

4-24-2019

## tRNA Methylation Is a Global Determinant of Bacterial Multi-drug Resistance.

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### Recommended Citation

Masuda, Isao; Matsubara, Ryuma; Christian, Thomas; Rojas, Enrique R.; Yadavalli, Srujana S.; Zhang, Lisheng; Goulian, Mark; Foster, Leonard J.; Huang, Kerwyn Casey; and Hou, Ya-Ming, "tRNA Methylation Is a Global Determinant of Bacterial Multi-drug Resistance." (2019). *Department of Biochemistry and Molecular Biology Faculty Papers*. Paper 152.  
<https://jdc.jefferson.edu/bmpfp/152>

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1 **tRNA Methylation Is a Global Determinant of**  
2 **Bacterial Multi-Drug Resistance**

3  
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22  
23 *Running Title: Role of tRNA methylation in bacterial antibiotic resistance*

25 **SUMMARY**

26 Gram-negative bacteria are intrinsically resistant to drugs, due to their double-membrane  
27 envelope structure that acts as a permeability barrier and as an anchor for efflux pumps.  
28 Antibiotics are blocked and expelled from cells, and cannot reach high enough intracellular  
29 concentrations to exert a therapeutic effect. Efforts to target one membrane protein at a time  
30 have been ineffective. Here, we show that m<sup>1</sup>G37-tRNA methylation determines the synthesis of  
31 a multitude of membrane proteins via its control of translation at proline codons near the start of  
32 open-reading frames. Decreases in m<sup>1</sup>G37 levels in *Escherichia coli* and *Salmonella* impair  
33 membrane structure and sensitize these bacteria to multiple classes of antibiotics, rendering  
34 them unable to develop resistance or persistence. Codon engineering of membrane-associated  
35 genes reduces their translational dependence on m<sup>1</sup>G37 and confers resistance. These findings  
36 highlight the potential of tRNA methylation in codon-specific translation to control the  
37 development of multi-drug resistance in Gram-negative bacteria.

38

39 **KEYWORDS:** membrane barrier, drug efflux, m<sup>1</sup>G37-tRNA, TrmD, proline codons, resistance,  
40 persistence, tRNA<sup>Pro</sup>

41

42 Multi-drug resistance of Gram-negative bacteria is a critical and expanding medical challenge.  
43 In many cases, antibiotics are blocked from entry and expelled from cells, and hence cannot  
44 reach high enough intracellular concentrations to exert a therapeutic effect. This problem is due  
45 in large part to the double-membrane structure of the cell envelope of Gram-negative bacteria,  
46 which acts both as a permeability barrier and as a platform for efflux machineries that export  
47 drugs (Payne et al., 2007; Silver, 2011). In previous efforts focusing on targeting one membrane  
48 protein or one efflux pump at a time, resistance mutations were quick to develop (Murakami et  
49 al., 2006). Such mutations are selected upon the antibiotic challenge during therapy, giving rise  
50 to a resistant population (Silver, 2011, 2012). Inhibition of a process that simultaneously controls  
51 the expression of multiple membrane-associated genes would be a more powerful strategy for  
52 enhancing antibiotic efficacy. Such a global mechanism, which has not yet been identified, could  
53 provide a new anti-bacterial strategy to enable multiple drugs to take action, render resistance  
54 less likely, and accelerate bactericidal action.

55

56 The cell envelope of Gram-negative bacteria consists of a plasma inner membrane (IM), a cell  
57 wall, and an outer membrane (OM). The IM is a fluid lipid bilayer, while the cell wall is a rigid  
58 and cross-linked matrix of peptidoglycan that endows the cell with mechanical strength (Holtje,  
59 1998). The OM is made up of phospholipids in the inner leaflet and lipopolysaccharides in the  
60 outer leaflet, forming an asymmetric bilayer that prevents compounds from diffusing into the  
61 periplasm or cytosol, and also expels compounds to the external medium through membrane-  
62 bound efflux transporters (Nikaido, 1998). We recently showed that, in addition to its barrier  
63 function, the OM of *Escherichia coli* confers mechanical stiffness to the cell on par with the cell  
64 wall (Rojas et al., 2018), indicating that robust OM biogenesis is important for cellular  
65 mechanical integrity. The biogenesis of both IM and OM requires extensive integration with  
66 protein components, which also regulate cell-wall synthesis (Typas et al., 2011). Thus, the  
67 production of membrane proteins determines the quality of the entire Gram-negative cell

68 envelope; it is essential for establishing a permeability barrier and efflux activity against drugs  
69 and for defining cell shape and stability during cell growth.

70

71 One mechanism for global coordination of protein biosynthesis is via codon-specific translation,  
72 which directly impacts the speed and quality of translation at specific codons and has the ability  
73 to reprogram gene expression for disease development and drug resistance (Rapino et al.,  
74 2018). This regulation is distinct from transcriptional regulation via promoters or translational  
75 regulation via ribosome-binding sites. Mechanistically, codon-specific translation is mediated by  
76 post-transcriptional modifications of the tRNA anticodon or adjacent nucleotides. For  
77 membrane-associated genes, the translation of proline (Pro) codons (CCN) is critical, because  
78 Pro is the unique amino acid that is required for the creation of kinks in polypeptides and for the  
79 structure and activity of trans-membrane domains (Schmidt et al., 2016). We previously showed  
80 that the translation of Pro codons, particularly CC[C/U] codons, requires the conserved *N*<sup>1</sup>-  
81 methylation of G37 on the 3'-side of the tRNA anticodon (Gamper et al., 2015a, b). Without  
82 m<sup>1</sup>G37, tRNA is highly prone to stalling and +1 frameshifting (Gamper et al., 2015a, b), which  
83 are errors that disrupt the reading frame and prematurely terminate protein synthesis. The  
84 synthesis of m<sup>1</sup>G37 in bacteria is by the conserved tRNA methyl transferase TrmD, using *S*-  
85 adenosyl-methionine as the methyl donor (Hou et al., 2017) (Figure 1A, B). Depletion of TrmD,  
86 and consequently m<sup>1</sup>G37-tRNA, accumulates ribosomal frameshifts and leads to cell death  
87 (Gamper et al., 2015a). We found that CC[C/U] codons are prevalent in Gram-negative  
88 membrane-associated genes (Figure 1C), raising the possibility that the m<sup>1</sup>G37 methylation of  
89 tRNA by TrmD can provide a general mechanism to control the biosynthesis of membrane  
90 proteins.

91

92 Here, we demonstrate that TrmD is a global determinant of membrane biosynthesis in *E. coli*  
93 and *Salmonella enterica* serovar Typhimurium (hereafter *Salmonella*), two major Gram-negative

94 pathogens. We show that m<sup>1</sup>G37 deficiency caused by TrmD depletion disrupts the OM  
95 structure and rigidity, sensitizes *E. coli* and *Salmonella* to various classes of antibiotics, and  
96 suppresses their development of resistance or persistence upon antibiotic exposure.  
97 Engineering of the CC[C/U] codon to the less vulnerable CCG codon in membrane-associated  
98 genes reduces the translational dependence on m<sup>1</sup>G37 and confers drug resistance to bacteria.  
99 We also show that the conservation of m<sup>1</sup>G37 is required for codon-specific translation of  
100 CC[C/U], and that the methylation cannot be substituted by any other nucleotides. These results  
101 demonstrate that by simultaneously affecting codon-specific translation of Pro in entire classes  
102 of genes encoding membrane-associated proteins, TrmD-mediated methylation of tRNA is a  
103 major determinant of multi-drug resistance in Gram-negative bacteria.

104

## 105 RESULTS

106

### 107 **m<sup>1</sup>G37-deficient *E. coli* and *Salmonella* have lower levels of membrane proteins**

108 We previously showed that m<sup>1</sup>G37 has the strongest effect on codon-specific translation of  
109 CC[C/U] at the 2<sup>nd</sup> codon position of an open reading frame, and that this effect gradually  
110 decreases over the next 15 codons (Gamper et al., 2015a). In an analysis of the *E. coli* MG1655  
111 genome, we found that the occurrence of CC[C/U] at the 2<sup>nd</sup> codon position is 2-fold higher for  
112 genes encoding membrane-associated proteins relative to non-membrane-associated proteins  
113 (1.8% vs. 0.8%, n = 4,289, *p* < 0.05, Fisher's exact test with Bonferroni correction) (Hou et al.,  
114 2017). This enrichment was also observed when considering both the 2<sup>nd</sup> and 3<sup>rd</sup> codon  
115 positions (3.7% vs. 1.5%, n = 4,289, *p* < 0.0005, Fisher's exact test with Bonferroni correction).  
116 The over-representation of CC[C/U] is also evident in the genome of *Salmonella* LT2 (Hou et al.,  
117 2017). Among genes with CC[C/U] at the 2<sup>nd</sup> codon position, 31% and 26% encode membrane-  
118 associated proteins in *E. coli* and *Salmonella*, respectively (Figures 1C and S1). The high  
119 prevalence of Pro near the N-terminus of membrane proteins is consistent with its role in  
120 creating turns of transmembrane domains that cross a lipid bilayer (Yohannan et al., 2004).

121

122 To determine how m<sup>1</sup>G37 controls codon-specific translation of membrane-associated genes,  
123 we created *trmD-KD* (knockdown) strains of *E. coli* and *Salmonella*. Since *trmD* is essential for  
124 cell viability (Gamper et al., 2015a) and cannot be deleted, we created each *trmD-KD* strain by  
125 deleting the chromosomal *trmD* (Figure S2A-B) while expressing the human counterpart *trm5*  
126 from a plasmid with an arabinose (Ara)-inducible promoter. We previously showed that Trm5 is  
127 capable of supplying m<sup>1</sup>G37-tRNA to support bacterial viability (Christian et al., 2004), but that it  
128 is unstable in bacteria and can be removed rapidly (Christian et al., 2013). In the *E. coli* and  
129 *Salmonella trmD-KD* strains, the level of human Trm5 upon Ara induction increased with time  
130 and reached a steady state in 1-2 h, but decreased rapidly within 30 min upon Ara removal



131 (Figure 1D). Cells with Trm5-produced m<sup>1</sup>G37 formed colonies up to a 10<sup>4</sup>-fold dilution, whereas  
132 m<sup>1</sup>G37-deficient cells were not viable even without dilution (Figure 1E). To determine  
133 intracellular m<sup>1</sup>G37 levels, cells were grown with 0.2% Ara to saturation and diluted 1:100 into  
134 fresh Luria broth (LB) with or without Ara for 4 h, followed by another dilution to OD<sub>600</sub> = 0.1 in  
135 fresh LB with or without Ara and grown for 3 h. These serial passages were necessary to  
136 deplete cells of pre-existing m<sup>1</sup>G37-tRNA (Figure S3A). Primer extension analysis validated that  
137 the UGG isoacceptor of tRNA<sup>Pro</sup> in *trmD-KD* cells contained m<sup>1</sup>G37 at 70% and 12% in cultures  
138 with and without Ara (Figure 1F). This pattern was preserved for the GGG isoacceptor (Figure  
139 S3B,C) and was consistent with quantitative mass spectrometry analyses of the UGG  
140 isoacceptor (Figure 1G).

141  
142 To determine the effect of m<sup>1</sup>G37 deficiency on the biosynthesis of membrane proteins, we  
143 used quantitative proteomics to measure protein levels in the membrane fraction of *E. coli trmD-*  
144 *KD* cells grown with or without Ara. A total of 226 membrane proteins, 47 of which were  
145 associated with the OM, were analyzed by label-free quantification to determine fold-changes  
146 between Ara+ and Ara- conditions. While non-OM proteins were on average up-regulated in the  
147 absence of Ara by 16% (median increase of 2<sup>0.22</sup> = 1.16), OM proteins were on average down-  
148 regulated by 21% (median decrease of 2<sup>-0.33</sup> = 0.79) (Figure 2A). Of interest were LolB and  
149 OmpA, responsible for stable anchoring of drug-efflux pumps to the OM (Hayashi et al., 2014;  
150 Tsukahara et al., 2009) and for anchoring the OM to the peptidoglycan cell wall, respectively.  
151 *lolB* and *ompA* are enriched with Pro codons relative to the average codon usage in *E. coli*  
152 protein-coding genes (Figure 2B, *lolB*: CCN (6.7% vs. 4.3%) and CC[C/U] (2.4 vs. 1.1%) and  
153 *ompA* CCN (5.5 vs. 4.3%)). This enrichment is specific, because their usage of Leu codons  
154 (CUN), which also require m<sup>1</sup>G37 for translation, is typical (Figure S3D). The enrichment of Pro  
155 codons in *lolB* and *ompA* supports the notion that their decrease in protein levels is correlated  
156 with the poor translation of Pro codons in m<sup>1</sup>G37-deficient cells. Western blot analysis showed

157 that the amount of LolB relative to the cytosolic cysteinyl-tRNA synthetase CysRS (Hou et al.,  
158 1991; Lipman and Hou, 1998) in m<sup>1</sup>G37-deficient cells decreased to 26% in *E. coli* and to 56%  
159 in *Salmonella* (Figures 2C and S3E), while relative mRNA levels were unaffected (Figure S3F),  
160 indicating that the reduction in protein levels was due to reduced translation. These data are  
161 consistent with the notion that translation of *lolB* involves a TrmD-dependent codon at the 2<sup>nd</sup>  
162 and 4<sup>th</sup> positions of the *E. coli* gene and at the 4<sup>th</sup> position of the *Salmonella* gene (Figure 1A),  
163 whereas translation of *cysS* (for CysRS) involves no such codons in the first 16 positions.  
164 Western blot analysis also showed that the amount of OmpA relative to CysRS decreased to  
165 72% in m<sup>1</sup>G37-deficient *E. coli* cells (Figure 2D), providing additional support for the notion that  
166 translation of membrane-associated genes that are enriched with Pro codons is sensitive to loss  
167 of m<sup>1</sup>G37.

168

### 169 **m<sup>1</sup>G37 deficiency causes membrane damage and reduces OM stiffness**

170 We hypothesized that the reduced biosynthesis of membrane proteins in m<sup>1</sup>G37-deficient cells  
171 would damage membrane structural integrity. We observed increased intracellular accumulation  
172 in m<sup>1</sup>G37-deficient bacteria of both the redox sensor AlamarBlue, which becomes fluorescent  
173 inside cells, and the DNA fluorescent stain Hoechst 33342, indicating increased membrane  
174 permeability (Figures 3A, B, S4A). The accumulation of each dye was measured during  
175 exponential growth, and dye exposure was initiated in the presence of carbonyl cyanide *m*-  
176 chlorophenyl hydrazine (CCCP) to inactivate membrane efflux. To validate that AlamarBlue  
177 fluorescence reflected the permeability of the OM, we treated *E. coli* and *Salmonella* m<sup>1</sup>G37+  
178 cells with sublethal doses of polymyxin B, which binds to lipopolysaccharide in the OM and  
179 permeabilizes the double-membrane envelope. We showed that intracellular AlamarBlue  
180 fluorescence increased as a function of polymyxin B dose (Figure S4B), and that the maximum  
181 increase (4- to 5-fold) at a lethal dose of polymyxin B was in the same range as the observed  
182 increases in m<sup>1</sup>G37-deficient cells relative to m<sup>1</sup>G37+ cells (2- to 3-fold, Figure 3A, B). We

183 further showed that the intracellular AlamarBlue increase due to m<sup>1</sup>G37 deficiency was similar  
184 to the increase in *E. coli* cells expressing a defective OM pore protein relative to the control  
185 (Figure S4C). This defective pore protein was created by mutations in the siderophore  
186 transporter protein FhuA to enlarge the pore size, rendering the OM hyperpermeable to a wide  
187 range of compounds without affecting efflux (Krishnamoorthy et al., 2016).

188

189 To further validate the significance of the AlamarBlue increase due to m<sup>1</sup>G37 deficiency, we  
190 created *proS-KD* and *cysS-KD* strains, in which the essential genes responsible for amino-acid  
191 charging of tRNA<sup>Pro</sup> (*proS*) and tRNA<sup>Cys</sup> (*cysS*), respectively, were deleted from the  
192 chromosome and cell viability was maintained by Ara-dependent, plasmid-borne expression of  
193 each native gene. The *proS-KD* strain was a positive control to determine whether the  
194 deficiency of Pro-tRNA<sup>Pro</sup> affected translation of Pro codons in a manner similar to the deficiency  
195 of m<sup>1</sup>G37, while the *cysS-KD* strain was a negative control for how depletion of an essential  
196 protein that is unlikely to be involved in OM protein biogenesis would affect membrane  
197 permeability. The relative AlamarBlue increase due to *proS* depletion (2- to 3-fold) was  
198 comparable to that due to m<sup>1</sup>G37 deficiency, whereas the relative change due to *cysS* depletion  
199 was not significant (<1.3-fold, Figure S4C). Together, these data show that m<sup>1</sup>G37 deficiency  
200 increases membrane permeability to the same extent as the deficiency caused by a  
201 hyperpermeable pore or by reduced levels of charged tRNA for translation of Pro codons.

202

203 m<sup>1</sup>G37 deficiency also reduced membrane efflux, as indicated by the increased time required to  
204 pump out 50% of pre-loaded Nile Red dye (from 36 ± 1 to 66 ± 2 s for *E. coli* and 32 ± 3 to 45 ±  
205 3 s for *Salmonella* in m<sup>1</sup>G37-deficient relative to m<sup>1</sup>G37+ cells, Figure 3C-E). The extensions of  
206 efflux time (1.8- and 1.4-fold for *E. coli* and *Salmonella*, respectively) were smaller than that due  
207 to deletion of *acrB* relative to wildtype (> 4-fold) (Figure S5A, B); this smaller effect is expected,  
208 because m<sup>1</sup>G37 deficiency reduces but does not eliminate levels of efflux pumps, whereas *acrB*

209 deletion ( $\Delta acrB$ ) eliminates a component of the AcrAB-TolC complex, which is the major efflux  
210 pump responsible for expelling most antibiotics. The reduction in efflux due to m<sup>1</sup>G37 deficiency  
211 was also observed by monitoring ethidium bromide (Figure S5C, D), which showed an increase  
212 in the efflux time as a function of polymyxin B dose (Figure S5E, F). As expected, the extension  
213 time required for expelling ethidium bromide was smaller compared with the effect of  $\Delta tolC$  on  
214 the AcrAB-TolC complex (Figure S5C, D). We also used Thioflavin T (ThT) to probe the  
215 membrane potential (Prindle et al., 2015) and confirmed that m<sup>1</sup>G37 deficiency reduced the  
216 fluorescence of ThT in *E. coli* and *Salmonella* (Figure 3F), further supporting our conclusion that  
217 the OM was impaired.

218  
219 To determine how m<sup>1</sup>G37 deficiency affected the cell envelope structure, we measured cellular  
220 mechanical stiffness using an assay that we recently developed and utilized to demonstrate that  
221 the OM makes a surprisingly large contribution to the overall stiffness of the *E. coli* cell envelope  
222 (Rojas et al., 2018). Perturbation of the OM by chemical agents or genetic mutations caused  
223 large reductions in stiffness and rendered cells susceptible to lysis under oscillatory osmotic  
224 shocks (Rojas et al., 2018). We previously showed that deletion of *ompA* and *lpp* and  
225 introduction of a mutant allele of *lptD* each decreased OM stiffness (Rojas et al., 2018). While  
226 *ompA* and *lpp* encode abundant OM proteins, the mutant *lptD* allele encodes a variant of the  
227 lipopolysaccharide assembly machinery that is known to increase the OM permeability to  
228 antibiotics (Ruiz et al., 2005). We thus hypothesized that the altered OM composition during  
229 m<sup>1</sup>G37 deficiency would decrease the stiffness of the cell envelope.

230  
231 Our assay involves application of force to the cell envelope by subjecting cells to oscillatory  
232 osmotic shocks using a microfluidic device and measurement of the resulting deformations of  
233 the cell envelope (Rojas et al., 2014; Rojas et al., 2018). For small shock magnitudes (100 mM  
234 sorbitol), the plasma membrane essentially remains in contact with the cell envelope (Rojas et

235 al., 2014), so that the boundary of the cytoplasm detected from phase-contrast images can be  
236 used to track the envelope contour. The degree to which the envelope deforms, as defined by  
237 the amplitude of the cell-length oscillations in response to oscillatory osmotic shocks, is  
238 inversely correlated with envelope stiffness (Rojas et al., 2018). During m<sup>1</sup>G37 deficiency due to  
239 growth without Ara for ~4 h, cells grew more slowly and were smaller than cells grown in the  
240 presence of Ara (Figure 3G). The amplitude of response to 100 mM oscillatory osmotic shocks  
241 increased substantially in Ara<sup>-</sup> cells relative to Ara<sup>+</sup> cells ( $n = 2$  experiments with 67-713 cells;  
242 Figure 3G-I), indicating a decrease in envelope stiffness. This increase in amplitude runs  
243 counter to the expectation based on the reduction in cell size alone, whereby the mechanical  
244 expansion of a thin shell under load is predicted to be larger for a cell with a larger radius than  
245 for a shell of the same material and thickness with a smaller radius. In sum, these data suggest  
246 that m<sup>1</sup>G37 deficiency changes the composition of the cell envelope, resulting in lower load-  
247 bearing capacity and higher permeability.

248

### 249 **m<sup>1</sup>G37 deficiency sensitizes Gram-negative bacteria to multiple antibiotics**

250 We hypothesized that m<sup>1</sup>G37 deficiency would sensitize Gram-negative cells to antibiotics due  
251 to compromised permeability and mechanics of the cell envelope. We assessed antibiotics with  
252 various mechanisms of action (Silver, 2011), including: the  $\beta$ -lactams ampicillin and  
253 carbenicillin, which target cell-wall biosynthesis; the aminoglycosides kanamycin and  
254 gentamicin, which inhibit protein synthesis; paromomycin, which reduces fidelity of the 30S  
255 ribosomal subunit; the ansamycin polyketide rifampicin, which targets RNA polymerase; and the  
256 quinolone ciprofloxacin, which targets DNA gyrase. This diverse collection of antibiotics  
257 accesses different mechanisms of membrane permeability and efflux pumps, allowing us to  
258 determine the general impact of m<sup>1</sup>G37 deficiency. We inoculated *E. coli* and *Salmonella* at 10<sup>6</sup>  
259 colony-forming units (CFUs)/mL and grew these cells with each antibiotic for 18 h. Defining  
260 growth as an increase in cell density above OD<sub>600</sub> of 0.15 for the purpose of determining the

261 minimum inhibitory concentration (MIC), we found that m<sup>1</sup>G37-deficient *E. coli* and *Salmonella*  
262 showed at least 2-fold lower MICs relative to controls for all antibiotics (Figures 4A, B, S6A, B).  
263 In most cases, these reductions were in the same range as those reported previously for  $\Delta toIC$   
264 cells (Krishnamoorthy et al., 2016), and also in the same range as the reductions in cells treated  
265 with a sublethal dosage of polymyxin B (Figure 4A, B). For example, the fold-changes in the  
266 MICs of ampicillin and carbenicillin between m<sup>1</sup>G37+ and m<sup>1</sup>G37-deficient cells of *E. coli* (2.0-  
267 and 2.7-fold) and *Salmonella* (2.7- and 3.0-fold) were similar to those between untreated and  
268 polymyxin-treated m<sup>1</sup>G37+ cells (1.5- and 2.0-fold and 0.8- and 1.0-fold, respectively). This  
269 similarity held generally for all tested antibiotics, indicating that m<sup>1</sup>G37 deficiency has similar  
270 effects as polymyxin on membrane permeability to antibiotics. To further validate the magnitude  
271 of m<sup>1</sup>G37 effects on antibiotic sensitivity, we showed that the fold-change in MIC of antibiotics  
272 during m<sup>1</sup>G37 deficiency was generally larger than the effect of  $\Delta efp$  (Figure S6C), the gene  
273 encoding protein-synthesis elongation factor P, which has a role in antibiotic susceptibility  
274 (Navarre et al., 2010). The broad spectrum of antibiotics exhibiting a reduction in MIC in m<sup>1</sup>G37-  
275 deficient cells indicates that multiple membrane proteins were affected, resulting in a generally  
276 compromised membrane similar to the damage caused by polymyxin B.

277

278 As an additional probe of membrane structure, we tested vancomycin, a linear hepta-peptide  
279 that inhibits cell-wall synthesis (Ruiz et al., 2005). Vancomycin is typically only active against  
280 Gram-positive bacteria, although disruption of the OM in Gram-negative bacteria permits its  
281 passage and action (Shlaes et al., 1989; Young and Silver, 1991). We observed a 2- to 4-fold  
282 reduction in the MIC of vancomycin in m<sup>1</sup>G37-deficient cells (Figure 4A, B), a 4- to 5-fold  
283 reduction in polymyxin-treated m<sup>1</sup>G37+ cells (Figure 4A, B), and a 2-fold reduction in  $\Delta efp$  cells  
284 (Figure S6C). These effects further highlight the damage to the OM in m<sup>1</sup>G37-deficient cells.

285

286 While we could not quantify the full extent of the effect of m<sup>1</sup>G37 deficiency on antibiotic  
287 sensitivity, due to the essentiality of TrmD, we were interested in determining whether the OM  
288 damage in m<sup>1</sup>G37-deficient cells increased intracellular drug concentrations sufficiently to  
289 accelerate bactericidal action. By incubating 10<sup>6</sup> CFUs of cells with increasing concentrations of  
290 each antibiotic and measuring CFUs/mL over time within the first 24 h of treatment, we  
291 demonstrated that m<sup>1</sup>G37-deficient cells were killed faster relative to controls. The concentration  
292 of each drug that displayed the strongest effect due to m<sup>1</sup>G37 deficiency was selected for in-  
293 depth analysis (Figure 4C, D). The time-kill kinetics of carbenicillin and ampicillin showed that  
294 the viability of both m<sup>1</sup>G37+ and m<sup>1</sup>G37-deficient cells remained relatively stable within 5-7 h of  
295 exposure, after which the viability of m<sup>1</sup>G37-deficient cells declined while m<sup>1</sup>G37+ cells regrew.  
296 By contrast, the time-kill kinetics of gentamicin and kanamycin showed a 10<sup>3</sup>- to 10<sup>4</sup>-fold  
297 decrease in viability immediately upon exposure, after which m<sup>1</sup>G37-deficient cells remained low  
298 in viability up to 24 h while m<sup>1</sup>G37+ cells recovered. The more robust regrowth of  
299 aminoglycoside-treated cells relative to carbenicillin- or ampicillin-treated cells is likely driven by  
300 the development of adaptive resistance through aminoglycoside-induced down-regulation of  
301 drug uptake and up-regulation of efflux (Mohamed et al., 2012). The presence of m<sup>1</sup>G37 may  
302 confer adaptive resistance by promoting biosynthesis of high-quality pumps. In the time-kill  
303 kinetics of vancomycin, m<sup>1</sup>G37 deficiency immediately decreased cell viability upon exposure,  
304 while m<sup>1</sup>G37+ cells simply increased in number over time.

305  
306 Our *cysS-KD* and *proS-KD* uptake data (Figure S4C) suggest that the reduced viability of  
307 m<sup>1</sup>G37-deficient cells was due to translational defects at Pro codons, and not to the nonspecific  
308 loss of an essential gene. Further supporting this conclusion, time-kill kinetics with carbenicillin  
309 and vancomycin revealed that m<sup>1</sup>G37-deficient *proS-KD* cells were killed faster and to a greater  
310 extent than *cysS-KD* cells (Figure 4E, F). To query whether the reduced cell viability during  
311 m<sup>1</sup>G37 deficiency was due to an unrelated stress response, we determined that m<sup>1</sup>G37+ and

312 m<sup>1</sup>G37-deficient cells had virtually identical time-kill kinetics when incubated with 2 mM H<sub>2</sub>O<sub>2</sub>  
313 (Figure S6E, F), indicating that the expression of genes in response to oxidative stress, unlike  
314 those for biosynthesis of the cell envelope, is not affected by m<sup>1</sup>G37 deficiency. Thus, m<sup>1</sup>G37  
315 deficiency has a specific effect on bacterial survival in antibiotic exposure, likely due to the  
316 reduced synthesis of membrane proteins.

317

### 318 **m<sup>1</sup>G37-deficient cells exhibit reduced resistance and persistence to antibiotics**

319 We hypothesized that the faster antibiotic killing of m<sup>1</sup>G37-deficient cells would preempt their  
320 ability to develop mutations that confer resistance. We chose a concentration for each drug near  
321 1X MIC for m<sup>1</sup>G37+ cells and determined the relative frequency of resistance in m<sup>1</sup>G37-deficient  
322 cells. Log-phase cells were grown on plates containing each antibiotic and the frequency of  
323 resistance was determined by the number of colonies that appeared after three days of  
324 incubation. Consistently across *E. coli* and *Salmonella*, analysis of a broad spectrum of  
325 antibiotics showed that m<sup>1</sup>G37-deficient cells produced significantly fewer resistant colonies  
326 than m<sup>1</sup>G37+ cells from an inoculum of 10<sup>5</sup> CFUs (Figure 5A, B). We confirmed that selected  
327 resistant colonies indeed exhibited an increase in MIC (by 3- to 6-fold) to the tested drug (Figure  
328 5C). When we tested each drug at 1X MIC for m<sup>1</sup>G37+ and m<sup>1</sup>G37-deficient cells, respectively,  
329 m<sup>1</sup>G37-deficient cells remained compromised in the frequency of resistance relative to m<sup>1</sup>G37+  
330 cells (Figure S6G, H).

331

332 Unlike resistance that arises from genetic mutations upon drug treatment, persistence arises  
333 from noise in gene expression that gives rise to drug tolerance in a subpopulation of isogenic  
334 cells (Brauner et al., 2016). This subpopulation of persisters typically survives for some time,  
335 contributing to the recurrence of chronic infections. Although the mechanisms underlying  
336 persistence are complex, one major pathway is to enhance efflux to pump out the drug (Pu et  
337 al., 2016) while shutting down all other biological processes. We hypothesized that by reducing



338 protein synthesis of efflux pumps and OM proteins (Figure 2C, D), m<sup>1</sup>G37 deficiency would  
339 reduce the frequency of persistence under antibiotic treatment.

340  
341 We studied persistence using *Salmonella*, which showed a greater response in uptake due to  
342 m<sup>1</sup>G37 deficiency than *E. coli* (Figure 3A, B) and hence was predicted to manifest a larger effect  
343 on persistence. *Salmonella* cells were treated with a lethal dosage (2-3X MIC) of gentamicin or  
344 paromomycin, and viability was measured over time after the start of treatment. While untreated  
345 cells maintained viability, drug-treated cells displayed bi-phasic time-kill curves (Figure 5D-F)  
346 that signify a heterogeneous response of persistent and non-persistent sub-populations  
347 (Balaban et al., 2004). The faster phase of the bi-phasic curve represented killing of the  
348 susceptible population, while the slower phase reflected killing of the persistent population. The  
349 greater extent of killing in the faster phase was consistent with the susceptible population being  
350 the larger fraction. After 6 h of treatment, m<sup>1</sup>G37-deficient cells exhibited a >10-fold reduction in  
351 the frequency of persistence relative to m<sup>1</sup>G37+ controls, indicating that m<sup>1</sup>G37 deficiency  
352 compromised *Salmonella*'s ability to tolerate high drug concentrations. Together, these data  
353 support the notion that, when the cell envelope was disrupted by m<sup>1</sup>G37 deficiency, more  
354 antibiotics penetrated into and accumulated inside cells to accelerate bactericidal action before  
355 resistance or persistence can develop.

356

### 357 **Codon composition determines the effect of m<sup>1</sup>G37 methylation**

358 We tested the hypothesis that the reduced synthesis of membrane proteins in m<sup>1</sup>G37-deficient  
359 cells was due to the poor translation of Pro codons by the unmethylated tRNA<sup>Pro</sup>. We examined  
360 the translation of *E. coli lolB*, which has a CCC-C sequence at the 2<sup>nd</sup> codon and a CCC-G  
361 sequence at the 4<sup>th</sup> codon (Figure 1C). To maintain the natural gene dosage, we changed the  
362 m<sup>1</sup>G37-dependent CCC at both positions on the chromosome to the less-dependent CCG  
363 codon. We used  $\lambda$ -Red recombination for codon engineering, which left a scar in the genome.

364 Western blot analysis of lysates of cells with the scar showed that, while m<sup>1</sup>G37 deficiency  
365 reduced the translation of the unedited *lolB* to 89%, it had the opposite effect on the translation  
366 of the edited gene by increasing it to 131% (Figure 6A). Each measurement of *lolB* translation  
367 was normalized to that of *cysS*. The increase in *lolB* translation by single-nucleotide  
368 synonymous changes illustrates the effect of m<sup>1</sup>G37 on codon-specific translation.

369  
370 As a second test, we changed the CCC codon at the 6<sup>th</sup> position of *tolC* in *Salmonella* (Figure 1)  
371 to CCG. This single-nucleotide synonymous change would lessen the translational dependence  
372 on m<sup>1</sup>G37 relative to the unedited gene, thereby increasing *tolC* translation and reducing  
373 susceptibility to antibiotics in m<sup>1</sup>G37 deficiency. We focused on novobiocin, which is cell-  
374 permeable but subject to TolC-mediated efflux (Kodali et al., 2005). Survival of m<sup>1</sup>G37-deficient  
375 cells under novobiocin treatment was 2.7-fold higher when expressing the edited *tolC* relative to  
376 cells expressing the unedited gene (Figure 6B), supporting the codon-specific effect of m<sup>1</sup>G37.  
377 The Pro at the 6<sup>th</sup> position of TolC is conserved among Gram-negative bacteria, and substitution  
378 of Pro with Ala by mutating the CCC codon to GCG reduced the protein to undetectable levels  
379 (data not shown), probably due to membrane mistargeting and destabilization (Masi et al.,  
380 2009). These data suggest that the conservation of Pro at the 6<sup>th</sup> position is critical for TolC  
381 structure and function, and that its incorporation into the protein is regulated at the codon level  
382 by m<sup>1</sup>G37.

383

#### 384 **The importance of m<sup>1</sup>G37 in the UGG isoacceptor of tRNA<sup>Pro</sup>**

385 *E. coli* and *Salmonella* both express three isoacceptors of tRNA<sup>Pro</sup> ([http://trna.bioinf.uni-](http://trna.bioinf.uni-leipzig.de/)  
386 [leipzig.de/](http://trna.bioinf.uni-leipzig.de/)), all of which contain m<sup>1</sup>G37. Of the three, the UGG isoacceptor is the most sensitive  
387 to loss of m<sup>1</sup>G37 (Gamper et al., 2015a). This isoacceptor is capable of reading all Pro codons  
388 via an additional cm<sup>o</sup>5U34 modification at the wobble position (Nasvall et al., 2004), and it is  
389 also the only one that is required for cell growth and survival. We tested whether an alternative

390 nucleotide could substitute for m<sup>1</sup>G37 in the UGG tRNA to eliminate the need for *trmD*. We  
391 created a derivative of *E. coli* MG1655 that lacked the tRNA gene on the chromosome and  
392 expressed the isoacceptor from a plasmid to maintain viability. This strain also lacked the gene  
393 for the GGG isoacceptor on the chromosome, so that the translation of CC[C/U] was completely  
394 dependent on the plasmid-borne UGG tRNA. While we designed strains with all three non-G  
395 substitutions on the plasmid-borne tRNA, we only recovered the C37 variant (data not shown),  
396 suggesting that the A37 and U37 variants were lethal. We previously showed that the C37  
397 variant is not methylated by TrmD (Christian et al., 2004).

398  
399 The strain expressing the C37 variant of the UGG tRNA was severely defective in growth  
400 relative to the G37 version (Figure 6C), even though *trmD* was intact. Cells expressing the C37-  
401 tRNA accumulated more Hoechst dye (Figure 6D), indicating the disruption of the membrane  
402 barrier. Cells expressing the C37-tRNA were also more sensitive to antibiotic killing than cells  
403 expressing the G37 version, with MIC decreases of 8.2-fold for gentamicin and 4.0-fold for  
404 vancomycin (Figure 6E). These decreases for two unrelated antibiotics suggest that the  
405 envelope structure is disrupted in cells expressing the C37-tRNA. Expression of the C37-tRNA  
406 led to more rapid killing upon exposure to gentamicin or vancomycin (Figure 6F). Collectively,  
407 these data indicate that C37-tRNA is unable to support the biosynthesis of membrane proteins  
408 at the levels of m<sup>1</sup>G37-tRNA, and that the single G37C substitution is sufficient to cause general  
409 damage to the cell envelope, leading to faster antibiotic killing. Thus, m<sup>1</sup>G37 methylation by  
410 TrmD is necessary for the function of UGG tRNA and cannot be replaced.

411

## 412 **DISCUSSION**

413

414 Multi-drug resistance among Gram-negative bacteria is a major human health problem. We  
415 report here the discovery of m<sup>1</sup>G37 methylation of tRNA as a global determinant of multi-drug  
416 resistance in *E. coli* and *Salmonella*. The mechanism of this methylation is at the codon level  
417 during the elongation phase of protein synthesis, rather than at the initiation of transcription or  
418 translation. Because protein synthesis is the last step of gene expression in a highly energy-  
419 demanding process, the control of its speed and quality at individual codons provides enormous  
420 capacity to influence the proteome of a cell. The m<sup>1</sup>G37 methylation is present in all  
421 isoacceptors of Pro, two isoacceptors (GAG and CAG) of Leu, and one isoacceptor (CCG) of  
422 Arg. The complete association of m<sup>1</sup>G37 with tRNA<sup>Pro</sup> species emphasizes its ability to regulate  
423 translation of genes enriched with Pro codons (particularly the CC[C/U] codons), which include  
424 many Gram-negative genes encoding OM proteins. With few exceptions, most of these genes  
425 are not operon-organized and cannot be simultaneously regulated by transcription or translation  
426 initiation. Instead, their dependence on translation of Pro codons to generate transmembrane  
427 domains provides a common thread that unites them under the control of m<sup>1</sup>G37 methylation.  
428 Our data support a model in which m<sup>1</sup>G37 ensures robust biosynthesis of Gram-negative OM  
429 membrane proteins to produce an effective envelope barrier and efflux activity, which confers  
430 multi-drug resistance, whereas m<sup>1</sup>G37 deficiency reduces the levels of OM proteins, thereby  
431 permeabilizing the OM structure and sensitizing cells to antibiotic killing (Figure 7A). While  
432 m<sup>1</sup>G37 deficiency does not act on all genes for membrane proteins, the effects are sufficiently  
433 widespread (e.g. *lolB*, *ompA*, and *tolC*) and impactful to accelerate bactericidal action of  
434 antibiotics and to halt resistance or persistence upon antibiotic exposure. Our data are generally  
435 consistent across *E. coli* and *Salmonella*, and are likely applicable to a broad spectrum of Gram-  
436 negative pathogens, including *Pseudomonas aeruginosa*, *Yersinia pestis*, *Serratia marcescens*,  
437 and *Shigella dysenteriae*, in which CC[C/U] codons are widely present near the start of

438 membrane-associated genes (Figure S7). Strikingly, the CC[C/U] codon at the 6<sup>th</sup> position of  
439 *tolC* is conserved among  $\gamma$ -proteobacteria (Figure 7B), indicating that the efflux activity of the  
440 gene and multi-drug resistance of these Gram-negative bacteria is determined by m<sup>1</sup>G37.

441  
442 m<sup>1</sup>G37 is distinct from the >100 post-transcriptional modifications that have been associated  
443 with tRNA to date (<http://modomics.genesilico.pl/>). Crucially, m<sup>1</sup>G37 is both essential and is  
444 conserved across all three kingdoms of life (Bjork et al., 2001). In bacteria, where m<sup>1</sup>G37 is  
445 synthesized by TrmD, its level is stable across various growth phases (Gamper et al., 2015a).  
446 Even when *E. coli* cells are deep in stationary phase, when glucose and all other nutrients are  
447 depleted, m<sup>1</sup>G37 levels remain at ~100% (Gamper et al., 2015a). By contrast, levels of most  
448 tRNA post-transcriptional modifications are variable depending on cellular conditions. The  
449 synthesis of m<sup>1</sup>A58, required for tRNA translation in eukaryotes, is subject to demethylation  
450 during glucose deprivation (Liu et al., 2016). The formation of s<sup>4</sup>U8 in bacteria is induced by  
451 near-UV radiation (Favre et al., 1971) and that of cm<sup>5</sup>U34 is activated by hypoxia (Chionh et  
452 al., 2016). The formation of m<sup>5</sup>C34 in yeast is induced by oxidative stress (Chan et al., 2012)  
453 and that of mcm<sup>5</sup>U34 and mcm<sup>5</sup>s<sup>2</sup>U34 is by alkylation damage (Begley et al., 2007). The  
454 stability of m<sup>1</sup>G37 levels emphasizes the potential of targeting TrmD for antibacterial therapies.

455  
456 TrmD is a high-priority antibacterial target (White and Kell, 2004). Besides its essentiality for  
457 bacterial growth and survival (Gamper et al., 2015a), TrmD is broadly conserved among  
458 bacterial species, has a methyl-donor binding site for drug targeting, and is fundamentally  
459 distinct from its human counterpart Trm5 in structure and mechanism (Christian et al., 2004;  
460 Christian and Hou, 2007; Christian et al., 2010; Christian et al., 2016; Lahoud et al., 2011;  
461 Sakaguchi et al., 2012; Sakaguchi et al., 2014), enabling the development of bacteria-selective  
462 compounds. However, while pharmaceutical companies have attempted to target TrmD,  
463 progress has stalled, because the isolated inhibitors have failed to overcome the OM barrier and

464 efflux activity (Hill et al., 2013). This obstacle resonates with the major challenge that confronts  
465 current antibacterial discovery – the inability to make compounds that penetrate bacteria,  
466 especially Gram-negative species (Tommasi et al., 2015). Our finding that TrmD is a global  
467 determinant of the biosynthesis of Gram-negative membrane proteins provides new insight into  
468 how to address this problem.

469  
470 To target TrmD, we suggest exploiting its ability to control the translation of CC[C/U] in  
471 membrane-associated genes. The CCC codon at the 6<sup>th</sup> position of *tolC* is an example, which is  
472 conserved among  $\gamma$ -proteobacterial pathogens and is required for protein stability, acting as an  
473 Achilles heel that is required for efflux activity of *tolC* but is also subject to regulation by TrmD  
474 for translation. While the AcrAB-TolC pump exports a wide range of antibiotics (Li et al., 1995;  
475 Okusu et al., 1996), it does not act on gentamicin-like aminoglycosides (Edgar and Bibi, 1997).  
476 Thus, primary inhibitors of TrmD should be gentamicin-like molecules, capable of entering cells  
477 without being expelled by AcrAB-TolC. Once inside cells, these inhibitors can target TrmD and  
478 reduce the synthesis of TolC, as well as many other membrane proteins and efflux pumps that  
479 depend on TrmD for translation. By targeting TrmD while exerting collateral damage on the cell  
480 envelope, primary inhibitors can destabilize the membrane barrier to allow secondary inhibitors  
481 with distinct mechanisms of action to enter cells and function. In this two-tiered strategy,  
482 accelerated bactericidal action should reduce the likelihood of resistance and persistence and  
483 improve the efficiency of antibacterial treatments, yielding a general strategy for mitigating  
484 bacterial multi-drug resistance. This study demonstrates that tRNA methylation events such as  
485 m<sup>1</sup>G37 have broad effects on cellular physiology and membrane biology, which can be exploited  
486 for novel drug discovery.

487

488 **STARMETHODS**

489 Detailed methods are provided in the online version of this paper and include the following:

- 490 • Key resources tables
- 491 • Contact for reagent and resource sharing
- 492 • Methods details
  - 493 ○ Construction of strains
  - 494 ○ MS analysis of membrane proteomes
  - 495 ○ Western blotting
  - 496 ○ Primer-extension analysis of m<sup>1</sup>G37
  - 497 ○ LC-MS/MS analysis of m<sup>1</sup>G37
  - 498 ○ Quantification of *lo/B* mRNA with a YFP reporter
  - 499 ○ AlamarBlue accumulation assay
  - 500 ○ Hoechst accumulation assay
  - 501 ○ Nile Red efflux assay
  - 502 ○ Ethidium bromide efflux assay
  - 503 ○ Thioflavin T fluorescence assay
  - 504 ○ Imaging in microfluidic devices
  - 505 ○ Cell tracking and analysis
  - 506 ○ Minimal inhibitory concentration (MIC) measurements
  - 507 ○ Time-kill analyses
  - 508 ○ Resistance analyses
  - 509 ○ Persistence analyses
  - 510 ○ Codon engineering
- 511 • Quantification and statistical analyses
- 512 • Data and software availability

513

514 **SUPPLEMENTAL INFORMATION**

515 Supplemental information includes 7 figures, which can be found with this article online at....

516 Table S1: Primers used in this study. (Related to STAR Methods)

517

518

519 **AUTHOR CONTRIBUTIONS**

520 I.M. and R.M. constructed strains, performed codon engineering, Western blotting, MIC  
521 determination, and time-kill, resistance, and persistence analyses. T.C. performed primer  
522 extension, E.R. performed oscillatory osmotic-shock analyses, S.S.Y. analyzed dye  
523 accumulation, and L.Z. quantified mass spectrometry data. All authors analyzed and interpreted  
524 the data. K.C.H. and Y.M.H. wrote the manuscript with comments provided by M.G. and L.F.

525

526 **ACKNOWLEDGMENTS**

527 We acknowledge support by grants from the US National Institutes of Health (GM108972 and  
528 GM114343 to YMH; GM080279 to MG) and the National Science Foundation (DMR-1120901 to  
529 MG; MCB-1149328 to KCH), the Allen Discovery Center at Stanford on Systems Modeling of  
530 Infection (to KCH), the Canadian Institutes of Health Research (CIHR MOP-77688 to LF), and a  
531 JSPS postdoctoral fellowship (to IM). KCH is a Chan Zuckerberg Biohub Investigator. We thank  
532 Chuan He, Mark Brynildsen, Hiroshi Nikaido, and Sean Moore for discussions, Hajime Tokuda  
533 and Glenn Björk for antibodies against LolB, OmpA, and TrmD, Roy Kishony for the YFP  
534 plasmid, Harvey Rubin, Trevor Selwood, Lynn Silver, and Hong-Suk Kim for discussion, Sunita  
535 Maharjan for help with experiments, Helen Zgurskaya for providing the *E. coli* hyper-permeable  
536 strain, and Laszlo Csonka for providing the *Salmonella* LT2 strain.

537

538 **DECLARATION OF INTERESTS**



539 The authors declare no competing interests.

540

541 **FIGURE LEGENDS**

542

543 **Figure 1: m<sup>1</sup>G37-tRNA is important for expression of membrane-associated genes.**

544 A) TrmD (PDB: 1UAK) synthesizes m<sup>1</sup>G37-tRNA.

545 B) Translation of CCC codon requires m<sup>1</sup>G37-tRNA<sup>Pro</sup> to suppress +1 frameshifts at the P-  
546 site.

547 C) Gram-negative genes for membrane-associated proteins often contain CC[C/U] codons  
548 (red) near the start of the ORF. Five examples from *E. coli* (*Ec*) and *Salmonella enterica*  
549 (*Se*) are shown.

550 D) Western blots of *trmD-KD* cells showed that human Trm5 is unstable upon removing the  
551 inducer Ara. Overnight cultures with 0.2% Ara were diluted 1:100 into fresh LB in Ara+/-  
552 conditions. Cells were sampled over time and levels of Trm5 and CysRS were  
553 determined using antibodies.

554 E) Expression of *trm5* is required for viability of *trmD-KD* cells. Overnight cultures in LB with  
555 0.2% Ara were maintained in a viable state by expression of the plasmid-borne P<sub>BAD</sub>-  
556 controlled human *trm5*. Cells were serially diluted and spotted on LB plates with or  
557 without 0.2% Ara. Growth was assayed after overnight incubation at 37 °C.

558 F) Primer extension analysis of m<sup>1</sup>G37 in tRNA<sup>Pro/UGG</sup>. Cells were prepared as in (D),  
559 diluted after 5 h to OD<sub>600</sub> = 0.1 in fresh LB (Ara+/-), incubated for another 2 h at 37 °C,  
560 and total small RNA was purified. (Top) Primer extension was blocked at m<sup>1</sup>G37 in cells  
561 grown with Ara+ (+), whereas the primer read through to nucleotide C1 in cells grown  
562 without Ara (Ara-). (Bottom) m<sup>1</sup>G37 levels are shown as mean ± standard error of the  
563 mean (SEM), n = 3. Welch's *t*-test: \*\**p* < 0.05, \*\*\**p* < 0.01.

564 G) Mass spectrometry analysis of m<sup>1</sup>G37 levels in tRNA<sup>Pro/UGG</sup>. Cells were prepared as in  
565 (D) and the tRNA was isolated by affinity purification. m<sup>1</sup>G37 levels are shown as mean  
566 ± SEM, n = 3. The fraction of m<sup>1</sup>G among total Gs in the tRNA was 0.055, representing

567 ~100% methylation as compared to the theoretical value (one m<sup>1</sup>G among 25 Gs = 0.04,  
568 Figure S3B). Welch's *t*-test: \*\**p* < 0.05, \*\*\**p* < 0.01.

569 See also Figures S1 and S2.

570

571 **Figure 2: m<sup>1</sup>G37 deficiency in *E. coli* and *Salmonella* affects cell viability.**

572 A) Quantitative mass spectrometry analysis of membrane proteins in *E. coli trmD-KD* cells  
573 isolated from Ara<sup>-</sup> and Ara<sup>+</sup> conditions. The label-free quantification intensity is  
574 compared to the signal of log<sub>2</sub> (fold-change) (Ara<sup>-</sup>/Ara<sup>+</sup>). OM proteins are plotted in  
575 black with a vertical line indicating the median of -0.33 (equivalent to a decrease of  
576 21%), while non-OM proteins are plotted in blue with a vertical line showing the median  
577 of 0.22 (equivalent to an increase of 16%). *p* < 0.001 by a Kolmogorov-Smirnov analysis.

578 B) Frequency of Pro codons CCN (top) and CC[C/U] (bottom) in genes whose OM proteins  
579 are reduced in Ara<sup>-</sup> vs. Ara<sup>+</sup> in (A). Each frequency is compared to the average  
580 frequency of respective Pro codons in *E. coli* protein-coding genes.

581 C,D) m<sup>1</sup>G37 deficiency decreased LolB levels (C) to 26% in *E. coli* and to 56% in  
582 *Salmonella*, and decreased OmpA levels in *E. coli* to 72% (D) in Western blots (top).  
583 Overnight cultures of *trmD-KD* cells were diluted 1:100 into fresh LB with or without 0.2%  
584 Ara and grown for 4 h at 37 °C. Cells were inoculated into fresh LB in Ara<sup>+/-</sup> conditions  
585 for another 3 h. Data and error bars represent mean ± SD, *n* = 4.

586 See also Figure S3.

587

588 **Figure 3: m<sup>1</sup>G37 deficiency weakens the cell envelope.**

589 A,B) *E. coli* (A) and *Salmonella* (B) *trmD-KD* cells in m<sup>1</sup>G37 deficiency (m<sup>1</sup>G37<sup>-</sup>) show  
590 increased membrane permeability relative to m<sup>1</sup>G37<sup>+</sup> cells. Cells were grown as in  
591 Figure 2C and the intracellular accumulation of AlamarBlue in m<sup>1</sup>G37<sup>+</sup> (Ara<sup>+</sup>, blue) and  
592 m<sup>1</sup>G37-deficient (Ara<sup>-</sup>, red) conditions was monitored in the presence of CCCP. Levels

593 of intracellular dye accumulation were normalized by OD<sub>600</sub>. Data and error bars are  
594 mean ± SD, n = 3.

595 C-E) *E. coli* (C) and *Salmonella* (D) *trmD-KD* cells showed reduced Nile Red efflux in  
596 m<sup>1</sup>G37<sup>-</sup> vs. m<sup>1</sup>G37<sup>+</sup> conditions. Cells pre-loaded with Nile Red were de-energized with  
597 CCCP for 100 s, followed by addition of 50 mM glucose (Glc) to activate efflux, and the  
598 time course of Nile Red efflux was monitored for 200 s in cells grown in Ara+/-  
599 conditions. The time required to efflux 50% pre-loaded Nile Red ( $t_{\text{efflux } 50\%}$ ) was longer for  
600 m<sup>1</sup>G37<sup>-</sup> relative to m<sup>1</sup>G37<sup>+</sup> cells (E). The lack of efflux in m<sup>1</sup>G37<sup>+</sup> cells in the presence  
601 of CCCP were negative controls. Data and error bars are mean ± SD, n > 3.

602 F) Membrane potential was reduced in m<sup>1</sup>G37<sup>-</sup> vs. m<sup>1</sup>G37<sup>+</sup> cells as measured by ThT  
603 fluorescence. *E. coli* and *Salmonella trmD-KD* cells were inoculated in LB from a 1:100  
604 dilution of an overnight culture without or with 0.2% Ara and grown for 4 h at 37 °C,  
605 followed by dilution in LB in Ara+/- conditions to OD<sub>600</sub> of 0.1 and grown for 3 h at 37 °C.  
606 ThT fluorescence was normalized by OD<sub>600</sub>. Data and error bars are mean ± SD, n > 3.

607 G) The population-averaged length of the cell envelope during 100-mM oscillatory osmotic  
608 shocks was shorter in Ara<sup>-</sup> (red) than Ara<sup>+</sup> (blue) *E. coli* cells. Data are mean ± SD, n =  
609 3. Inset: Phase-contrast microscopy showed that *E. coli trmD-KD* cells were smaller in  
610 m<sup>1</sup>G37<sup>-</sup> (red) relative to m<sup>1</sup>G37<sup>+</sup> (blue) conditions. Scale bars: 2 μm.

611 H) The fractional extension of the cell envelope was larger in m<sup>1</sup>G37<sup>-</sup> relative to m<sup>1</sup>G37<sup>+</sup>  
612 cells. The extension was calculated as  $(l - l_{\text{av}})/l$ , where  $l$  is the effective population-  
613 averaged envelope length and  $l_{\text{av}}$  is the time-averaged value of  $l$  using the period of the  
614 oscillatory cycles as an averaging window. Data are mean ± SD, n = 3.

615 I) The amplitude of length oscillations in (H) averaged over oscillatory cycles was larger in  
616 m<sup>1</sup>G37<sup>-</sup> relative to m<sup>1</sup>G37<sup>+</sup> cells, averaged over oscillatory cycles. Data and error bars  
617 are mean ± SD from n > 67 cells. \*\*\*:  $p < 0.0001$  by Student's  $t$ -test. In a replicate  
618 experiment, the ratio of the amplitude of length oscillations between m<sup>1</sup>G37<sup>+</sup> and

619 m<sup>1</sup>G37<sup>-</sup> cells measured after sufficient m<sup>1</sup>G37 depletion to reduce growth rate to < 0.2  
620 h<sup>-1</sup> was 1.43 (n > 609 cells).

621 See also Figures S4 and S5.

622

623

624 **Figure 4: m<sup>1</sup>G37 deficiency sensitizes *trmD-KD* cells to multiple antibiotic classes.**

625 A,B) m<sup>1</sup>G37<sup>-</sup> cells had at least 2-fold lower MICs than m<sup>1</sup>G37<sup>+</sup> cells. The fold-decrease in  
626 MIC of each antibiotic was calculated for *E. coli* (A) and *Salmonella* (B) *trmD-KD* cells as  
627 the ratio of the MIC in m<sup>1</sup>G37<sup>+</sup> and m<sup>1</sup>G37<sup>-</sup> cells (red) and was compared with the  
628 relative decrease of m<sup>1</sup>G37<sup>+</sup> cells upon treatment with polymyxin B (PMB) at 0.25X MIC  
629 (blue). Overnight cultures were inoculated into fresh LB at 10<sup>6</sup> CFUs/mL and incubated  
630 with an antibiotic in serial dilutions. After 18 h of incubation at 37 °C, cell densities lower  
631 than OD<sub>600</sub> = 0.15 were scored as no growth. Fold-changes are taken from Figure S6A,  
632 where data and errors are mean ± SD, n > 4. Amp, ampicillin; Cbc, carbenicillin; Rif,  
633 rifampicin; Kan, kanamycin; Gen, gentamicin; Par, paromomycin; Cip, ciprofloxacin; Van,  
634 vancomycin.

635 C,D) Time-kill analyses of *E. coli* (C) and *Salmonella* (D) *trmD-KD* cells indicate that  
636 m<sup>1</sup>G37<sup>+</sup> cells (blue) recovered from antibiotic exposure, but that m<sup>1</sup>G37<sup>-</sup> (red) cells did  
637 not. Overnight cultures (10<sup>6</sup> CFUs/mL) were inoculated into fresh LB with an antibiotic at  
638 the indicated concentration and grown at 37 °C. Data and error bars show mean ± SD, n  
639 > 3.

640 E,F) Percent survival of m<sup>1</sup>G37<sup>-</sup> *E. coli trmD-KD* cells upon exposure to 25 µg/mL  
641 carbenicillin (E) or 256 µg/mL vancomycin (F), showing a decrease in survival  
642 comparable to *proS-KD* cells but faster and to a greater extent compared with *cysS-KD*  
643 cells. Data and error bars show mean ± SD, n > 3.

644 See also Figure S6.

645

646 **Figure 5: m<sup>1</sup>G37 deficiency decreases resistance and persistence to antibiotic treatment.**

647 A-C) Resistance arises less frequently in m<sup>1</sup>G37<sup>-</sup> (red) *E. coli* (A) and *Salmonella* (B) *trmD*-  
648 *KD* cells than in m<sup>1</sup>G37<sup>+</sup> (blue) cells. An overnight culture of cells at 10<sup>5</sup> CFUs was  
649 plated onto an LB agar plate containing the indicated concentration of gentamicin (Gen),  
650 kanamycin (Kan), ampicillin (Amp), or vancomycin (Van). Each concentration was near  
651 1X MIC for m<sup>1</sup>G37<sup>+</sup> cells. Resistant colonies were counted after incubation at 37 °C for 3  
652 days. Mutants were verified to have an increase in MIC to the respective antibiotic (C).  
653 Data and error bars are mean ± SD, n = 3. Welch's *t*-test: \**p* < 0.1, \*\**p* < 0.05, \*\*\**p* <  
654 0.01.

655 D-F) Persistence of *Salmonella trmD-KD* cells, showing CFUs/mL over time (left) and the  
656 average CFUs/mL at 6 h post-treatment in m<sup>1</sup>G37<sup>+</sup> and m<sup>1</sup>G37<sup>-</sup> conditions (right).  
657 Untreated *Salmonella trmD-KD* cells had similar CFUs/mL in the two conditions (D),  
658 while persistence arose more frequently in m<sup>1</sup>G37<sup>+</sup> than m<sup>1</sup>G37<sup>-</sup> cells treated with 20  
659 µg/mL Gen (3.7X and 8.5X MIC for m<sup>1</sup>G37<sup>+</sup> and m<sup>1</sup>G37<sup>-</sup> conditions) (E) and with 100  
660 µg/mL paromomycin (Par; 2.7X and 10.7X MIC for m<sup>1</sup>G37<sup>+</sup> and m<sup>1</sup>G37<sup>-</sup> conditions) (F).  
661 An overnight culture in LB with 0.2% Ara was diluted 1:100 into fresh LB with or without  
662 0.2% Ara and incubated at 37 °C for 3 h. Cells were treated with water (no drug), Gen or  
663 Par for 0, 1, 2, 4, and 6 h, collected, washed, and plated on LB with Ara. Horizontal lines  
664 on the right represent the median, n = 5. Mann-Whitney U test: \*\**p* < 0.05, \*\*\**p* < 0.01.

665

666 **Figure 6: m<sup>1</sup>G37-tRNA is required for translation of CC[C/U] codons.**

667 A) Western blot analysis showed that m<sup>1</sup>G37<sup>-</sup> (red) *E. coli trmD-KD* cells had lower *lolB*  
668 expression relative to *cysS* from the native gene than m<sup>1</sup>G37<sup>+</sup> (blue) cells, but higher  
669 expression from the codon-engineered gene. Data and error bars are mean ± SD, n = 6.  
670 Welch's *t*-test: \*\**p* < 0.05.

- 671 B) *m*<sup>1</sup>G37– *Salmonella trmD-KD* cells survived better in novobiocin treatment when  
672 expressing the engineered CCG codon at the 6<sup>th</sup> position of *toIC* than when expressing  
673 the natural CCC codon. Cells were grown in the presence of 12.5 µg/mL novobiocin for  
674 24 h and the fold-change in CFUs relative to *t* = 0 was compared. One sample *t*-test: \**p*  
675 < 0.1, *n* = 5.
- 676 C) *E. coli* cells expressing C37-tRNA<sup>Pro/UGG</sup> (red) grew poorly compared to cells expressing  
677 the G37 version (blue). Data and error bars are mean ± SEM, *n* = 3.
- 678 D) Cells expressing C37-tRNA<sup>Pro/UGG</sup> (red) accumulated more Hoechst 33342 dye than cells  
679 expressing G37-tRNA<sup>Pro/UGG</sup> (blue). Data and error bars are mean ± SEM, *n* = 3.
- 680 E) Cells expressing C37-tRNA<sup>Pro/UGG</sup> (red) showed lower MICs than cells expressing G37-  
681 tRNA<sup>Pro/UGG</sup> (blue). Data and error bars are mean ± SEM, *n* = 3. Welch's *t*-test: \*\**p* <  
682 0.05, \*\*\**p* < 0.01.
- 683 F) Cells expressing C37-tRNA<sup>Pro/UGG</sup> (red) died faster than cells expressing G37-tRNA<sup>Pro/UGG</sup>  
684 (blue) after exposure to gentamicin or vancomycin. Data and error bars are mean ±  
685 SEM, *n* = 3.

686

687 **Figure 7: *m*<sup>1</sup>G37-dependent regulation of bacterial multi-drug resistance.**

- 688 A) Gram-negative membrane-associated genes are enriched with CC[C/U] codons, which  
689 depend on TrmD synthesis of *m*<sup>1</sup>G37-tRNA for translation. In the *m*<sup>1</sup>G37+ condition  
690 (top), translation of CC[C/U] is active to establish a robust envelope barrier and efflux  
691 activity that confers multi-drug resistance. In the *m*<sup>1</sup>G37-deficient condition (*m*<sup>1</sup>G37–,  
692 bottom), translation of CC[C/U] is impaired, decreasing the barrier and efflux activity,  
693 permitting intracellular accumulation of multiple drugs, accelerating bactericidal action,  
694 and inhibiting the development of resistance and persistence.
- 695 B) The *toIC* gene is conserved with the CC[C/U] codon at the 6<sup>th</sup> position among many  
696 Gram-negative γ-proteobacterial pathogens.

697

See also Figure S7.

698



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868

869

870 **Key Resources Table**

871

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Rabbit polyclonal anti-LolB antibodies	(Matsuyama et al., 1997)	N/A
Rabbit polyclonal anti-CysRS antibodies	This paper	N/A
Rabbit polyclonal anti-TrmD antibodies	(Li and Bjork, 1999)	N/A
Rabbit polyclonal anti-hTrm5 antibodies	Sigma-Aldrich	Cat.#SAB2102581; QC18187
Rabbit polyclonal anti-OmpA antibodies	(Tani et al., 1990)	N/A
Goat polyclonal anti-rabbit IgG antibodies peroxidase conjugate	Sigma-Aldrich	Cat. #A0545
<b>Bacterial and Virus Strains</b>		
<i>Escherichia coli</i> strain K-12 substrain MG1655	ATCC	700926
<i>E. coli</i> strain BW25113	The Coli Genetic Stock Center (CGSC)	CGSC#: 7636
<i>E. coli</i> $\Delta$ <i>acrB</i>	CGSC	JW0451-2
<i>E. coli</i> $\Delta$ <i>efp</i>	CGSC	JW4107-1
<i>E. coli</i> $\Delta$ <i>tolC</i>	CGSC	JW5503-1
<i>Salmonella enterica</i> serovar Typhimurium strain LT2	ATCC	700720
Bacteriophage P1vir	Goulian lab collection	N/A
Bacteriophage P22	ATCC	97540
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
L-(+)-arabinose	Acros Organics	Cat. #365181000
D-(+)-glucose	MG Scientific	Cat. #MAL4912
EcoRI	New England BioLabs	Cat. #R0101S



PstI	New England BioLabs	Cat. #R0140S
PfuUltraII fusion HS DNA polymerase	Agilent Technologies	Cat. #600670
Nuclease P1	Sigma-Aldrich	Cat. #N8630
Alkaline phosphatase	Sigma-Aldrich	Cat. #P5931
1-methylguanosine (QQQ standard)	Boc Sciences	Cat. #2140-65-0
Guanosine (QQQ standard)	Sigma-Aldrich	Cat. #G6752
[ $\gamma$ - <sup>32</sup> P]-ATP	PerkinElmer	Cat. #NEG002A
T4 polynucleotide kinase	New England Biolabs	Cat. #M0201
AlamarBlue Dye	Invitrogen	Cat. #DAL1025
Hoechst 33342 (H33342)	Sigma-Aldrich	Cat. #B2261
Carbonyl cyanide <i>m</i> -chlorophenyl hydrazine (CCCP)	Sigma-Aldrich	Cat. #C2759
Ethidium bromide	Sigma-Aldrich	Cat. #E7637
Nile Red	Acros Organics	Cat. #415711000
Thioflavin T	Sigma-Aldrich	Cat. #T3516
Sorbitol	Sigma-Aldrich	Cat. #S1876
Ampicillin	Fisher Scientific	Cat. #BP1760
Carbenicillin	Fisher Scientific	Cat. #BP2648
Chloramphenicol	Gold Biotechnology	Cat. #G-105
Ciprofloxacin	Sigma-Aldrich	Cat. #17850
Gentamicin	Gold Biotechnology	Cat. #G-400
Kanamycin	Gemini Bio-products	Cat. #400-114P
Novobiocin	Sigma-Aldrich	Cat. #N1628
Paromomycin	Sigma-Aldrich	Cat. #P5057
Polymyxin B	Sigma-Aldrich	Cat. #P4932
Rifampicin	Sigma-Aldrich	Cat. #R3501
Vancomycin	Sigma-Aldrich	Cat. #SBR00001
Critical Commercial Assays		

SuperSignal West Pico Chemiluminescent Substrate	Thermo Fisher Scientific	Cat. #34080
Experimental Models: Organisms/Strains		
<i>E. coli</i> BL21(DE3) <i>trmD-KD</i>	(Gamper et al., 2015a)	N/A
<i>E. coli</i> MG1655 <i>trmD-KD</i>	This paper	N/A
<i>Salmonella enterica</i> serovar Typhimurium LT2 <i>trmD-KD</i>	This paper	N/A
<i>E. coli</i> MG1655 <i>cysS-KD</i>	This paper	N/A
<i>E. coli</i> MG1655 <i>proS-KD</i>	This paper	N/A
<i>E. coli proM-KD</i> C37-UGG tRNA	This paper	N/A
<i>E. coli trmD-KD</i> codon-engineered <i>lolB</i>	This paper	N/A
<i>Salmonella trmD-KD</i> codon-engineered <i>tolC</i>	This paper	N/A
Oligonucleotides		
Oligo DNA primers for strain construction, plasmid construction, codon engineering, primer extension and affinity tRNA purification	Table S1	N/A
Recombinant DNA		
pKD4	CGSC	CGSC #7632
pKD46	CGSC	CGSC #7634
PCP20	CGSC	CGSC #7629
pZS2R	(Kelsic et al., 2015)	N/A
pACYC- <i>araC</i> -P <sub>C</sub> -P <sub>BAD</sub> -human <i>trm5</i>	(Gamper et al., 2015a)	N/A
pACYC- <i>araC</i> -P <sub>C</sub> -P <sub>BAD</sub> - <i>Ec cysS-His-deg</i>	This paper	N/A
pACYC- <i>araC</i> -P <sub>C</sub> -P <sub>BAD</sub> - <i>Ec proS-His-deg</i>	This paper	N/A
pKK223-3 <i>E. coli</i> G37-UGG tRNA	This paper	N/A
pKK223-3 <i>E. coli</i> C37-UGG tRNA	This paper	N/A
pZS2R-P <sub>lolB</sub> -YFP	This paper	N/A
Software and Algorithms		

MaxQuant v. 1.5.3.30	(Tyanova et al., 2016)	<a href="http://www.biochem.mpg.de/5111795/maxquant">http://www.biochem.mpg.de/5111795/maxquant</a>
tRNA MS analysis software v.B.07.00	MassHunter Workstation, qualitative analysis	N/A
Image Lab v. 6.0	BIO-RAD	<a href="http://www.bio-rad.com/en-us/product/image-lab-software">http://www.bio-rad.com/en-us/product/image-lab-software</a>
ImageJ v. 1.51	NIH	<a href="https://imagej.nih.gov">https://imagej.nih.gov</a>
Felix32	Photon Technology International	N/A
µManager v. 1.4	(Edelstein et al., 2014)	N/A
MATLAB 2016b	MathWorks	<a href="https://www.mathworks.com/products/matlab.html">https://www.mathworks.com/products/matlab.html</a>
Other		
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel	Cat. #740609
Gibson Assembly Master Mix	New England BioLabs	Cat. #E2611L
MicroPulser Electroporator	BIO-RAD	Cat. #1652100
NucleoBond AX 2000	Macherey-Nagel	Cat. #740525
Impact II Qtof Mass Spectrometer	Bruker Daltonics	N/A
0.22-µm filter	Millipore	SLGV004SL
6410 QQQ triple-quadrupole LC mass spectrometer	Agilent	N/A
Immobilon-P PVDF Membrane	Millipore	IPVH00010
Branson 1210 Ultrasonic Cleaner	Branson	N/A

Chemi-Doc XRS+ System	BIO-RAD	Cat. #1708265
Typhoon IP Imaging system	GE Healthcare	N/A
Infinite M200 PRO plate reader	Tecan	N/A
Black opaque 96-well microplate	Greiner Bio-One	Cat. #655077
Transparent sealing film	Excel Scientific	Cat. #STR-SEAL-PLT
Synergy H1 Hybrid Multi-Mode Reader	BioTek	N/A
Quartz cuvette	Starna Cells	Cat. #3-Q-10
QuantaMaster 220 spectrofluorometer	Photon Technology International	N/A
Microfluidic perfusion plates	CellASIC	Cat. #B04a
ONIX microfluidic platform	CellASIC	N/A
Nikon Eclipse Ti-E inverted fluorescence microscope	Nikon	N/A
DU885 electron multiplying charged coupled device camera	Andor	N/A
Active-control environmental chamber	Haison Technology	N/A

872

873

874 **Methods Details**

875

876 **Strain constructions**

877 The *Escherichia coli* MG1655 *trmD-KD* (*E. coli trmD-KD*) strain was made via P1 transduction of  
878 *E. coli* K-12 MG1655, using phage lysate prepared from an *E. coli* BL21(DE3) *trmD-KD* strain  
879 (Gamper et al., 2015a, b). The *Salmonella enterica* serovar Typhimurium LT2 *trmD-KD*  
880 (*Salmonella trmD-KD*) strain was made using the  $\lambda$ -Red recombinase system (Datsenko and  
881 Wanner, 2000). A kanamycin resistance marker (*kan<sup>R</sup>*) was amplified from pKD4 using primers  
882 in Table S1 and purified using a PCR clean-up kit (Macherey-nagel). *Salmonella* LT2 cells were  
883 transformed with the  $\lambda$ -Red recombinase plasmid pKD46 and also with a pACYC-*araC*-P<sub>C</sub>-P<sub>BAD</sub>-  
884 human *trm5* that encodes human *trm5* under the arabinose (Ara)-controlled P<sub>BAD</sub> promoter and  
885 the repressor *araC* under the P<sub>C</sub> promoter. *Salmonella* cells were grown with expression of  $\lambda$ -  
886 Red recombinase, harvested in mid-log phase, and made electro-competent after two washes  
887 with cold 10% glycerol. Cells were electroporated with the indicated PCR-amplified and purified  
888 fragment using MicroPulser Electroporator (BIO-RAD), and cells exhibiting *kan<sup>R</sup>* were analyzed  
889 for marker insertion to the chromosomal *trmD* locus by PCR using primers in Table S1. Insertion  
890 was confirmed via sequencing (data not shown). After overnight growth at 43 °C to remove  
891 pKD46, cells were transformed with the FLP-recombinase plasmid pCP20 at 30 °C and removal  
892 of the *kan<sup>R</sup>* marker and the remaining ~100 bp scar sequence was confirmed via PCR and  
893 subsequent sequencing (data not shown). Finally, pCP20 was cured from cells by incubating  
894 them at 43 °C overnight. After confirmation of the *trmD-KD* genotypes via PCR using primers at  
895 flanking regions of the *trmD* locus (Figure S2A), cells were grown in Luria broth (LB)  
896 supplemented with 0.2% Ara overnight at 37 °C. Cells were inoculated at a 1:100 dilution into  
897 fresh LB without Ara but with 0.2% D-glucose (Glc) and grown for 3 h at 37 °C to deplete pre-  
898 existing Trm5 and methylated tRNAs. A 10-fold serial dilution of cells was spotted onto LB

899 plates with 0.2% Ara or 0.2% Glc for m<sup>1</sup>G37<sup>+</sup> and m<sup>1</sup>G37<sup>-</sup> conditions, respectively, and growth  
900 was examined after overnight incubation at 37 °C.

901  
902 To create *E. coli cysS-KD* and *proS-KD* strains, we first created the maintenance plasmids that  
903 expressed *E. coli cysS* and *proS* respectively with a C-terminal degron tag for rapid depletion.  
904 The ORFs were each amplified from extracted genomic DNA of *E. coli* MG1655 using primers in  
905 Table S1. These PCR products encoded a C-terminal 6x His tag followed by a GGS linker and a  
906 degron tag YALAA. The plasmid backbone sequence was amplified from the pACYC-*araC*-P<sub>C</sub>-  
907 P<sub>BAD</sub> maintenance plasmid that already encoded a GGS linker and a degron tag using primers in  
908 Table S1. Each PCR product was ligated to the linearized plasmid using a Gibson cloning kit  
909 (New England Biolabs) and the correct clone was confirmed by sequencing analysis. *E. coli*  
910 MG1655 harboring pKD46 λ-Red recombinase plasmid and a *cysS* or *proS* maintenance  
911 plasmid was prepared as electrocompetent cells. A kanamycin marker targeting the  
912 chromosomal *cysS* or *proS* was amplified from pKD4 using primers in Table S1 and  
913 electroporated into competent cells of MG1655 for recombination and gene deletion. Cells were  
914 screened for kanamycin resistance and the chromosomal locus was confirmed by PCR using  
915 primers in Table S1. The kanamycin marker was then removed by FLP recombination using  
916 pCP20 and removal was confirmed by sequencing.

917  
918 The *E. coli* MG1655 strain expressing the variant C37-tRNA<sup>Pro/UGG</sup> was constructed using the λ-  
919 Red system to remove the native tRNA gene (*proM*) from the chromosome. A *kan<sup>R</sup>* marker was  
920 amplified via PCR with homologous extensions to the flanking regions of the *proM* locus using  
921 primers in Table S1. *E. coli* MG1655 was transformed with pKD46 and the maintenance plasmid  
922 pKK223-3 *E. coli* G37-UGG tRNA expressing *E. coli proM* at the EcoRI and PstI sites. Cells  
923 were electroporated with the PCR-amplified *kan<sup>R</sup>* to introduce the marker into the chromosomal  
924 *proM* locus. Non-G37 (namely, A37, C37, and U37) variants of *E. coli* tRNA<sup>Pro/UGG</sup> were created

925 using Quikchange mutagenesis (Agilent) of pKK223-3 *E. coli* G37-UGG tRNA. Transformation  
926 of *E. coli* MG1655 with these variants, followed by P1 transduction of the *proM-KD* locus,  
927 recovered only the C37 variant. For cells expressing the G37 or C37 version of the UGG tRNA  
928 from the maintenance plasmid, the GGG tRNA gene (*proL*), which reads CCC and CCU codons  
929 but is not essential for growth, was removed via  $\lambda$ -Red recombination in *E. coli* MG1655,  
930 followed by P1 transduction into the respective strain (see Table S1 for primers). After selection  
931 for *kan<sup>R</sup>*, the marker was removed with FLP recombinase from pCP20 and purified. Each  
932 purified G37 and C37 clone was grown overnight and inoculated into fresh LB to OD<sub>600</sub> = 0.05  
933 with 100  $\mu$ g/mL ampicillin and growth in a 40-mL culture was monitored by OD<sub>600</sub> for 13 h at  
934 37 °C.

935

#### 936 **MS analysis of membrane proteomes**

937 An overnight *E. coli trmD-KD* culture was inoculated at a 1:100 dilution into fresh LB with or  
938 without 0.2% Ara and grown for 5 h at 37 °C. Cells were then diluted to OD<sub>600</sub> = 0.1 in fresh LB  
939 with or without 0.2% Ara and grown for another 2 h. Cells were harvested and a membrane  
940 fraction was prepared by method 4 in (Thein et al., 2010). Extracted membrane proteins (30-40  
941  $\mu$ g) were boiled in 4% SDS in 100 mM Tris pH 6.8, separated into three technical replicates, and  
942 run on a 10% SDS-PAGE gel. Proteins were visualized, digested with trypsin, and analyzed on  
943 an Impact II QTOF mass spectrometer (Bruker Daltonics) (Gibbs et al., 2017). Mass  
944 spectrometry data were analyzed with MaxQuant v. 1.5.3.30 (Tyanova et al., 2016) against the  
945 UNIPROT *Escherichia coli* K12 protein sequence database (downloaded on May 12, 2015;  
946 4,481 entries) plus common contaminants (245 entries) with variable modifications of  
947 methionine oxidation, N-acetylation of proteins, and fixed modification of cysteine  
948 carbamidomethylation. The false discovery rate was set to 1% for both proteins and peptides.  
949 Technical replicates of the two treatments were searched together using MaxQuant's "match  
950 between run" and label-free quantification options.

951

952 **Western blotting**

953 *E. coli trmD-KD* and *Salmonella trmD-KD* cells were grown in LB supplemented with 0.2% Ara  
954 overnight at 37 °C. Cells were inoculated at a 1:100 dilution into fresh LB with 0.2% Ara or 0.2%  
955 Glc and grown at 37 °C. To monitor the depletion of Trm5 (the maintenance protein), cells were  
956 sampled over 3 h and whole-cell lysates were prepared via repeated heating at 95 °C and  
957 vortexing. Cell lysates containing 15-20 µg proteins were separated via 12% sodium dodecyl  
958 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to an Immobilon-P  
959 PVDF membrane (Millipore). The membrane was incubated with primary rabbit antibodies  
960 against human Trm5 (Sigma-Aldrich) at a 1:1,000 dilution or against *E. coli* CysRS at a 1:10,000  
961 dilution and secondary goat antibody against rabbit IgG (Sigma-Aldrich), followed by incubation  
962 with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and  
963 imaging with Chemi-Doc XRS+ (BIO-RAD). The absence of chromosomally expressed TrmD  
964 was confirmed by Western blotting using rabbit antibodies against *E. coli* TrmD (a gift from Dr.  
965 Glenn Bjork). For LolB and OmpA quantification, after inoculation into fresh LB, cells were  
966 grown for 4 h at 37 °C, diluted to OD<sub>600</sub> = 0.1 into fresh LB with or without 0.2% Ara, and grown  
967 for another 3 h. Cells were harvested, precipitated with 10% (w/v) trichloroacetic acid (TCA),  
968 washed with ice-cold acetone, and sonicated using a Bransonic 1210 Ultrasonic Cleaner  
969 (Branson) until the pellet was dissolved. Proteins were pelleted by centrifuge at 16,000g for 10  
970 min at 4 °C, dried and resuspended in water, then boiled in 1x SDS buffer at 95 °C for 5 min;  
971 total protein content was analyzed via 12% SDS-PAGE. Rabbit polyclonal antibodies against  
972 LolB and OmpA were kind gifts from Dr. Hajime Tokuda (Morioka University). Levels of LolB  
973 and OmpA relative to CysRS and total membrane proteins, respectively, were quantified using  
974 Image Lab v. 6.0 (BIO-RAD).

975

976 **Primer-extension analysis of m<sup>1</sup>G37**



977 *E. coli trmD-KD* and *Salmonella trmD-KD* cells were grown in LB overnight as for Western  
978 blotting. Cells were diluted 1:100 into fresh LB with or without 0.2% Ara and grown for 4 h at  
979 37 °C. Cells were then diluted to OD<sub>600</sub> = 0.1 in fresh LB with or without 0.2% Ara and grown for  
980 another 3 h. To analyze the initial pre-depletion of methylated tRNA (Figure S3A), the overnight  
981 culture was diluted 1:100 into fresh LB without Ara and cells were collected every hour up to  $t =$   
982 3 h. Cells were harvested via centrifugation at 4,000g for 10 min at 4 °C and pellets were stored  
983 at -20 °C until use. Total small RNA was extracted from cell pellets as described previously  
984 (Frenkel-Morgenstern et al., 2012). Briefly, cell pellets were suspended in buffer A (1 mM Tris-  
985 HCl [pH 7.5] and 10 mM MgCl<sub>2</sub>), mixed with an equal volume of water-saturated phenol, and  
986 vortexed three times each for 45 s. After centrifugation at 12,000g for 5 min, the aqueous phase  
987 was collected and the phenol phase was extracted three times with an equal volume of buffer A.  
988 Total small RNAs in the pooled aqueous phase were pelleted via ethanol precipitation and  
989 centrifugation. RNA pellets were dissolved in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM  
990 ethylenediaminetetraacetic acid) and stored at -20 °C. The level of m<sup>1</sup>G37 in tRNA<sup>Pro/UGG</sup> was  
991 quantified via primer extension on 2 µg of total small RNA with Superscript III reverse  
992 transcriptase (Invitrogen) at 50 °C for 40 min as described previously (Christian et al., 2004).  
993 The primer (Table S1) was designed to hybridize to the tRNA to enable a 2-nucleotide extension  
994 to m<sup>1</sup>G37 and was labeled at the 5'-end with [ $\gamma$ -<sup>32</sup>P]-ATP (PerkinElmer) using T4 polynucleotide  
995 kinase (New England Biolabs). The same primer was used for analysis of tRNA<sup>Pro/UGG</sup> in *E. coli*  
996 and *Salmonella*, which share an identical sequence. Primer extension was stopped via heating  
997 at 65 °C for 5 min and separation was achieved on 12% polyacrylamide/7 M urea gels. Gels  
998 were imaged via phosphorimaging using a Typhoon IP Imaging system (GE Healthcare) and  
999 analyzed with ImageJ v. 1.51 (NIH). A similar analysis was performed for tRNA<sup>Pro/GGG</sup> (Table S1,  
1000 Figure S3B, S3C). The amount of m<sup>1</sup>G37 was calculated as the percentage by the band  
1001 intensity of the primer stop at position 37 over the sum of stops and read-through to nucleotide 1.  
1002

1003 **LC-MS/MS analysis of m<sup>1</sup>G37**

1004 *E. coli trmD-KD* and *Salmonella trmD-KD* cells were grown in Ara<sup>+</sup> and Ara<sup>-</sup> conditions and  
1005 total small RNA was prepared as in primer-extension analysis. The tRNA fraction was enriched  
1006 using NucleoBond AX 2000 (Macherey-nagel). Briefly, the column was first equilibrated with a  
1007 buffer containing 100 mM Tris-acetate [pH 6.3], 15% EtOH, and 200 mM KCl. The RNA sample  
1008 was loaded and washed with a buffer containing 100 mM Tris-acetate [pH 6.3], 15% EtOH, and  
1009 400 mM KCl. The enriched tRNA fraction was then eluted from the column with a buffer  
1010 containing 100 mM Tris-acetate [pH 6.3], 15% EtOH, and 750 mM KCl, and tRNA<sup>Pro/UGG</sup>  
1011 isoacceptor was affinity-purified (Masuda et al., 2018) and the salt adducts were removed by  
1012 repeated ethanol-precipitation in the presence of a high concentration of NH<sub>4</sub>OAc.  
1013 Approximately 200-300 ng tRNA was digested with nuclease P1 (1 U, Sigma-Aldrich) in 20 µL  
1014 reaction buffer containing 10 mM of NH<sub>4</sub>OAc [pH 5.3] at 42 °C for 2 h. With the addition of 2.5  
1015 µL NH<sub>4</sub>HCO<sub>3</sub> (1M, freshly prepared in water), 1 U of alkaline phosphatase (Sigma-Aldrich) was  
1016 added and the sample was incubated at 37 °C for 2 h. After the incubation, the sample was  
1017 diluted with an additional 40 µL water and filtered with 0.22-µm filters (4 mm diameter, Millipore)  
1018 and 8 µL of the entire solution was injected into an LC-MS/MS. Nucleosides were separated by  
1019 reverse phase ultra-performance liquid chromatography on a C1 column with on-line mass  
1020 spectrometry detection by an Agilent 6410 QQQ triple-quadruple LC mass spectrometer in  
1021 positive electrospray ionization mode. The nucleosides were quantified with retention time and  
1022 the nucleoside-to-base ion mass transition of 284-152 (G), 268-136 (A), and 298.1-166.1 (m<sup>1</sup>G).  
1023 Quantification was performed in comparison with a standard curve, obtained from pure  
1024 nucleoside standards running with the same batch of samples. The m<sup>1</sup>G level was calculated as  
1025 the ratio of m<sup>1</sup>G to G based on calibrated concentration curves.

1026

1027 **Quantification of *lolB* expression with a YFP reporter**

1028 The native promoter of *lolB* was PCR-amplified from *E. coli* MG1655 genomic DNA and inserted  
1029 into the pZS2R plasmid, a vector of 4.3 kb in length that carries a Kan<sup>R</sup> marker, is amplified from  
1030 the pSC101 replication origin, and contains YFP under the control of the strong and constitutive  
1031  $\lambda$  phage promoter R (a kind gift from Dr. Roy Kishony) (Kelsic et al., 2015)). The insertion  
1032 replaced the original promoter with the  $P_{lolB}$  promoter to generate pZS2R- $P_{lolB}$ -YFP for  
1033 transcriptional analysis of YFP. *E. coli trmD-KD* cells harboring this plasmid were grown in LB,  
1034 diluted 1:100 into fresh LB, and grown for 4 h at 37 °C with or without 0.2% Ara. Cells were  
1035 diluted to OD<sub>600</sub> = 0.1 in fresh LB with or without 0.2% Ara and grown for another 3 h. Cells were  
1036 then harvested by centrifugation at 7,000g for 1 min and suspended in M9, and the YFP  
1037 intensity from the suspension was measured in an Infinite M200 PRO (Tecan) plate reader at  
1038 excitation and emission wavelengths of 500 nm and 540 nm, respectively. After normalization  
1039 based on OD<sub>600</sub>, the signal intensity was calculated for the m<sup>1</sup>G37-deficient condition relative to  
1040 the m<sup>1</sup>G37+ condition (Figure S3F).

1041

#### 1042 **AlamarBlue accumulation assay**

1043 *E. coli trmD-KD* and *Salmonella trmD-KD* cells were grown in Ara+ and Ara- conditions as in  
1044 RNA analyses above. Cells were washed with and resuspended in 150  $\mu$ L of 20 mM potassium  
1045 phosphate buffer pH 7.0 containing 1 mM MgCl<sub>2</sub> (PPB) at 4 x 10<sup>8</sup> CFU/mL in a 96-well plate. At  
1046  $t = 0$ , a 1/10 volume of AlamarBlue (Invitrogen) in the stock concentration was added and  
1047 fluorescence signal at Ex565nm/Em590nm was monitored over 30 min as the uptake of  
1048 AlamarBlue. The signal was normalized by OD<sub>600</sub> and plotted over time. *E. coli cysS-KD* and *E.*  
1049 *coli proS-KD* cells were grown in the same way and used for the assay. As a control, an *E. coli*  
1050 hyper-permeable strain (Krishnamoorthy et al., 2016) (a gift from Dr. Helen Zgurskaya) was  
1051 used. This strain has a highly permeable outer membrane, due to a mutant form of the outer  
1052 membrane protein FhuA that is driven from an arabinose promoter. The hyper-permeable strain  
1053 was grown for 5 h in the presence or absence of arabinose and AlamarBlue uptake was

1054 monitored as described above. Another control was the use of polymyxin B (PMB), which  
1055 disrupts and permeabilizes the outer membrane by binding to lipids. *E. coli trmD-KD* and  
1056 *Salmonella trmD-KD* cells grown with Ara were used for the assay, and the AlamarBlue uptake  
1057 was monitored for 10 min without PMB, followed by an additional 30 min of incubation in the  
1058 presence of varying concentrations of PMB (1 to 20 µg/mL).

1059

### 1060 **Hoechst accumulation assay**

1061 Hoechst H33342 dye is an intercalating agent that fluoresces when bound to DNA (van den  
1062 Berg van Saparoea et al., 2005), and hence is commonly used for measuring outer-membrane  
1063 permeability. The fluorescence intensity of the dye accumulated in the cell serves as a proxy for  
1064 cellular uptake and efflux. Accumulation of H33342 was monitored in the presence of cyanide 3-  
1065 chlorophenylhydrazine (CCCP) to inhibit energy-dependent efflux. In accordance with a  
1066 protocol adapted from a previous assay (Murata et al., 2007), cells were grown to saturation  
1067 overnight with shaking at 37 °C in LB with chloramphenicol (34 µg/mL) and Ara (0.2% w/v),  
1068 diluted 1:100 into fresh LB with chloramphenicol in the presence of Ara or Glc (0.2% w/v), and  
1069 grown for 3 h to OD<sub>600</sub>~0.8 at 37 °C until cells reached exponential phase. Cells were then  
1070 diluted 1:10 into fresh LB with chloramphenicol in the presence of the same carbon source (Ara  
1071 or Glc) as in the first round of culturing and grown for 5 h. Cells were harvested via  
1072 centrifugation (5,400 g for 5 min) at room temperature, washed with 1X phosphate-buffered  
1073 saline (PBS) [pH 7.4], suspended in PBS, and adjusted to OD<sub>600</sub>~0.6. Each cell suspension  
1074 (100 µL) was transferred to a well in a black opaque 96-well microplate (Greiner Bio-One) and  
1075 mixed with 100 µL of 3 µM H33342 dye (Sigma-Aldrich) in 1X PBS [pH 7.4] to a final  
1076 concentration of 1.5 µM. The plate was covered with a transparent sealing film (Excel Scientific)  
1077 and fluorescence intensity was monitored every minute for 30 min with shaking in a Synergy H1  
1078 Hybrid Multi-Mode Reader (Biotek) or Infinite M200 PRO (Tecan) plate reader at 37 °C.  
1079 Fluorescence was recorded with excitation and emission wavelengths of 355 and 460 nm,

1080 respectively. In experiments in which ATP-dependent efflux was abolished, freshly prepared  
1081 CCCP (Sigma-Aldrich) was added to a final concentration of 50  $\mu$ M (from a 50 mM stock  
1082 solution prepared in dimethyl sulfoxide (DMSO)) together with 1.5  $\mu$ M H33342. Each experiment  
1083 was repeated at least three times. For *E. coli* C37-tRNA<sup>Pro/UGG</sup> cells, a mid-log culture was used  
1084 for the assay.

1085

### 1086 **Nile Red efflux assay**

1087 The Nile Red efflux assay was modified from a previous protocol (Bohnert et al., 2010). Cells  
1088 were grown as for Hoechst assays for 5 h and then shifted to room temperature and prepared  
1089 as follows: each culture (6 mL) was centrifuged for 10 min at 3,829g and the pellet was  
1090 suspended in PPB. After another round of centrifugation and resuspension, cells were adjusted  
1091 to OD<sub>600</sub>~0.9-1.0 in PPB (potassium phosphate buffer: 20 mM potassium phosphate buffer pH  
1092 7.0 containing 1 mM MgCl<sub>2</sub>) and mixed with CCCP (1 mM stock solution in 50% DMSO) to a  
1093 final concentration of 5  $\mu$ M. After incubation at room temperature for ~20 min, cells were  
1094 transferred to 10 mL glass tubes. Nile Red (Acros Organics; 1 mM stock in anhydrous DMSO)  
1095 was added to a final concentration of 5  $\mu$ M, and the tubes were incubated at 37 °C and shaken  
1096 at 140 rpm for 3 h. Cells were shifted to room temperature for 1 h without shaking and then  
1097 centrifuged for 5 min at 3,829g. The supernatant was discarded, any droplets left clinging to the  
1098 tube walls were removed with Kimwipes, and cells were suspended in PPB at OD<sub>600</sub>~0.9-1.0.  
1099 Cell suspensions (0.2 mL) were quickly transferred to a quartz cuvette (Starna Cells Inc.)  
1100 containing 1.8 mL PPB. Fluorescence emission was recorded with a QuantaMaster 220  
1101 spectrofluorometer (Photon Technology International) using the PTI Felix32 software. Cell  
1102 suspensions were continuously stirred with a magnetic stirrer inside the cuvette. The slit width  
1103 was set to 10 nm and the excitation and emission wavelengths were set to 552 nm and 636 nm,  
1104 respectively. The fluorescence of each cell suspension was followed over 100 s, and Nile Red  
1105 efflux was triggered via rapid energization with the addition of 100  $\mu$ L of 1 M glucose.

1106 Fluorescence was monitored for another 200 s. Trials displaying no pre-energization efflux were  
1107 included in the analysis, whereas trials that showed substantial pre-energization efflux were  
1108 discarded. The time required for 50% Nile Red efflux ( $t_{\text{efflux } 50\%}$ ) was calculated for at least three  
1109 independent measurements per sample as described previously (Bohnert et al., 2010). A  $\Delta\text{acrB}$   
1110 strain from the Coli Genetic Stock Center (CGSC) at Yale University was tested as a control.

1111

### 1112 **Ethidium bromide efflux assay**

1113 *E. coli trmD-KD* and *Salmonella trmD-KD* cells were grown in Ara+ and Ara- conditions as in  
1114 RNA analyses above. Cells were adjusted to OD<sub>600</sub>~0.9-1.0 in PPB and incubated with 20  $\mu\text{M}$   
1115 CCCP and 10  $\mu\text{g/mL}$  ethidium bromide (EtBr) for 2 h at 30 °C. Cells were spun, washed and  
1116 resuspended in a fresh PPB at  $5 \times 10^8$  CFU/mL in a 96-well plate. The fluorescence signal of  
1117 EtBr at Ex530nm/Em600nm was monitored for the first 3 min, then efflux was activated by  
1118 addition of 50 mM Glc, and the signal was monitored for 30 min. The EtBr signal was  
1119 normalized by OD<sub>600</sub> and plotted over time. An *E. coli toIC-KO* strain was purchased from CGSC,  
1120 and after the kanamycin marker was removed by a pCP20 plasmid transformation, it was used  
1121 for the assay as a control. Efflux was also assayed for *E. coli trmD-KD* and *Salmonella trmD-KD*  
1122 cells with various concentrations of polymyxin B added to the cell resuspension 5 min prior to  
1123 Glc addition.

1124

### 1125 **Thioflavin T fluorescence assay**

1126 Overnight cultures of *E. coli* and *Salmonella trmD-KD* cells were inoculated in LB at 1:100  
1127 dilution without or with 0.2% Ara and grown for 4 h at 37 °C. Cells were diluted in LB in Ara+/-  
1128 conditions to OD<sub>600</sub> = 0.1 and grown for another 3 h at 37 °C. Cells were then washed with M9  
1129 medium and incubated in M9 containing 20  $\mu\text{M}$  Thioflavin T (ThT) for 2 h at 37 °C with Ara or  
1130 Glc for m<sup>1</sup>G37+ and m<sup>1</sup>G37-deficient conditions, respectively, and the ThT fluorescence was  
1131 measured at Ex446nm/Em482nm and normalized by OD<sub>600</sub> (Prindle et al., 2015).

1132

### 1133 **Imaging in microfluidic devices**

1134 Overnight *E. coli trmD-KD* cultures were grown in LB + 0.2% Ara and 30 ug/mL chloramphenicol.  
1135 These cultures were diluted 1:100 into 1 mL fresh LB with 0.2% Ara or 0.2% Glc (to deplete the  
1136 pre-existing Trm5 and m<sup>1</sup>G37 tRNA) and grown with shaking at 37 °C for 3.5 h. Cells were then  
1137 transferred to B04A microfluidic perfusion plates (CellASIC Corp.) that had been loaded with  
1138 medium and pre-warmed to 37 °C, and cells were incubated at 37 °C for >1 h before imaging.  
1139 The medium was exchanged using the ONIX microfluidic platform (CellASIC Corp.). The  
1140 osmolarity of the growth medium or phosphate-buffered saline (PBS) was modulated with  
1141 sorbitol (Sigma). For oscillatory osmotic shocks, cells were allowed to grow for 5 min in medium  
1142 in the imaging chamber before being subjected to 100-mM oscillatory osmotic shocks by  
1143 switching between LB and LB + 100 mM sorbitol.

1144

1145 Cells were imaged on a Nikon Eclipse Ti-E inverted fluorescence microscope with a 100X (NA  
1146 1.40) oil-immersion objective. Images were collected on a DU885 electron multiplying charged  
1147 couple device camera (Andor) using µManager v. 1.4 (Edelstein et al., 2014). Cells were  
1148 maintained at 37 °C during imaging with an active-control environmental chamber (HaisonTech).

1149

### 1150 **Cell tracking and analysis**

1151 To calculate the amplitude of length oscillations during oscillatory osmotic shocks, cells were  
1152 tracked over time using custom MATLAB algorithms, similar to previous studies (Rojas et al.,  
1153 2014). First, cell-wall lengths (*l*) were automatically identified. The effective population-averaged  
1154 length  $l_{\text{eff}}$  at time  $t_1$  was calculated by integrating the population-averaged elongation rate over  
1155 time (Rojas et al., 2014):

1156

$$l_{\text{eff}} = \int_{t_0}^{t_1} \dot{e} dt + l_0,$$

1157 where  $l_0$  is the mean initial cell length at time  $t_0$ , and  $\dot{e}$  is the instantaneous growth rate. The  
1158 effective population-averaged length was then smoothed with a mean filter with window size  
1159 equal to the period of oscillation, and subtracted from the unsmoothed trace to obtain the  
1160 deviation of the length oscillations around the smoothed trace. The peak-to-peak amplitude was  
1161 calculated for each cycle. The mean amplitude was calculated by averaging the peak-to-peak  
1162 amplitude over cycles. Uncertainty was estimated as the standard deviation of the mean  
1163 amplitude over cycles.

1164

### 1165 **Minimal inhibitory concentration (MIC) measurements**

1166 Overnight cultures of *trmD-KD* cells with 0.2% Ara were diluted 1:100 into LB without Ara and  
1167 grown at 37 °C for 1 and 3 h for *Salmonella* and *E. coli*, respectively, to pre-deplete Trm5 and  
1168 methylated tRNAs (Figure S3). This short pre-depletion was appropriate for MIC analysis,  
1169 because longer pre-depletion made cells in m<sup>1</sup>G37-deficient conditions too weak to distinguish  
1170 death by antibiotic killing from death by lack of m<sup>1</sup>G37 (data not shown). After pre-depletion,  
1171 cells for all but ciprofloxacin analysis were diluted to 10<sup>6</sup> CFU/mL and grown in 96-well plates  
1172 with 0.2% Ara or Glc in the presence of an antibiotic across a 2-fold serial dilution. After 18 h of  
1173 incubation at 37 °C, OD<sub>600</sub> was measured and the MIC was determined based on a threshold for  
1174 growth of OD<sub>600</sub> = 0.15 (Kim et al., 2010). For analysis of MIC of ciprofloxacin, 10<sup>5</sup> CFU/mL cells  
1175 were inoculated and grown for 24 h at 37 °C. The MIC of polymyxin B for *E. coli trmD-KD* and  
1176 *Salmonella trmD-KD* cells grown in the Ara+ condition was determined as 0.5 and 1.0 µg/mL,  
1177 respectively. To test the effect of polymyxin B on the permeability of each antibiotic, we used  
1178 polymyxin B at 0.25X MIC for *E. coli trmD-KD* and *Salmonella trmD-KD* cells. For *E. coli* C37-  
1179 tRNA<sup>Pro/UGG</sup> cells, overnight cultures were inoculated into fresh LB at 10<sup>6</sup> CFU/mL and the MICs  
1180 were determined for gentamicin and vancomycin. A  $\Delta efp$  strain was purchased from CGSC and  
1181 the kanamycin marker was removed by transformation of the pCP20 plasmid. The MICs were  
1182 determined for ampicillin, gentamicin and vancomycin.



1183

#### 1184 **Time-kill analyses**

1185 Cells depleted of Trm5 and m<sup>1</sup>G37-tRNA were prepared as for MIC analyses and were  
1186 inoculated into fresh LB at 10<sup>6</sup> CFU/mL with 0.2% Ara or 0.2% Glc in the presence of an  
1187 antibiotic. Several concentrations were tested for each drug, ranging from 0.6X to 6.4X MIC  
1188 (Figure S6D); the concentration that yielded the largest difference between m<sup>1</sup>G37+ and  
1189 m<sup>1</sup>G37-deficient conditions was selected (12.5 µg/mL carbenicillin, 3.125 µg/mL ampicillin, 6.25  
1190 µg/mL gentamicin, 12.5 µg/mL kanamycin, and 256 µg/mL vancomycin). In the presence of the  
1191 chosen concentration of each drug, cells were grown at 37 °C and sampled up to 18-24 h. At  
1192 each time point, 10-fold serial dilutions of cells were spotted onto LB plates with 0.2% Ara and  
1193 grown overnight. The number of viable colonies was counted and converted to CFU/mL. For  
1194 analysis of *E. coli cysS-KD* and *proS-KD* cells, cells depleted of the protein product of each  
1195 gene were prepared in a similar manner as for *trmD-KD* cells and the time-kill curve was  
1196 determined for 25 µg/mL carbenicillin and 256 µg/mL vancomycin in the absence of Ara. After  
1197 counting viable colonies from an LB plate, the survival rate was calculated relative to  $t = 0$ . The  
1198 same CFU counting method was used for a control experiment with 2 mM H<sub>2</sub>O<sub>2</sub> (Fig. S6); the  
1199 time course was followed up to 6 h. For analysis of *E. coli* C37-tRNA<sup>Pro/UGG</sup> cells, overnight  
1200 cultures were inoculated into fresh LB at 10<sup>6</sup> CFU/mL with 3.125 µg/mL gentamicin or 512  
1201 µg/mL vancomycin and analyzed as above.

1202

#### 1203 **Resistance analyses**

1204 Cells depleted of Trm5 and m<sup>1</sup>G37-tRNA were cultured as for MIC and time-kill assays. Cells  
1205 were diluted to 10<sup>5</sup> CFU based on the calibration that OD<sub>600</sub> = 1 corresponds to 8 x 10<sup>8</sup> CFU/mL,  
1206 and plated on LB with 0.2% Ara or 0.2% Glc in the presence of an antibiotic at a concentration  
1207 near 1X MIC of m<sup>1</sup>G37+ cells or at a concentration of 1X MIC for each specific type of cells:  
1208 gentamicin at 2.7 µg/mL (m<sup>1</sup>G37+) and 0.88 µg/mL (m<sup>1</sup>G37-deficient) for *E. coli* and 5.5 µg/mL

1209 (m<sup>1</sup>G37+) and 2.3 µg/mL (m<sup>1</sup>G37-deficient) for *Salmonella*; ampicillin at 9.4 µg/mL (m<sup>1</sup>G37+) and 4.7 µg/mL (m<sup>1</sup>G37-deficient) for *E. coli* and 2.4 µg/mL (m<sup>1</sup>G37+) and 0.78 µg/mL (m<sup>1</sup>G37-deficient) for *Salmonella*; vancomycin at 341 µg/mL (m<sup>1</sup>G37+) and 128 µg/mL (m<sup>1</sup>G37-deficient) for *E. coli* and 512 µg/mL (m<sup>1</sup>G37+) and 192 µg/mL (m<sup>1</sup>G37-deficient) for *Salmonella*. After incubation at 37 °C for 3 days, CFUs were counted. A representative gentamicin-resistant clone was purified and an increase in MIC was confirmed (Fig. 5C).

1215

### 1216 **Persistence analyses**

1217 *Salmonella trmD-KD* cells were grown in LB with 0.2% Ara overnight, diluted 1:100 into fresh LB with 0.2% Ara or 0.2% Glc, grown at 37 °C for 3 h, and challenged with a specific antibiotic for 6 h. At each time point, cells were washed three times with saline (0.9% NaCl) and 10-fold dilutions were spotted on LB plates with 0.2% Ara at 37 °C. CFUs were counted the next day.

1221

### 1222 **Codon engineering**

1223 Codon engineering of *E. coli lolB* on the chromosome was performed with the λ-Red recombinase system. The 5' end of *lolB* with the flanking sequence was amplified via PCR using primers with mutations to change the second and fourth codons in the MG1655 genome AUG-CCC-CUG-CCC-GAU to AUG-CCG-CUG-CCG-GAU. The PCR product was connected with the *kan<sup>R</sup>* sequence of pKD4 via a second PCR, followed by a third PCR to expand coverage to the entire *lolB* sequence for homologous recombination. The resultant PCR product was introduced into *E. coli* MG1655 cells expressing λ-Red recombinase from pKD46. After selection for the *kan<sup>R</sup>* marker and confirmation via sequencing (data not shown), the mutated locus was moved into *E. coli trmD-KD* with the maintenance plasmid expressing human *trm5*. The desired clone was selected with the *kan<sup>R</sup>* marker, which was subsequently removed via pCP20-mediated FLP recombination to leave a scar. The scar-carrying mutant with the engineered codon was purified from single colonies. An isogenic strain carrying the wild-type sequence was also created from

1235 *trmD-KD* cells with the scar sequence. LolB protein levels were determined through Western  
1236 blotting.

1237 Codon engineering of *Salmonella toIC* on the chromosome was accomplished with  $\lambda$ -Red  
1238 recombination. The 5' end of *toIC* with the flanking sequence was amplified by PCR with primers  
1239 containing a mutation to change the sixth codon from AUG-AAG-AAA-UUG-CUC-CCC-AUC to  
1240 AUG-AAG-AAA-UUG-CUC-CCG-AUC. After the second and third PCRs, recombination was  
1241 performed in *Salmonella* LT2 cells expressing  $\lambda$ -Red recombinase from pKD46. The mutation  
1242 was confirmed via sequencing (data not shown). A clone containing the mutation but without  
1243 phage contamination was isolated using a green plate (Chan et al., 1972) and the mutated locus  
1244 was transferred to *Salmonella trmD-KD* with the maintenance plasmid expressing human *trm5*.  
1245 The desired clone was selected with the *kan<sup>R</sup>* marker, which was removed via pCP20-mediated  
1246 FLP recombination to leave a scar. An isogenic strain carrying the wild-type sequence was  
1247 isolated from *trmD-KD* cells and grown in LB at 37 °C along with the mutant clone with 0.2% Ara.  
1248 Cells were inoculated into fresh Ara-free LB at 10<sup>6</sup> CFU/mL, supplemented with 12.5  $\mu$ g/mL  
1249 novobiocin, and grown without pre-depletion at 37 °C. After 24 h of growth, 10-fold dilutions  
1250 were spotted onto LB plates with 0.2% Ara for CFU analysis. The fold-increase of CFUs after 24  
1251 h at 37 °C relative to  $t = 0$  was calculated and normalized to growth of the wild-type clone.

1252

### 1253 **Quantification and statistical analyses**

1254 All experiments were repeated at least three times with biological replicates; mean or median  
1255 values are shown. Statistical significance was determined using an unpaired, two-tailed Welch's  
1256 *t* test, a one-sample Student's *t* test, a Wilcoxon rank-sum test, or Fisher's exact test. Statistics  
1257 were computed with R v. 3.1.3 (R Core Team, Vienna, Austria) or Microsoft Excel. Statistical  
1258 significance was defined as  $p < 0.05$ .