A bacterial regulatory uORF senses multiple classes of ribosome-targeting antibiotics

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ABSTRACT

Expression of many bacterial genes is regulated by cis- and trans-acting elements in their 5’ upstream regions (URs). Cis-acting regulatory elements in URs include upstream ORFs (uORF), short ORFs that sense translation stress that manifests as ribosomes stalling at specific codons within the uORF. Here, we show that the transcript encoding the *Escherichia coli* TopAI-YjhQ toxin-antitoxin system is regulated by a uORF that we name “toIL”. We propose that in the absence of translation stress, a secondary structure in the UR represses translation of the *topAI* transcript by occluding the ribosome-binding site. Translation repression of *topAI* leads to premature Rho-dependent transcription termination within the *topAI* ORF. At least five different classes of ribosome-targeting antibiotics relieve repression of *topAI*. Our data suggest that these antibiotics function by stalling ribosomes at different positions within toIL, thereby altering the RNA secondary structure around the *topAI* ribosome-binding site. Thus, toIL is a multipurpose uORF that can respond to a wide variety of translation stresses.
Expression of many bacterial genes is regulated by elements in their upstream regions (URs) (Adams et al., 2021). UR RNA can contain binding sites for trans-acting factors such as regulatory RNAs or RNA-binding proteins. UR RNAs can also include cis-acting elements, such as sequences that form secondary structures, and short ORFs (sORFs) known as upstream ORFs (uORFs). Regulatory elements in URs can modulate transcription termination within the UR, RNase accessibility within the UR, or translation initiation of the downstream gene.

uORFs are typically <50 codons in length, and sense translational stress to regulate expression of the downstream gene. Environmental perturbations promote ribosome stalling within uORFs, often in a sequence-specific manner (Ramu et al., 2009); stalled ribosomes alter the UR RNA secondary structure, modulating the formation of transcription terminators, the accessibility of the ribosome binding site for the downstream gene, or loading sites for the Rho transcription termination factor. Ribosome stalling within uORFs can be mediated by limiting concentrations of charged tRNAs (Landick and Yanofsky, 1987), or by small molecules that bind the translating ribosomes, including amino acids and antibiotics (reviewed by (Seip and Innis, 2016)).

Translation repression mediated by regulatory elements in URs can lead to an additional level of repression at the level of transcription termination (Adams et al., 2021; Baniulyte et al., 2017; Bastet et al., 2017; Bossi et al., 2012; Yakhnin et al., 2001). This occurs because Rho-dependent transcription termination is inhibited by translation (Adhya and Gottesman, 1978; Richardson, 2002). Reduced translation mediated by regulatory elements in URs allows Rho to load onto Rho utilization (Rut) sequences in the nascent RNA for the ORF that would otherwise be occluded by ribosomes. Ruts are enriched for ‘YC’ dinucleotides, lack extensive secondary structure, and have a high C:G ratio (Alifano et al., 1991; Nadiras et al., 2018; Rivellini et al., 1991; Schneider et al., 1993). The relatively low information content required for a Rut means that many sequences within ORFs can function as a Rut when translation is repressed. Translating ribosomes may also inhibit Rho termination by
preventing association of Rho with RNA polymerase (RNAP) and the associated transcription elongation factor NusG (Burmann et al., 2010).

Genome-wide studies of transcription termination *Escherichia coli* and *Mycobacterium tuberculosis* have identified many Rho termination events within ORFs (Adams et al., 2021; Cardinale et al., 2008; Dar and Sorek, 2018; D’Halluin et al., 2022; Peters et al., 2012, 2009), suggesting that modulation of premature Rho termination is a widespread regulatory mechanism. Here, we characterize the mechanism of regulated Rho termination within the *E. coli* *topAI* gene that is operonic with two additional genes, *yjhQ* and *yjhP* (Mao et al., 2015). The *topAI* and *yjhQ* genes encode a type II toxin-antitoxin system, where *topAI* encodes a topoisomerase A inhibitor, and *yjhQ* encodes the cognate antitoxin (Yamaguchi and Inouye, 2015). We show that Rho-dependent transcription termination within *topAI* is a consequence of translation repression. We further show that the long 5′ UR of *topAI* encodes a regulatory uORF, *toiL*, that acts as a sensor for translation stress caused by multiple classes of ribosome-targeting antibiotics. We propose that these antibiotics promote ribosome stalling at various sites within *toiL*, and that ribosome stalling anywhere within *toiL* unmasks the ribosome binding site of *topAI*. Thus, *toiL* is a multipurpose regulatory uORF that can sense a wide variety of translational stresses.
RESULTS

Translational repression of *topAI* leads to intragenic Rho-dependent transcription termination

Genome-scale analysis of Rho termination identified a putative termination site in the coding region of *topAI* (Adams et al., 2021; Peters et al., 2012). The *topAI* transcript has an unusually long UR (171 nt) (Thomason et al., 2015), suggesting that Rho termination within *topAI* may be regulated by sequences in the UR. To independently assess whether Rho prematurely terminates *topAI* transcription, we constructed a *lacZ* transcriptional reporter fusion that included the *topAI* promoter and UR, and 42 nt of the *topAI* coding region (Figure 1A). We measured expression of this reporter fusion in wild-type cells and cells expressing the R66S Rho mutant that is defective for RNA binding and termination (Baniulyte et al., 2017; Martinez et al., 1996). We observed ~7-fold higher expression in *rho* mutant cells than in wild-type cells (Figure 1B), consistent with a Rho termination site upstream of position 42 of the *topAI* gene.

The precise length and position of a *rut* is difficult to predict, since Rho binds RNA with relatively low specificity; however, Rho is believed to favor pyrimidine-rich, unstructured RNA regions (Chhakchhuak et al., 2018; Nadiras et al., 2018). The most pyrimidine-rich region of the *topAI* UR is at the very 5’ end. Mutating just four C residues in the Rho-terminated construct of *topAI* (Figure 1A) caused a 4-fold increase in expression in wild-type cells, but no change in expression in *rho* mutant cells (Figure 1B). We conclude that the *rut* includes the pyrimidine-rich sequence at the very 5’ end of the *topAI* transcript.

To determine the position of the Rho termination site, we measured transcriptional activity of a “short” *topAI::lacZ* fusion that includes only the first 10 bp of the *topAI* ORF (Figure 1A). The effect of mutating *rho* on expression of this short reporter fusion was substantially lower than that observed for the longer reporter fusion, with no difference in expression observed for the short reporter fusion containing the mutant *rut* sequence (Figure 1B). This suggests that most of the transcription termination occurs between nucleotides 10
and 42 of the topAI coding region. We conclude that Rho loads onto the RNA early in the topAI transcript, but does not trigger termination until RNAP is within the ORF, >180 nt further downstream.

Translating ribosomes protect nascent mRNA in protein-coding regions from Rho (de Smit et al., 2009). Given that Rho termination occurs within the topAI ORF, we speculated that topAI is translationally repressed. To test whether topAI is actively translated, we constructed a topAI-lux translational reporter fusion (Figure 2A; Figure 2 – figure supplement 1A). We compared expression of this reporter fusion in wild-type and rho mutant cells. Although transcriptional repression of topAI is relieved in the rho mutant background (Figure 1), we observed strong repression of the topAI-lux translation in both wild-type and rho mutant cells (Figure 2 – figure supplement 1B). These data suggest that Rho termination within the topAI coding region is a consequence of translational repression.

**topAI repression is relieved under conditions of translation stress**

Translation repression often occurs by binding of a trans-acting factor (e.g., protein, sRNA, small molecule) overlapping the ribosome-binding site (Breaker, 2018; Kriner et al., 2016). To identify trans-acting regulators of topAI, we used a genetic selection for spontaneous mutants with increased topAI expression. Briefly, the mutant selection used a topAI-lacZ construct in a ΔlacZ background; overnight cultures were plated on minimal medium with lactose as the only carbon source, only allowing growth of spontaneous mutants with upregulated topAI expression (cis-acting mutants were discarded). We isolated 42 mutants with upregulated topAI expression. 39 of the mutants had a mutant rho gene, suggesting that the screen was saturating. We isolated three additional mutants that each carry single base mutations in one of the seven copies of the 23S rRNA gene, in domain IV (rrlA ΔG1911, rrlC ΔT1917) or domain V (rrlA G2253T) (Table S1). Mutations in these regions of 23S rRNA have been suggested to cause nonsense and frameshift readthrough, defects in translation fidelity and ribosome assembly, and reduced binding of ribosome release factors (Feinberg and Joseph, 2006; Gregory et al., 1994; Hirabayashi et al., 2006; Kipper et al., 2011; Liiv et al., 2005). To test whether the rRNA mutations are sufficient to upregulate topAI translation, we expressed wild-type or mutant (23S rRNA ΔT1917) rRNA
operons in *trans* in an otherwise wild-type strain, and measured expression of the *topAI-lux* translational reporter fusion. For these and all additional assays of *topAI-lux* reporter fusions, we used a strain lacking *topAI* and *yjhQ*, to rule out the possibility of autoregulation. Supplying the mutant 23S rRNA in *trans* resulted in increased *topAI* expression, whereas wild-type 23S rRNA did not (Figure 2B). Thus, the 23S rRNA mutation is dominant over the seven chromosomal rRNA operons, suggesting that the effect of the mutations in 23S rRNA genes on *topAI* expression is not simply due to a reduction in levels of active ribosomes.

Expression of toxin-antitoxin genes is often induced by environmental stresses (Page and Peti, 2016). We next aimed to identify an environmental condition(s) that *topAI* responds to. Given the effect of mutating 23S rRNA on *topAI* expression, we speculated that perturbing translation with ribosome-targeting antibiotics might induce expression of *topAI*. Moreover, increases in *topAI* mRNA levels upon treatment with erythromycin or clindamycin have been reported previously (Dzyubak and Yap, 2016). We measured expression of the *topAI* translational reporter fusion in cells grown with each of 12 ribosome-targeting antibiotics at sub-inhibitory concentrations. Tetracycline, spectinomycin, retapamulin, tylosin and erythromycin caused increases in expression from 250-fold to 4,000-fold of the *topAI-lux* translational fusion (Figure 2C; Table S2). We observed no change in expression in response to treatment with chloramphenicol (Figure 2C), kasugamycin, gentamicin, amikacin, streptomycin, apramycin, or hygromycin (Table S2), although we cannot rule out that these antibiotics might induce *topAI* expression at concentrations other than those tested here. Regardless, *topAI* expression is modulated by a wide variety of mechanistically distinct ribosome-targeting antibiotics, reinforcing the idea that expression of this toxin-antitoxin system responds to the translation status of the cell.

To determine whether the relief of translation repression by ribosome-targeting antibiotics also prevents premature Rho-dependent transcription termination, we used ChIP-qPCR to measure the association of RNAP (β subunit) across the *topAI-yjhQP* operon in wild-type or *rho* mutant cells grown in the presence or absence of a sub-inhibitory concentration of tetracycline. As a control, we measured the association of RNAP within *rho*, where premature Rho-dependent transcription termination has been previously described (Bastet et al., 2017;
Matsumoto et al., 1986). For analysis of RNAP association within rho, we normalized RNAP occupancy values to those in the rhoL region upstream; for topAI-yjhQP, we normalized RNAP occupancy values within the transcribed regions to those in the promoter region (Figure 3A). Mutation of rho led to a significant, 12.0-fold increase in RNAP occupancy within rho (t-test $p = 0.001$), consistent with Rho-dependent transcription termination early in the rho transcript (Figure 3B). Tetracycline treatment in wild-type cells did not lead to a significant increase in RNAP occupancy within rho (t-test $p = 0.1$). The combination of mutating rho and treating with tetracycline led to a significant, 5.9-fold increase in RNAP occupancy within rho (t-test $p = 0.0009$).

Mutation of rho led to significant, 3.6-, 18.9-, and 17.8-fold increases in RNAP occupancy at three positions across the topAI-yjhQP operon (Figure 3C; t-test $p = 0.009, 0.004, 0.001$, respectively). These data are consistent with Rho-dependent transcription termination within topAI. The larger increases in RNAP occupancy at later positions within the transcribed region are likely due to a combination of the limited spatial resolution of ChIP-qPCR, and the potential for multiple termination sites within the transcribed region. Tetracycline treatment in wild-type cells led to significant, 1.7-, 7.3-, and 6.7-fold increases in RNAP occupancy at the three positions across the topAI-yjhQP operon (t-test $p = 0.03, 0.0009, 0.001$, respectively), consistent with tetracycline treatment inhibiting Rho-dependent transcription termination within topAI. The combination of mutating rho and treating with tetracycline led to 2.0-, 5.7-, and 9.9-fold increases in RNAP occupancy at the three positions across the topAI-yjhQP operon (t-test $p = 0.0004, 0.006, 0.005$, respectively).

To determine whether the relief of translation repression by ribosome-targeting antibiotics affects the topAI-yjhQP RNA level, we used qRT-PCR to measure RNA levels at positions across the topAI-yjhQP operon in wild-type or rho mutant cells grown in the presence or absence of a sub-inhibitory concentration of tetracycline. Mutation of rho led to significant, 7.5-, 5-, and 8.4-fold increases in RNA levels at three positions across the topAI-yjhQP operon (Figure 3D; t-test $p = 0.002, 0.002, 3.5e^{-7}$, respectively). These data are consistent with Rho-dependent transcription termination within topAI. Given that the most upstream region tested is upstream
of the likely Rho termination site, these data suggest that premature Rho-dependent transcription termination destabilizes the topAI-yjhQP mRNA. Tetracycline treatment in wild-type cells led to significant, 4.9-, 2.0-, and 8.0-fold increases in RNA levels at the three positions across the topAI-yjhQP operon (t-test \( p = 0.008, 0.0007, 8.2e^{-6} \), respectively), consistent with tetracycline treatment inhibiting Rho-dependent transcription termination within topAI. The combination of mutating rho and treating with tetracycline led to 18.8-, 5.5-, and 19.9-fold increases in RNA levels at the three positions across the topAI-yjhQP operon (t-test \( p = 0.01, 0.006, 0.005 \), respectively). Together, our data support a model in which tetracycline treatment induces translation of topAI, which in turn prevents premature Rho-dependent transcription termination within topAI.

**Expression of a uORF, toiL, is required for antibiotic-mediated topAI regulation**

Long 5′ URS often contain regulatory elements such as uORFs that contribute to regulation of the downstream genes (Kriner et al., 2016). Given that topAI regulation responds to translation perturbation, we searched for a potential uORF that could act as a regulatory module in the topAI UR. Through manual inspection of available ribosome profiling data, we identified an 8-codon putative uORF that starts 139 nt upstream of topAI (Figure 4A) (Meydan et al., 2019; Wang et al., 2015; Weaver et al., 2019). A small protein (Mia-127) with near-identical amino acid sequence and relative genome position was described in Salmonella enterica (Figure 4, figure supplement 1) (Baek et al., 2017). To experimentally determine the frame and position of this uORF, which we renamed toiL (topoisomerase inhibitor leader) in E. coli K-12, we measured expression of a toiL-lacZ translational fusion. Replacing native codons with stop codons in the predicted CDS prevented expression of this fusion, unlike the equivalent substitution upstream of the ORF (Figure 4), supporting our ORF prediction. The first 7 amino acids of the ToiL protein are conserved across bacteria that encode the topAI-yjhQP operon (Figure 4 – figure supplement 1).

To determine if toiL is a regulatory uORF, we tested whether expression of the topAI-lux translational reporter fusion is induced by ribosome-targeting antibiotics if toiL translation is impaired. We first mutated the start codon of toiL from ‘ATG’ to ‘gTa’ (Figure 2A; the specific substitutions were made to preserve the predicted
secondary structure of the 5′ UR; Figure 4 – figure supplement 2A). The start codon mutation (Figure 2A; Figure 4 – figure supplement 2B) did not impact expression of a topAI-lux transcriptional reporter fusion that used the entire UR for topAI (Figure 4 – figure supplement 2C), indicating that the mutation does not cause premature Rho termination. We measured expression of the wild-type and mutant topAI-lux translational fusions in the presence or absence of sub-inhibitory concentrations of ribosome-targeting antibiotics. The toiL start codon mutation caused a 12- to 110-fold decrease in topAI induction by antibiotics (Figure 2C). Nonetheless, antibiotics were still able to modestly induce topAI expression. The toiL start codon is flanked by in-frame TTG and CTG trinucleotides that could potentially function as secondary start codons, perhaps explaining why mutation of the ATG start codon did not completely abolish the effect of antibiotic treatment. To test this possibility, we disrupted the predicted toiL Shine-Dalgarno (S-D) sequence (Figure 2A) and measured topAI-lux induction by ribosome-targeting antibiotics. Disruption of the toiL S-D site abolished induction of topAI expression by all antibiotics tested (Figure 2C), supporting the idea that active translation of toiL is absolutely required for topAI regulation by ribosome-targeting antibiotics.

**Tetracycline causes de-repression of topAI translation by disrupting base-pairing around the topAI ribosome-binding site**

Long URs that encode uORFs often utilize alternating RNA structures to modulate expression of the downstream gene (Kriner et al., 2016). For topAI, the class of regulatory element (i.e., a uORF) and the type of inducer (i.e., ribosome-targeting antibiotics) closely resembles the regulation of rRNA methyltransferase genes ermB/ermC (Subramanian et al., 2012), where stalling of the ribosome within a uORF in the presence of certain macrolide antibiotics alters the downstream RNA structure, facilitating ermB/ermC translation. Computational prediction of the topAI UR RNA secondary structure suggests that sequences within toiL base-pair with the topAI ribosome-binding site (Figure 5A). Consistent with this prediction, the 5′ UR of toiL has been reported to form an extended region of dsRNA (Lybecker et al., 2014). We used in-cell SHAPE-seq (Watters et al., 2016a) to experimentally assess the topAI UR RNA secondary structure, and to investigate changes in secondary structure that occur upon tetracycline treatment. The SHAPE reagent, 1M7, penetrates live cells and modifies...
the backbone of accessible RNA nucleotides. In the subsequent RNA library preparation steps, modified nucleotides block reverse transcription (RT), creating RT-stop points that are detected bioinformatically after the 1M7-treated and untreated libraries are sequenced. Lower SHAPE reactivity is an indication of increased base-pairing interactions or nucleotide occlusion by other cellular factors such as ribosome binding. The SHAPE-seq data for untreated cells were in good agreement with the computationally predicted structure of the topAI UR (Figure 5A; Table S3): the beginning of the 5’ mRNA end is highly reactive, as are predicted loops. We speculate that moderate reactivity in the toiL coding region is the result of translating ribosomes that temporarily disrupt the predicted long-range base-pairing interactions. Tetracycline treatment significantly altered reactivity ($p < 0.05$; see Methods for details) in two distinct regions in the 5′ region in comparison to the untreated control: sequence from the toiL S-D to the toiL start codon became less reactive, whereas the topAI S-D became more reactive (Figure 5B; Table S3). We propose that, in the presence of tetracycline, initiating ribosomes stall at the start of toiL, which leads to a decrease in base-pairing between toiL and the topAI S-D. No significant changes in the toiL ORF region were observed as a result of tetracycline treatment ($p > 0.05$), likely due to these nucleotides switching from occlusion by base-pairing to occlusion by a stalled ribosome.

**Tetracycline stalls ribosomes at the start codon of toiL and at start codons across the transcriptome**

To directly measure ribosome positioning within toiL in vivo, we used ribosome profiling to map ribosome occupancy across the E. coli transcriptome in untreated cells, and cells treated with tetracycline. A comparison of ribosome occupancy across all genes showed that topAI and its operonic genes yjhQ and yjhP are among the most strongly induced by tetracycline (Figure 6A; Table S4; note that induction could be at the level of RNA abundance, translation, or both). For cells treated with tetracycline, ribosome occupancy throughout toiL was substantially higher than in untreated cells. When mapping the 3’ ends of ribosome-protected RNA fragments, we observed a strong peak at position +18 of toiL, a location consistent with the downstream edge of a ribosome stalled at the toiL start codon (Figure 6B). To determine whether tetracycline induces ribosome stalling in other ORFs, we compared ribosome occupancy in the region around the start codons of all annotated ORFs. Tetracycline induced stalling at positions +16 to +18 relative to start codons (Figure 6C), consistent with
ribosomes stalled at start codons, as has been described for tetracycline in an earlier study (Nakahigashi et al., 2016). We conclude that tetracycline induces expression of \textit{topAI} by stalling ribosomes at the start codon of \textit{toiL}. We reasoned that retapamulin, a ribosome-targeting antibiotic that traps initiating ribosomes on start codons (Meydan et al., 2019; Yan et al., 2006) would induce \textit{topAI} expression by the same mechanism. Consistent with this prediction, retapamulin strongly induced (~600-fold) expression of the \textit{topAI-lux} translational reporter fusion, and induction by retapamulin depended upon active translation of \textit{toiL} (Figure 2C).

\textbf{Mapping ribosome stalling sites induced by different antibiotics}

To further assess potential ribosome stalling at \textit{toiL in vivo}, we used a modified version of a previously described stalling reporter construct (Bailey et al., 2008). Macrolide-mediated induction of this reporter relies on ribosome stalling at codon Ile9 of the \textit{ermCL} uORF. We designed a hybrid construct where progressively longer segments of the \textit{toiL} ORF, extending towards the 3’ end, are fused to the \textit{ermCL} 10\textsuperscript{th} codon and the remaining downstream sequence, followed by a luciferase reporter gene (Figure 7A-B; Figure S4). These constructs include the RNA structural features from the \textit{ermCL-ermC} intergenic region that are altered in response to ribosome stalling upstream. Thus, ribosome stalling within \textit{toiL} sequences, close to the junction with \textit{ermCL}, is expected to induce expression of the luciferase reporter gene (Figure 7A; Figure 7 – figure supplement 1). We measured expression of the stalling reporter constructs in a strain lacking \textit{topAI} and \textit{yjhQ}.

As a control, we replaced \textit{toiL} with the native \textit{ermCL} sequence to show that this reporter is responsive to erythromycin treatment, but not other antibiotics (Figure 7 – figure supplement 2). Tetracycline and retapamulin induced expression most strongly with the fusion to the start codon of \textit{toiL} (Figure 7C), consistent with ribosome stalling at the start codon. Tylosin induced expression of only the 5-codon fusion reporter construct, suggesting tylosin-induced stalling at Val5. The effect of tylosin on expression of this reporter was abolished by the Val5→Leu mutation (Figure 7C). Interestingly, tetracycline, retapamulin and erythromycin also showed some induction with the 5-codon \textit{toiL} fusion, with the effect reduced by the Val5→Leu mutation (Figure 7C); additional stalling at this codon might have an additive effect on \textit{topAI} induction. Erythromycin induced expression most strongly with the construct that has the complete \textit{toiL} ORF, including the stop codon (Figure
7C), suggesting that ribosomes stall during translation termination. Spectinomycin induced expression of several constructs, but the level of induction was lower than for other antibiotics (Figure 7C), despite being able to induce topAI to a similar extent to tetracycline (Figure 2C). Chloramphenicol did not induce expression of any of the constructs, consistent with its inability to induce expression of topAI (Figure 2C).
DISCUSSION

Model for topAI regulation

We propose the following model for topAI regulation (Figure 8): In the absence of translation stress, a hairpin that encompasses the toiL uORF and the topAI ribosome-binding side prevents initiation of topAI translation, which in turn promotes premature Rho-dependent transcription termination within the topAI ORF. Under these repressive conditions, toiL is presumably being efficiently translated, but ribosomes translating toiL only transiently disrupt the hairpin. Upon induction of certain types of translation stress, notably ribosome inhibition by select antibiotics, ribosomes stall within toiL, disrupting the repressive hairpin and leading to translation of topAI, which in turn prevents Rho-dependent transcription termination within topAI. TopAI-yjhQP RNA levels in the rho R66S mutant increase ~2-fold upon tetracycline treatment (Figure 3D), suggesting an additional, albeit modest level of regulation at the level of mRNA stability. This could be due to a stabilizing effect of translation (Deana and Belasco, 2005), or direct regulation of RNase accessibility in the 5' UR.

toiL is a multipurpose sensory uORF

Sensory uORFs are versatile regulators that have evolved to rapidly alter physiology in response to a wide variety of environmental stresses that impact translation. However, most regulatory uORFs characterized to date have been proposed to respond to a single translational stress. By contrast, toiL senses a wide variety of ribosome-targeting antibiotics with unrelated mechanisms of action. The ability of toiL to transduce a wide range of translational stress signals is likely due to the fact that base-pairing with the topAI ribosome-binding site occurs across the length of the toiL ORF; hence, ribosome stalling anywhere within toiL would likely prevent hairpin formation, relieving topAI repression. Many antibiotics have been shown to stall ribosomes in ORFs in a sequence-specific manner (Orelle et al., 2013; Vázquez-Laslop and Mankin, 2018a). We speculate that the toiL sequence has evolved to stall ribosomes in a sequence-specific manner in response to a variety of antibiotics.
There are a few other examples of uORFs where more than one class of antibiotic can induce ribosome stalling. *Clostridiodes difficile cplR* is regulated by a uORF that senses retapamulin and lincomycin (Obana et al., 2023). *Bacillus subtilis vmlR* is regulated by a uORF that senses retapamulin, lincomycin, iboxamycin (Takada et al., 2022). And *Staphylococcus haemolyticus vga(A)* is regulated by a uORF that senses lincosamides, streptogramin A, and pleuromutins (Vimberg et al., 2020). However, in these cases, the antibiotics all likely function to stall ribosomes at start codons (Takada et al., 2022). Hence, even though the antibiotics have different targets within the ribosome, the position of ribosome stalling is the likely to be the same. By contrast, our data strongly suggest that while tetracycline and retapamulin stall ribosomes at the *stoiL* start codon, tylosin, spectinomycin and erythromycin stall ribosomes further downstream.

Another uORF that is known to sense multiple classes of ribosome-targeting antibiotics is found upstream of the actinobacterial gene *whiB7* (Burian and Thompson, 2018; Lee et al., 2022). Whether antibiotics induce ribosome stalling within the *whiB7*-associated uORF has not been tested. Intriguingly, *whiB7* expression can also be induced by amino acid starvation (Lee et al., 2022), suggesting that non-antibiotic stresses that lead to ribosome stalling within the uORF can also contribute to regulation. An artificial uORF-regulated reporter construct has also been generated that responds to a variety of ribosome-targeting antibiotics, albeit to lesser extent than *topAI* or *whiB7*; the mechanism of uORF regulation in response to antibiotic treatment was presumed to involve ribosome stalling in the uORF, although this has not been tested (Osterman et al., 2012).

**Understanding how different antibiotics stall ribosomes within *stoiL***

The mechanism by which tetracycline stalls ribosomes at start codons has not been determined. Tetracycline binds to the 30S ribosomal subunit in the decoding center, and likely functions by preventing association of aminoacyl-tRNAs with the A-site (Nguyen et al., 2014). We speculate that tetracycline binds to initiating ribosomes when the A-site is free, preventing association of an aminoacyl-tRNA in the A-site, an essential step prior to formation of the first peptide bond in the nascent peptide. Intriguingly, regulation of tetracycline
resistance can involve a uORF (Chopra and Roberts, 2001), including one case where the uORF is only four codons long (Wang et al., 2005), consistent with regulation by ribosome stalling at the start codon.

Macrolides such as erythromycin function by binding in the nascent peptide exit tunnel of the ribosome (Vázquez-Laslop and Mankin, 2018b). Ribosome stalling induced by erythromycin is well-established for uORFs upstream of macrolide-resistance genes (Ramu et al., 2009), where the nascent peptide in the exit tunnel senses the presence of erythromycin, leading to structural changes that prevent peptide bond formation. Our data are consistent with erythromycin inducing ribosome stalling at the end of toiL (Figure 7C), suggesting that the nascent peptide senses erythromycin, leading to structural changes that inhibit the action of release factors. Indeed, erythromycin was recently shown to stall ribosomes at the penultimate codon of the Streptococcus msrDL uORF by altering the 23S rRNA structure to prevent release factor binding (Fostier et al., 2023). When ribosomes stall on msrDL in the presence of erythromycin, the N\(^{-3}\) and N\(^{-2}\) amino acids with respect to the C-terminal residue are leucine and isoleucine respectively, and the stop codon is UAA. The N\(^{-3}\) and N\(^{-2}\) amino acids are the closest to erythromycin in the peptide exit tunnel (Fostier et al., 2023). Assuming erythromycin induces stalling of ribosomes on the penultimate codon of toiL, the N\(^{-3}\) and N\(^{-2}\) amino acids amino acids would be valine and isoleucine, respectively, and the stop codon is UAA, suggesting a similar interaction of the ToiL and MsrDL nascent peptides with erythromycin, and a similar mechanism of stalling.

Tylosin is also a macrolide, and stalling induced by tylosin has also been observed previously at an RYR sequence in Bacillus subtilis (Yakhnin et al., 2019). This suggests a similar mechanism of action to erythromycin, which can stall ribosomes at R/K-X-R/K motifs (Davis et al., 2014; Ramu et al., 2009). By contrast, our data suggest that tylosin induces ribosome stalling at Val5 of toiL (Figure 7C). Despite the apparent preference for R/K-X-R/K motifs for tylosin-induced ribosome stalling, tylosin has been observed to stall ribosomes at other sequences (Orelle et al., 2013), suggesting that the sequence requirements for tylosin-induced stalling are complex, as they are for erythromycin (Ramu et al., 2009).
Spectinomycin also induces expression of topAI in a toiL-dependent manner, although our data suggest that spectinomycin does not lead to ribosome stalling at a single codon position in toiL (Figure 7C). Little is known about the effects of spectinomycin on ribosome stalling, but it has been shown to stall ribosomes at specific sequences within ORFs (Orelle et al., 2013).

The link between translation attenuation and premature Rho-dependent termination

In addition to directly regulating translation of topAI, toiL indirectly regulates topAI transcription by modulating Rho-dependent transcription termination within the topAI ORF. We speculate that Rho prematurely terminates transcription of other uORF-regulated genes where the uORF modulates translation, similar to the function of some riboswitches (Bastet et al., 2018). Other studies of premature Rho termination events within coding regions suggested that termination occurs due to the unmasking of Ruts within the ORF that are otherwise occluded by translating ribosomes (Bastet et al., 2017; Ben-Zvi et al., 2019; Bossi et al., 2012; de Smit et al., 2008). By contrast, the Rut for topAI is located within the 5′ UR. This suggests that the mechanism by which translation of topAI prevents Rho termination is distinct to that of other characterized examples of prematurely Rho-terminated genes. Specifically, ribosomes translating topAI likely prevent Rho from catching the elongating RNAP, rather than preventing Rho loading onto the nascent RNA. If there are indeed two distinct mechanisms by which ribosomes can prevent Rho termination, the level of translation required for each mechanism may be different. An alternative possibility is that the topAI Rut is very long and extends into the topAI ORF, in which case ribosomes could prevent Rho termination by preventing Rho loading.

We identified elements of the topAI Rut that are located a short distance upstream of toiL. Given the length of RNA required to constitute a Rut, the topAI Rut must overlap toiL. Thus, the Rut is functional despite the presence of an overlapping ORF that is actively translated. We propose that (i) Rho can only load onto the topAI 5′ region after translation of toiL is terminated, or (ii) Rho can loop the RNA around the toiL region to access upstream and downstream elements of the Rut. It has previously been proposed that Rho can step over an RNA
roadblock to access available Ruts (Kriner and Groisman, 2017; Schwartz et al., 2007), thereby helping Rho to overcome roadblocks in the 5′ region.

**Concluding remarks**

uORFs permit rapid regulation in response to translational stress. For almost all previous uORFs shown to sense antibiotics, the downstream gene is functionally related to antibiotic resistance, often directly promoting resistance to the antibiotic(s) that induce expression. Although topAI has been characterized as a DNA gyrase inhibitor (Yamaguchi and Inouye, 2015), we speculate that topAI and/or yjhQ/P, which are transcribed from the same operon, encode proteins involved in resistance to antibiotic-targeting ribosomes, or survival under another form of translation stress.

There has been a recent explosion in the discovery of bacterial sORFs, many of which are located short distances upstream of canonical ORFs on the same strand (Meydan et al., 2019; Smith et al., 2022; Stringer et al., 2021; Venturini et al., 2020; Weaver et al., 2019). We speculate that there are large numbers of regulatory uORFs even in a well-characterized bacterium like *E. coli*. Indeed, analysis of ribosome profiling data for cells treated with tetracycline revealed >5-fold increases in ribosome-associated RNA levels relative to untreated cells for several genes that are known to be associated with a uORF (Table S4), including mgtA, corA and speC.
MATERIALS AND METHODS

Strains and plasmids
All strains and plasmids used in this study are listed in Table S5. All oligonucleotides used in this study are listed in Table S6. An E. coli MG1655 ΔlacZ ΔtopAI-yjhQ::thyA strain (GB001) was constructed using the FRUIT recombineering method (Stringer et al., 2012). Briefly, the thyA gene was amplified using primers JW7676 + JW7677 and electroporated into strain AMD189 (MG1655 ΔlacZ ΔthyA) (Stringer et al., 2012) to replace the topAI gene from 379 bp upstream of topAI to 74 bp into the yjhQ coding region.

All topAI and toiL lacZ and luciferase reporter plasmids (pJTW100, pGB164, pGB182, pGB196, pGB197, pGB200, pGB201, pGB202, pGB215, pGB217, pGB297, pGB305, pGB306, pGB313) were made to include sequence starting at -400 bp upstream of topAI and were presumed to include the native topAI promoter. Fusions to lacZ are derivatives plasmid pAMD033 (Dornenburg et al., 2010). Fusions to luciferase are derivatives of pGB135, which includes the luxCDABE operon from Photorhabdus luminescens. Constructing pGB135 first required construction of plasmid pGB96. pGB96 was made by PCR-amplification of a region from plasmid pJTW064 (Stringer et al., 2014) using primers JW8044 + JW8045, PCR-amplification of a region from pCS-PesaRlux (Shong and Collins, 2013) using primers JW8046 + JW8047, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pET-PesaRlux cut with XhoI and XbaI (Shong and Collins, 2013). pGB135 was made by PCR-amplification of two regions from pGB96 using primers JW8044 + JW8522 and JW8523 + JW8047, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pCS-PesaRlux cut with XhoI and XbaI (Shong and Collins, 2013). pGB135 has the PesaR fragment from pCS-PesaRlux replaced with a constitutive promoter and convenient restriction sites for transcriptional or translation fusions to the luxC gene; transcriptional reporter fusions included a Shine-Dalgarno sequence, whereas translational fusions used the sequence up to the initiation codon of the gene of interest followed by the second codon of the reporter gene.
Plasmid pJTW100 is a derivative of pAMD033 that includes a full-length topAI gene and the first 90 bp of yjhQ translationally fused to lacZ. pJTW100 was constructed by PCR-amplifying from an E. coli MG1655 colony using primers JW5638 + JW5639, and cloning the resultant PCR product into pAMD033 (Dornenburg et al., 2010) cut with SphI and HindIII.

Plasmids pGB215 and pGB217 are derivatives of pAMD033 with sequence up to position +10 or +42 of topAI (relative to the start codon) fused transcriptionally to lacZ. pGB215 and pGB217 were made by PCR-amplification from an E. coli MG1655 colony using primers JW5638 + JW9453 or JW5638 + JW9154, respectively, and cloning of the result PCR products into the SphI and NheI sites of pAMD033 (Dornenburg et al., 2010). Plasmids pGB305 and pGB306 are equivalent to plasmids pGB215 and pGB217, except that they have a mutated rut in the 5′ UR (4C → 4A). They were constructed identically to pGB215 and pGB217 except that the PCRs used primers JW5638 + JW9453/JW9154, and the template for the PCR was plasmid pGB299. Plasmid pGB299 is a derivative of pAMD033 (Dornenburg et al., 2010) with the complete topAI gene (not including the stop codon) fused translationally to lacZ, with a mutated rut in the 5′ UR (4C → 4A). pGB299 was constructed by PCR-amplifying from an E. coli MG1655 colony using primers JW5638 + JW9803 and JW9804 + JW8023, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pAMD033 cut with SphI and HindIII.

Plasmid pGB214 is equivalent to pJTW100 except that it has a mutation in the toiL start codon (ATG→gTa). pGB214 was constructed by PCR-amplifying from an E. coli MG1655 colony using primers JW5638 + JW9139 and JW9138 + JW5639, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant PCR product into pAMD033 (Dornenburg et al., 2010) cut with SphI and HindIII. Plasmids pGB182 (wild-type) and pGB297 (toiL ATG→gTa) include the complete topAI 5′ UR transcriptionally fused to lacZ. pGB182 and pGB297 were made by PCR-amplifying from an E. coli MG1655
colony or pGB214, respectively, using primers JW5638 + JW8809, and cloning the resultant PCR product into pAMD033 (Dornenburg et al., 2010) cut with SphI and NheI.

Plasmid pGB164 is a derivative of pAMD033 with the full toiL gene (not including the stop codon) fused translationally to lacZ. Plasmids pGB196 (AAT→tga), pGB197 (CTG→tga), pGB200 (ATG→tga), and pGB201 (TTG→tga) are mutant derivatives of pGB164. pGB164 was made by PCR-amplifying from an E. coli MG1655 colony using primers JW5638 + JW8741, and cloning the resultant PCR product into pAMD033 (Dornenburg et al., 2010) cut with SphI and HindIII. pGB196, pGB197, pGB200, and pGB201 were cloned identically except that primer JW8741 was replaced with JW8999, JW8998, JW9013, and JW9014, respectively.

Plasmid pGB202 is a derivative of pGB135 with a translational fusion of the topAI start codon to luciferase. pGB313 is a derivative of pGB202 with a toiL start codon mutation. pGB202 and pGB313 were made by PCR-amplifying from an E. coli MG1655 colony or pGB214, respectively, using primers JW9288 + JW9289, and cloning the resultant PCR product into pGB135 cut with BamHI and EcoRI. Plasmid pGB366 is a derivative of pGB202 with a toiL Shine-Dalgarno sequence mutation. pGB366 was made by PCR-amplifying from an E. coli MG1655 colony using primers JW9288 + JW10873 and JW10874 + JW9289, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pGB135 cut with BamHI and EcoRI.

Plasmids pGB323, pGB324, pGB325, pGB326, pGB327, pGB328, pGB329, pGB346, and pGB347 include sequences that extend to different positions within toiL fused translationally to the ermCL gene from the 10th codon, which is fused translationally to luciferase. pGB323, pGB324, pGB325, pGB326, pGB327, pGB328, pGB329, pGB346, and pGB347 were made by PCR-amplifying (i) from an E. coli MG1655 colony using primer JW9288 and each of JW9989, JW10122, JW10144, JW10145, JW10146, JW10147, JW10148, JW101848, and JW10849, respectively, and (ii) from geneBlock GB007 (Supplementary Table 6) using primers JW10123 + JW9965. Pairs of PCR products were combined using splicing by overlap extension (Horton et al.,
1990), and the resultant products were cloned into pGB135 cut with *Bam*HI and *Eco*RI. pGB308 is equivalent to these plasmids except *toiL* coding sequence was replaced with the beginning of the *ermCL* sequence (codons 1-9). pGB308 was made by PCR-amplifying (i) from an *E. coli* MG1655 colony using primer JW9288 + JW9989, and (ii) from geneBlock GB007 (Supplementary Table 6) using primers JW9990 + JW9965. Pairs of PCR products were combined using splicing by overlap extension (Horton et al., 1990), and the resultant products were cloned into pGB135 cut with *Bam*HI and *Eco*RI.

Plasmids pGB322 and pGB318 are derivatives of pPro24 (Lee and Keasling, 2005), which contains a propionate-inducible promoter. pGB322 and pGB318 include a complete rRNA operon, either wild-type or mutant (ΔT1917 23S rRNA), respectively. The rRNA operons in both plasmids are fusions of the *rrnB* (upstream of 23S position +1917) and *rrnC* (position +1917 and downstream) operons. pGB318 was made first by PCR-amplifying from an *E. coli* MG1655 colony using primers JW10036 + JW9737 and JW9740 + JW10037, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pPro24 cut with *Nhe*I and *Bam*HI. pGB322 was made by PCR-amplifying from pGB318 using primers JW10036 + JW10104 and JW9740 + JW10105, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pPro24 cut with *Nhe*I and *Bam*HI.

Plasmid pGB72 is a derivative of pET-PesaRlux (Shong and Collins, 2013) that has a constitutive promoter (Burr et al., 2000) followed by 568 bp of a reverse-complemented *Hind*III fragment from the *rrsB* gene that is known to function as a Rho terminator (Li et al., 1984). pGB72 was made by annealing and extending primers JW7977b+7979b, PCR-amplifying from plasmid pSL103 (Li et al., 1984) using primers JW7994 and J7995, combining the two PCR products using splicing by overlap extension (Horton et al., 1990), and cloning the resultant product into pET-PesaRlux cut with *Xho*I and *Bam*HI.

**Isolation and identification of trans-acting mutants**
The trans-acting mutant genetic selection was performed as described previously (Baniulyte et al., 2017). Briefly, cultures of MG1655 ΔlacZ with pJTW100 were grown at 37 °C in LB medium. 100 μl of overnight culture was washed and plated on M9 + 0.2% lactose agar. Spontaneous survivors were tested for increased plasmid copy number or cis-acting mutations near topAI; these mutants were eliminated. Chromosomal mutations were identified either by PCR-amplification and sequencing of rho or by whole-genome sequencing. Rho mutants were also detected by transducing a wild-type rho locus and looking for phenotypic reversion, or by introducing and assaying a Rho-dependent termination luciferase reporter plasmid (pGB72). Total genomic DNA was extracted from the three remaining mutants following the manufacturer’s protocol (Qiagen, catalog # 69504). SNPs were identified using whole-genome sequencing, as described previously (Singh et al., 2016).

Quantification of RNAP association with DNA by chromatin immunoprecipitation coupled with real-time PCR (ChIP-qPCR)

20 ml E. coli wild-type (MG1655) or rho mutant (CRB016) cells were grown in LB at 37 °C with shaking. When the cultures reached an OD_{600} of ~1.0, tetracycline (Sigma #T3383) was added to one set of samples at a final concentration of 0.5 μg/ml. All untreated and tetracycline-treated samples (three biological replicates) were left at 37 °C with shaking for 17 min, after which 500 μl of each sample was pelleted and frozen for total RNA extraction later. For ChIP, the remainder of each sample was crosslinked with 1% formaldehyde (final concentration), and processed as previously described (Stringer et al., 2014). For immunoprecipitation, 800 μl of crosslinked chromatin was incubated with 25 μl Protein A Sepharose slurry (50%) in TBS and 1 μl of antibody against RNAP β (Biolegend, #663003, clone: NT63) for 90 minutes at room temperature while rotating. RNAP enrichment was normalized using the 2^{-ΔΔCt} method to the corresponding “input” control sample and either the rhoL region (for measurements within rho) or the topAI promoter region (for measurements within topAI-yjhQP), as previously described (Bastet et al., 2017). All real-time PCR samples were set up in technical duplicates.

Quantification of RNA levels by reverse transcription coupled with real-time PCR (RT-qPCR)
Total RNA was extracted from frozen pellets (see above) using a Quick-RNA Miniprep Kit (Zymo Research, #R1054) following the manufacturer’s protocol. Approximately 136 ng of total RNA for each sample was reverse-transcribed using MultiScribe Reverse Transcriptase (Invitrogen, #4311235) following the manufacturer’s protocol. A “no reverse transcriptase” control was also included to assess DNA contamination. The resulting cDNA was diluted 1:3 and used as a template for quantitative real-time PCR. RNA expression values were obtained using the $2^{\Delta\Delta Ct}$ method, normalizing to a region within the $mreB$ gene. Values were further normalized to those in wild-type cells (MG1655) without tetracycline treatment. All real-time PCR samples were set up in technical duplicates.

**SHAPE-seq**

A total of three biological replicates of the strain GB001 + pJTW100 were grown to an OD$_{600}$ of ~1.2 in LB medium at 37 °C. Cultures were split, and one set of two replicates was treated with 0.2 μg/ml tetracycline (T3383, Sigma) for 90 min. Cultures were subjected to the in-cell SHAPE-seq procedure previously described for poorly expressed mRNAs (Watters et al., 2016a). The SHAPE reagent 1M7 was purchased from MedChem Express (HY-D0913). SHAPE-seq libraries were sequenced using an Illumina MiSeq instrument (251 nt, paired-end reads). Sequencing data were analyzed using the *spats* pipeline (Watters et al., 2016a, 2016b). Raw reactivities are listed in Table S3. Significant changes in reactivity and corresponding adjusted $p$-values upon tetracycline treatment were determined using the DESeq2 R package (Love et al., 2014), comparing untreated and tetracycline treated sample reactivities at each position using three (“Untreated”) and two (“Treated”) biological replicates from each sample. The mRNA structure of the topAI UR was predicted using mFOLD (Zuker, 2003) and drawn using StructureEditor (https://rna.urmc.rochester.edu/GUI/html/StructureEditor.html).

**Reporter assays**

Bacterial cultures for luciferase or β-galactosidase assays were grown at 37 °C in LB medium to an OD$_{600}$ of 0.5–0.6 unless otherwise indicated in the figure legend. Cultures were grown in 15 ml round-bottom tubes except for antibiotic titration assays (Table S2; see below). Tetracycline (Sigma #T3383), spectinomycin
(Sigma-Aldrich #S4014), erythromycin (Sigma-Aldrich #E5389), retapamulin (Sigma-Aldrich #CDS023386),
tylosin (Sigma-Aldrich #T6134), chloramphenicol (Sigma-Aldrich #C0378), kasugamycin, gentamicin (Sigma-
Aldrich #G1272), amikacin, streptomycin, apramycin, or hygromycin (Millipore #400052) antibiotics were
added at indicated concentrations and timepoints. Plasmid-encoded wild-type and mutant rRNA overexpression
(Figure 2B) was induced by adding 12 mM sodium propionate (Sigma-Aldrich #P1880) to bacterial cultures at
an OD_{600} of ~0.2. β-galactosidase assays were performed as previously described (Baniulyte et al., 2017). For
luciferase assays, each cell culture (200 μl) was aliquoted into a 96-well plate with four technical replicates each.
Luminescence readings for were taken using a Biotek Synergy 2 instrument. Luminescence counts (RLU) were
adjusted for OD_{600} and reported as RLU/OD_{600}. Antibiotic concentrations and lengths of treatments are
indicated in the figure legends. Antibiotic titration assays (Table S2) were performed in 96-well deep-well
plates. Briefly, 1.5 ml of GB001 cells containing the pGB202 plasmid was grown to an OD_{600} of ~1.0, aliquoted
into a 96-well deep-well plate, and treated with antibiotics at the indicated concentrations for 90 min. 100 μL of
each bacterial culture was used for luminescence detection, as described above.

Ribosome profiling (Ribo-seq)

200 ml of *E. coli* MG1655 cells were grown in LB ± 1 μg/ml tetracycline to an OD_{600} of ~1.1. Filtering was
started at 17 min post-treatment. Monosomes were isolated and libraries were constructed as previously
described (Smith et al., 2022), with minor modifications: the 3’-linked oligonucleotide ligation reaction was
carried out overnight at 16 °C; circularized, reverse-transcribed cDNA was PCR-amplified for 6-8 cycles using
universal primer JW8835 and sample-specific primers JW3249 (no antibiotic) or JW10755 (tetracycline).

Determination of Ribo-seq sequence read coverage was performed as described previously (Smith et al., 2022).
Briefly, sequence reads were trimmed using a custom Python script to remove the ligated adaptor sequence, and
mapped to reference genome MG1655 NC_000913.3 using Rockhopper (McClure et al., 2013). Sequence read
coverage for sequence 3’ ends (presumed to correspond to the positions of the downstream edge of ribosomes)
was determined for the whole genome, normalizing to total read count (reads per million; RPM). The metagene
plot showing sequence read coverage around start codons (Figure 6C) was generated as described previously (Smith et al., 2022). Normalized Ribo-seq coverage for all annotated ORFs was determined using differential expression analysis with Rockhopper (McClure et al., 2013), excluding the first and last 30 nt of each gene.
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DATA AVAILABILITY

Raw Illumina sequencing data are available from EBI ArrayExpress using accession numbers E-MTAB-8365 (SHAPE-seq) and E-MTAB-12242 (Ribo-seq).
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FIGURE LEGENDS

Figure 1. Rho-dependent transcription termination within the *topAI* gene. (A) Schematic representation of *topAI-lacZ* transcriptional reporter fusions, indicating the mutation in the putative *rut*. (B) β-galactosidase activity of *topAI-lacZ* transcriptional fusions in wild-type (strain AMD054) or *rho* mutant (R66S amino acid substitution in Rho; strain GB004) cells. Reporter fusions with wild-type sequence or mutation of the predicted *rut* (*rut*) extend to position +42 or +10 of *topAI* (wild-type +42, plasmid plasmid pGB217; wild-type +10, plasmid pGB215; *rut* +42, plasmid pGB306; *rut* +10, plasmid pGB305). Error bars represent ±1 standard deviation from the mean (n = 3).

Figure 2. *topAI* expression is induced in response to translation stress. (A) Schematic representation of *topAI-lux* translational reporter fusions, indicating mutations in the start codon and Shine-Dalgarno sequence of the *toiL* uORF. (B) Luciferase activity of the wild-type *topAI-lux* translational reporter fusion (pGB202) in Δ*topAI-yjhQ* cells (strain GB001) expressing either wild-type 23S rRNA (plasmid pGB322) or ΔT1917 23S rRNA (plasmid pGB318) in *trans*. Activity was measured four hours post-induction with propionate. (C) Luciferase activity of wild-type and mutant *topAI-lux* translational reporter fusions in Δ*topAI-yjhQ* cells (strain GB001) grown in the presence of ribosome-targeting antibiotics. Cells were grown in LB medium to an OD₆₀₀ of ~1.0. Indicated samples were treated with tetracycline (0.5 μg/ml), spectinomycin (90 μg/ml), retapamulin (4 μg/ml), erythromycin (100 μg/ml), tylosin (400 μg/ml), or chloramphenicol (1 μg/ml). Luminescence was measured 90 min after antibiotic treatment. Dark gray bars show data for the wild-type *topAI-lux* translational reporter fusion (plasmid pGB202); light gray bars show data for the *topAI-lux* translational reporter fusion with a mutated *toiL* start codon (plasmid pGB313); white bars show data for the *topAI-lux* translational reporter fusion with
a mutated *toIL* Shine-Dalgarno sequence (pGB366). Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 2, Figure Supplement 1. topAI is translationally repressed.** (A) Schematic representation of the *topAI-lux* translational reporter fusion. (B) Luciferase activity of the *topAI-lux* translational fusion (plasmid pGB202) in Δ*topAI-yjhQ* cells (“Wild-type”; strain GB001) or in Δ*topAI-yjhQ rho* mutant (R66S amino acid substitution in Rho; strain GB004). Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 3. Tetracycline treatment reduces premature Rho-dependent transcription termination within topAI-yjhQ and increases abundance of the associated mRNA.** (A) Schematic of the *rhoL-rho* and *topAI-yjhQP* operons showing the positions of PCR amplicons used for ChIP-qPCR and/or qRT-PCR. (B) Occupancy of RNAP measured by ChIP-qPCR in the *rho* gene. Occupancy values are normalized to those in *rhoL* (control PCR amplicon “C” in panel A). Data are shown for wild-type (strain MG1655) or *rho* mutant (R66S amino acid substitution in Rho; strain CRB016) cells grown in the presence or absence of tetracycline, as indicated. The x-axis label corresponds to the PCR amplicon shown in panel A. (C) Occupancy of RNAP measured by ChIP-qPCR at positions across the *topAI-yjhQ* transcript. Occupancy values are normalized to those in the *topAI* upstream region (control PCR amplicon “C” in panel A). (D) RNA levels measured by qRT-PCR at positions across the *topAI-yjhQ* transcript, normalized to the *mreB* gene. Error bars represent ±1 standard deviation from the mean (n = 3). Statistical significance is indicated as follows: n.s. = not significant (*p > 0.05*), *p < 0.05*, **p < 0.01**, ***p < 0.001.
**Figure 4.** A uORF, *toiL*, is located within the *topAI* 5’ upstream region. (A) Schematic representation of *toiL-lacZ* translational reporter fusions, indicating mutations upstream and within the *toiL* uORF. (B) β-galactosidase activity of the wild-type *toiL-lacZ* translational reporter fusion (wt; plasmid pGB164), and fusions with mutations immediately upstream of *toiL* (TTG→tga; plasmid pGB201), at the *toiL* start codon (ATG→tga; plasmid pGB200), second codon (CTG→tga; plasmid pGB197), or fourth codon (AAT→tga; plasmid pGB196), in wild-type cells (strain MG1655). Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 4, Figure Supplement 1.** ToiL conservation across *Enterobacteriaceae* species. Predicted ToiL sequences were aligned with ClustalO (Madeira et al., 2022). Asterisks indicate fully conserved positions.

**Figure 4, Figure Supplement 2.** Mutation of the *toiL* start codon does not alter RNA secondary structure and does not lead to Rho-dependent transcription termination in the *topAI* 5’ upstream region. (A) Predicted minimum free-energy secondary structures of the *topAI* 5’ UR for wild-type sequence and sequence where the *toiL* start codon (ATG) is mutated (GTA). (B) Schematic representation of *topAI-lacZ* transcriptional reporter fusions indicating the start codon mutation of *toiL*. (C) β-galactosidase activity of the wild-type *topAI-lacZ* transcriptional reporter (plasmid pGB182) and the *toiL* start codon mutant reporter (plasmid pGB297) in ΔtopAI-*yjhQ* cells (strain GB001). Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 5.** Structural changes in the *topAI* 5’ upstream region induced by tetracycline treatment. (A) Predicted RNA secondary structure of the *topAI* 5’ UR, indicating in-cell SHAPE reactivities (ρ) of each
base from an untreated sample. Values of $\rho$ are indicated on a yellow-to-green gradient; white circles indicate no detected reactivity. The position of the toiL uORF is indicated by a red arrow, and the position of the start of topAI is indicated by a gray rectangle. Numbers indicate position relative to the mRNA 5’ end. (B) Filled red or green circles indicate bases with significant changes in SHAPE reactivity when cells were treated with tetracycline ($p < 0.05$).

**Figure 6. Tetracycline stalls ribosomes on start codons.** (A) Normalized Ribo-seq coverage for all annotated ORFs for cells (strain MG1655) grown ± tetracycline. (B) Heatmap showing normalized Ribo-seq coverage in the region around the toiL uORF in untreated cells (no antibiotic) or tetracycline-treated cells. The color indicates the sequence-read coverage for RNA fragment 3’ ends that are presumed to represent the downstream edge of ribosome footprints. The toiL uORF position and encoded amino acid sequence is indicated. (C) Average of normalized Ribo-seq coverage for the regions around start codons for all annotated ORFs, for untreated cells (gray line; no antibiotic) or tetracycline-treated cells (orange line). Sequence-read coverage was calculated for RNA fragment 3’ ends that are presumed to represent the downstream edge of ribosome footprints.

**Figure 7. Ribosome stalling at the topAI leader in vivo.** (A) Schematic showing the toiL-ermCL-lux stalling reporter in the translationally inactive conformation (“OFF”) that is expected in the absence of ribosome stalling, and the translationally active conformation (“ON”) that is expected when ribosomes stall within toiL sequence close to the junction of toiL with ermCL. (B) Schematic showing toiL-ermCL stalling reporters where toiL is progressively extended by one codon at the 3’ end and fused to part of ermCL. toiL sequence is indicated by red arrows; ermCL sequence is indicated by green arrows. The last indicated stalling reporter extends to position 5 of toiL but has a Val5→Leu substitution (blue). (C)
Luciferase activity of the *toiL-ermCL-lux* stalling reporters (plasmids pGB323, pGB346, pGB325, pGB326, pGB324, pGB328, pGB329, pGB307, pGB347, and pGB327) in Δ*topAI-yjhQ* cells (strain GB001). Cells were grown to an OD$_{600}$ of ~1.0 and treated with the indicated ribosome-targeting antibiotics at the same concentrations as in Figure 2C. Luminescence was measured three hours post-treatment. Horizontal dashed lines indicate luminescence activity of 1, to facilitate comparison between panels with different y-axis scales. Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 7, Figure Supplement 1. Predicted RNA structures for translationally inactive and active conformations of *toiL* stalling reporters.** Predicted RNA structures are shown for *toiL-ermCL-lux* stalling reporters where increasing lengths of *toiL* are fused to part of *ermCL*. *toiL* is indicated by red arrows. *ermCL* sequence is indicated by green arrows. Pausing of ribosomes within *toiL*, close to the junction with *ermCL* sequence, is expected to promote formation of the active (“ON”) conformation, whereas the absence of ribosome stalling within *toiL* is expected to promote formation of the inactive (“OFF”) conformation.

**Figure 7, Figure Supplement 2. Expression of an *ermCL* stalling reporter is specifically induced by erythromycin.** (A) Schematic showing the *ermCL-lux* stalling reporter in the translationally inactive conformation (“OFF”) that is expected in the absence of erythromycin, and the translationally active conformation (“ON”) that is expected when ribosomes stall within *ermCL* in the presence of erythromycin. (B) Predicted RNA secondary structures of the inactive (“OFF”) and active (“ON”) conformations of the *ermCL-lux* stalling reporter. (C) Luciferase activity of the *ermCL-lux* stalling reporter (plasmid pGB308) in Δ*topAI-yjhQ* cells (strain GB001) treated with the indicated ribosome-targeting antibiotics. Cells were grown to an OD$_{600}$ of ~1.0 and treated with the indicated ribosome-
targeting antibiotics at the same concentrations as in Figure 2C. Luminescence was measured three hours post-treatment. Error bars represent ±1 standard deviation from the mean (n = 3).

**Figure 8. Model for regulation of topAI expression.** Schematic showing a model for regulation of topAI expression. In the repressed state, a hairpin forms between the toiL sequence and the ribosome-binding site of topAI, repressing translation, which in turn promotes premature Rho-dependent transcription termination within the topAI gene. When cells are treated with certain ribosome-targeting antibiotics such as tetracycline, ribosomes stall within toiL in a sequence-specific manner, preventing formation of the repressive hairpin.
Figure 3

A

B

C

D

**Wild-type, no tetracycline**

**rho mutant, no tetracycline**

**Wild-type, + tetracycline**

**rho mutant, + tetracycline**

Normalized RNAP Occupancy

Normalized RNA Level

PCR amplicon

PCR amplicon
Figure 4

A

TGGATGCTGGAGAATGTAATCATCCGATAA
TGGATGCTGGAGAATGTAATCATCCGATAA
TGGTGAATGCTGGAGAATGTAATCATCCGATAA
TGGTGAATGCTGGAGAATGTAATCATCCGATAA
TGGATGCTGGAGAGAATGTAATCATCCGATAA
TGGATGCTGGGATGTAATCATCCGATAA

\textcolor{red}{\text{topAI}}

\textcolor{green}{\text{lacZ}}

B

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{beta-galactosidase_activity.png}
\caption{\textbf{β-galactosidase Activity}
\begin{itemize}
\item \texttt{toil_wt}
\item \texttt{TTG→tga}
\item \texttt{ATG→tga}
\item \texttt{CTG→tga}
\item \texttt{AAT→tga}
\end{itemize}
}
\end{figure}
Figure 5

A

SHAPE reactivity ($\rho$)

B

Tetracycline vs No Antibiotic

- **N** SHAPE $\rho$ decrease ($p < 0.01$)
- **N** SHAPE $\rho$ increase ($p < 0.01$)
- **N** SHAPE $\rho$ increase ($p < 0.05$)
Figure 7

A

B

M1  L2  E3  N4  V5  I6  I7  R8  Stop9  V5L

C

Luciferase Activity

No Antibiotic

Tetracycline

Retapamulin

Tylosin

Erythromycin

Spectinomycin

Chloramphenicol
