at 0.1 Å intervals along the
30 vector of the proposed methylation. Means and standard deviations were calculated for densities at the
31 four positions tested by qPTxM on each nucleotide, from which Z-scores were then calculated for
32 selected nucleotides. To measure densities for both the best tested rotamer of m(2'O)C 2498 and the
33 modeled rotamer, densities along the 2'O-methyl bond were compared between the files generated by
34 qPTxM run twice as described above, once with prune=True (removing the modeled methyl group and
35 placing the rotameric methyl with the strongest density) and once with prune=False (leaving the
36 modeled methyl group intact).

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