Posttranscriptional Regulation of *tnaA* by Protein-RNA Interaction Mediated by Ribosomal Protein L4 in *Escherichia coli*

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ABSTRACT  *Escherichia coli* ribosomal protein (r-protein) L4 has extraribosomal biological functions. Previously, we described L4 as inhibiting RNase E activity through protein-protein interactions. Here, we report that from stabilized transcripts regulated by L4-RNase E, mRNA levels of *tnaA* (encoding tryptophanase from the *tnaCAB* operon) increased upon ectopic L4 expression, whereas TnaA protein levels decreased. However, at nonpermissive temperatures (to inactivate RNase E), *tnaA* mRNA and protein levels both increased in an *rne* temperature-sensitive (*rne(Ts)*) mutant strain. Thus, L4 protein fine-tunes TnaA protein levels independently of its inhibition of RNase E. We demonstrate that ectopically expressed L4 binds with transcribed spacer RNA between *tnaC* and *tnaA* and downregulates TnaA translation.

We found that deletion of the 5’ or 3’ half of the spacer compared to the wild type resulted in a similar reduction in TnaA translation in the presence of L4. *In vitro* binding of L4 to the *tnaC-tnaA* transcribed spacer RNA results in changes to its secondary structure. We reveal that during early stationary-phase bacterial growth, steady-state levels of *tnaA* mRNA increased but TnaA protein levels decreased. We further confirm that endogenous L4 binds to *tnaC-tnaA* transcribed spacer RNA in cells at early stationary phase. Our results reveal the novel function of L4 in fine-tuning TnaA protein levels during cell growth and demonstrate that r-protein L4 acts as a translation regulator outside the ribosome and its own operon.

IMPORTANCE Some ribosomal proteins have extraribosomal functions in addition to ribosome translation function. The extraribosomal functions of several r-proteins control operon expression by binding to own-operon transcripts. Previously, we discovered a posttranscriptional, RNase E-dependent regulatory role for r-protein L4 in the stabilization of stress-responsive transcripts. Here, we found an additional extraribosomal function for L4 in regulating the *tna* operon by L4-intergenic spacer mRNA interactions. L4 binds to the transcribed spacer RNA between *tnaC* and *tnaA* and alters the structural conformation of the spacer RNA, thereby reducing the translation of TnaA. Our study establishes a previously unknown L4-mediated mechanism for regulating gene expression, suggesting that bacterial cells have multiple strategies for controlling levels of tryptophanase in response to varied cell growth conditions.

KEYWORDS extraribosomal function, gene regulation, mRNA stability, posttranscriptional regulation, protein-RNA interactions

Some ribosomal proteins (r-proteins) in eukaryotes and prokaryotes exhibit extraribosomal functions (1–4). Several r-proteins autogenously control the expression of their operons by binding to their own mRNAs. Additional extraribosomal functions arise from interactions with nonribosomal components, such as specific proteins or RNA molecules. Although the molecular mechanisms of autogenous r-protein synthesis...
control have been studied extensively (5, 6), other extraribosomal functions are less understood.

R-protein L4, a member of the S10 r-protein operon, autogenously controls the synthesis of its own ribosomal proteins in *E. coli* (7, 8). As is the case for many other r-proteins, feedback regulation by L4 operates either by inhibiting translation of its polycistronic mRNA (9) or inhibiting transcription of its S10 operon by binding to independent but overlapping determinants within the 5′ untranslated region (5′-UTR) (6, 10, 11). Apart from its RNA-binding sites, L4 hosts multifunctional domains for interactions with other proteins (12, 13). Indeed, L4 was found to interact with 64 proteins in *E. coli* (14). We previously reported that L4 interacts with the C-terminal region of RNase E to regulate its activity, leading to the stabilization of specific stress-responsive mRNAs critical for cell survival in *E. coli* (4). In eukaryotes, L4 has been shown to functionally interact with the RNA helicase II/Grx1 in mammalian cells (15) and with calnexin in Madin-Darby canine kidney cells (16). In plants, plastid L4 might play a role in plastid transcriptional regulation (17). Despite the specific protein-RNA interactions demonstrated for many r-proteins in ribosomes (18), their RNA-based extraribosomal regulatory mechanisms are still being discovered.

RNase E-dependent and specific RNA targets of L4 have been reported, and it was revealed that the targets most affected were transcripts from the tryptophanase (*tnaCAB*) operon (4). This operon consists of a transcribed leader peptide, TnaC, followed by two structural genes, i.e., tryptophanase (TnaA, an enzyme involved in amino acid degradation) and tryptophan-specific permease (TnaB) (19). Expression of the *tnaCAB* operon is controlled by catabolite repression (20) and tryptophan-specific induction (21). Here, we show that steady-state levels of mRNA transcripts from *tnaCAB* increased upon ectopic expression of L4, whereas levels of TnaA protein decreased. We found that L4 specifically binds to the region upstream of the *tnaA* coding sequence, potentially affecting the structural conformation of the RNA region upstream of TnaA to reduce its translation. L4 binding does not affect translation of *tnaC*, which lies upstream of *tnaA*. We discuss the biological significance of the molecular mechanism by which L4 inhibits *tnaA* translation.

**RESULTS**

**L4 alters *tnaA* mRNA and protein output in the wild type but not an RNase E temperature-sensitive strain.** In a previous study (4), we found that a subset of transcripts showed differentially increased mRNA stability/abundance upon ectopic L4 expression. Therefore, we first examined whether mRNA stabilization/increased transcript abundance is correlated with the levels of protein synthesis by using Western blotting to assess final gene products. We found ectopic L4 induction increased protein levels of RNaseE (−1.8-fold) and RpoS (−1.6-fold) and very mildly affected Lon (−1.3-fold) and CspE (−1.2-fold) proteins in *E. coli* wild-type strain N3433 compared to cells containing a control plasmid (Fig. 1A). Unexpectedly, ectopic L4 expression resulted in decreased levels of endogenous tryptophanase (TnaA) (Fig. 1B), though steady-state levels of *tnaA* mRNA increased. The reduced levels of TnaA upon L4 expression could be the result of rapid TnaA degradation. To investigate this possibility, we arrested translation with chloramphenicol and then compared the protein stability of TnaA upon L4 expression with that of control plasmid (i.e., lacking inducible L4). Our data indicate that ectopic L4 expression does not alter TnaA protein half-life (>128 min) and does not promote faster TnaA degradation (see Fig. S1 in the supplemental material). Thus, we observed a decrease in TnaA protein levels despite an increase in *tnaA* transcript abundance (4), suggesting the existence of a novel regulatory mechanism that is independent of the L4-RNase E regulatory pathway. Furthermore, we analyzed expression levels of TnaA protein in an *rne* temperature-sensitive [rne(Ts)] mutant strain. RNase E inactivation in the *rne(Ts)* strain at a nonpermissive temperature resulted in an expected increase in TnaA protein levels (TnaA levels increased −2.2-fold after shifting to 44°C) (Fig. 1C), suggesting that the *tna* transcript is also regulated by RNase E in a manner independent of L4-specific regulation of TnaA protein levels. These
results indicate that expression of the TnaA protein of the tnaCA8 operon is reduced upon ectopic expression of L4 in wild-type cells.

To address this, we investigated the steady-state levels of tnaC and tnaA transcripts in the wild-type (WT) N3433 strain upon ectopic L4 expression. We used three specific probes (Fig. 2A) derived from the tna operon (probe A [tnaA], probe B [tnaB], and probe C [tnac]) and detected a sharp increase in the levels of 3.1-kb (~3- to 5-fold) and 1.9-kb (~3-fold) transcripts relative to basal expression with the control plasmid (Fig. 2B, compare lanes 1 and 2). The 3.1-kb transcript was detected by all three probes, whereas only probes A and C strongly detected the 1.9-kb RNA transcript, indicating that the 1.9-kb RNA fragment is at the 5’ end of the tna transcript and encodes TnaC and TnaA but not TnaB. Next, we determined the impact of ectopic L4 expression on transcript stability. We found that the half-lives of the 3.1-kb and 1.9-kb RNA species were more stable upon L4 expression (half-lives increased from ~6 to ~9 min and ~13 to >13 min, respectively) (Fig. 2C, Fig. S2A, and Table S1). These data indicate that the L4-RNase E pathway can also regulate the stability of these RNA species.

Next, we characterized tna mRNA stability by rifampin treatment and determined the rate at which tna transcript disappeared over time by Northern blotting analysis. We used isogenic strains containing or lacking RNase E activity (i.e., under nonpermissive temperatures). We found that after shifting the temperature-sensitive N3431 strain, but not the WT N3433 strain, to a nonpermissive temperature (44°C), the stability of tna transcripts (the 3.1-kb and 1.9-kb RNA species) increased compared to the status of the strain at 32°C (Fig. 2D, compare lanes 7 to 12 with lanes 1 to 6; Fig. S2B; Table S1). In contrast, the 3.1-kb and 1.9-kb RNA species were quite unstable in the N3433 wild-type strain at 44°C (Fig. 2E, compare lanes 7 to 12 with lanes 1 to 6; Fig. S2C; Table S1). These data show that the 3.1-kb and 1.9-kb RNA species are stabilized in the RNase E temperature-sensitive strain under a nonpermissive temperature (44°C), indicating that degradation of the tna transcripts (3.1 and 1.9 kb) is dependent on RNase E enzymatic activity. Thus, the half-life of tna mRNA is longer when the activity of RNase E is reduced/depleted either by L4 inhibition or by temperature inactivation.
FIG 2 Northern blot analysis of tna transcripts in the presence or absence of ectopically expressed L4 in an RNase E wild-type E. coli strain (N3433) or an RNase E temperature-sensitive mutant [rne(Ts)] strain (N3431) grown at 32°C or 44°C. (A) The positions of the transcription unit and individual probes of the tnaCAB operon are shown. The antisense RNA probes A, B and C were synthesized using T7 RNA polymerase and used for Northern blot detection of their respective transcripts. The wavy lines show the extent of the full-length (3.1 kb) and intermediate (1.9 kb) tna transcripts. (B) Northern blot analysis of
L4 does not affect expression of tna transcriptional fusion constructs but downregulates translational fusion of the tnaC-tnaA transcribed spacer mRNA. We found that L4 expression increased steady-state levels of tna transcripts while simultaneously reducing TnaA protein levels. Whereas the increased transcript levels might be due to increased promoter activity and mRNA stabilization (Fig. 2), the decreased TnaA protein levels could result from L4-mediated translational repression. We investigated this mechanism by creating transcriptional and translational tnaCAB fusion operons with lacZ to determine β-galactosidase activity *in vivo* upon L4 ectopic expression.

We used a single copy number pMU575 vector for transcriptional fusion (22) of the tna promoter (nucleotides [nt] −123 to +1, Ptna-galK’-’lacZ’). For translational fusion of the tnaCAB operon with lacZ, a pMU2386 vector derived from pMU525 that lacks an appropriate translation start signal (23) was fused with the tna operon (nt −123 to +36, Ptna-tnaC’-’lacZ’) (Fig. 3A and B). We determined β-galactosidase activity in cells harboring the Ptna-galK’-’lacZ’ transcriptional fusion construct and found no significant differences in the presence or absence of ectopic L4 expression (Fig. 3A). Furthermore, there was no difference in β-galactosidase activity for bacterial cells carrying the translational fusion lacZ plasmid lacking the tnaC-tnaA spacer region, with or without ectopic L4 expression (Fig. 3B). This outcome suggests that regulatory elements downstream of the promoter in the tnaC-tnaA transcribed spacer region are responsible for L4-mediated downregulation of TnaA translation. We constructed an additional translational fusion plasmid, Ptna-tnaC-tnaA’-’lacZ’ (Fig. 3Ca), which produces mRNA transcripts encoding TnaC and a TnaA’-β-galactosidase fusion protein. The transcript also contains a spacer between tnaC and tnaA. We determined and compared β-galactosidase activities in cells harboring this translational fusion plasmid in the presence or absence of ectopic L4 expression. The results (Fig. 3Cb) show that upon ectopic L4 expression in cells harboring the Ptna-tnaC-tnaA’-’lacZ’ translational fusion construct β-galactosidase activity was significantly reduced by ~2.3-fold (Fig. 3Cb and quantitative data in Fig. 3E, compare control versus L4). In all cell cultures used for this study, levels of ectopically expressed L4 (tagged with an N-terminal Flag peptide) are similar (Fig. 3D). Our results indicate that ectopically expressed L4 can downregulate the Ptna-tnaC-tnaA’-’lacZ’ but not Ptna-tnaC’-’lacZ’ translational fusion construct and that L4 has no significant effect on the Ptna-galK’-’lacZ’ transcriptional fusion construct, together demonstrating that L4 regulation of TnaA synthesis requires the tnaCA spacer region.

L4 binds specifically to the tnaC-tnaA transcribed spacer *in vitro* and *in vivo*. L4 has multiple functional domains and is known to repress both transcription and translation of its S10 operon by binding to its 5’-UTR and 23S rRNA (7, 24, 25). Accordingly, it can potentially interact with diverse specific targets. Using purified FLAG-L4 protein and an [α-32P]UTP-labeled tnaC-tnaA spacer RNA fragment (~220 nt) in an electrophoretic mobility gel shift assay, we observed a slower-migrating band of stable L4-tnaC-tnaA transcribed spacer RNA complexes (Fig. 4Aa, lanes 2 to 7). L4 binds...
Determination of β-galactosidase activity in tna transcriptional and translational fusion constructs in the presence or absence of ectopically expressed L4.

(A) Schematic representations of the plasmids used for transcriptional (Aa) or translational (Ba and Ca) fusion studies and β-galactosidase assays to study the effect of L4 on transcription (Ab) or translation (Bb and Cb) of the β-galactosidase fusion constructs. We used pMU575 (22) for transcriptional fusion of the tna promoter (−123 to +1 of the Ptna-galK′-lacZ′ transcriptional fusion construct or TRC in panel Aa). Plasmid pMU2386 derived from pMU525 (23) was used for the construction of the tna translational fusion construct Ptna-tnaC−tnaA′-lacZ′, which lacks the tnaC-tnaA spacer RNA (TSL-I in panel Ba), or Ptna-tnaC-tnaA′-lacZ′ with the tnaC-tnaA spacer RNA (TSL-II in panel Ca). Derived tna operon coordinators of their respective translational fusion constructs are also indicated. RBS, ribosomal binding site. Transcriptional (pTRC) or translational (pTSL-1 or pTSL-II) fusion vectors were cotransformed with either pBAD-Flag-L4 or pBAD-Flag in N3433 cells. Plasmid-free N3433 was used as a control for background β-galactosidase activity. Flag-L4 was induced with 0.2% arabinose for 30 min (cultured at 32°C, 180 rpm). The β-galactosidase assay was performed according to the method of Miller (53) and is described in Materials and Methods.

β-Galactosidase activity was measured every 10 min for 80 min after adding the β-galactosidase substrate o-nitrophenyl-β-D-galactopyranoside (ONPG). N3433 with the plasmids for each assay are indicated. Error bars indicate ±SEM. (D) Western blot (a) and quantification (b) of ectopically expressed Flag-L4 protein levels in the above strains. TolB was used for normalization. n = 3; mean ± SEM; n.s., no significance after one-way ANOVA and Tukey’s post hoc test. (E) Quantification of β-galactosidase activity for transcriptional and translational fusion constructs. For quantification, data were taken at the 60-min time point after adding ONPG. Background β-galactosidase activity in N3433 was subtracted from each assay. n = 3; mean ± SEM; ****, P < 0.0001; n.s., no significance after one-way ANOVA and Tukey’s post hoc test.
FIG 4  L4 specifically binds to the transcribed spacer between tnaC and tnaA in vitro and in vivo. (A) Mobility gel shift assay of L4 and tnaC-tnaA spacer RNA interactions. In vitro transcription of [α-32P]UTP-labeled tnaC-tnaA spacer RNA (220 nt, 30,000 cpm, ~2 ng) and mobility gel shift assays were performed as described in Materials and Methods. (Aa) Mobility gel shift assay in the absence (lane 1) or increasing amounts (lanes 2 to 7; 0.7, 1.4, 2.1, 2.7, 4.1, and 5.5 μM, respectively) of FLAG-L4, mixed at 30°C for 20 min and separated in a nondenaturing 5% polyacrylamide gel. The positions of bound and free RNA species are indicated. (Ab) Binding affinity of the L4-tnaC-tnaA complex. L4 binds to tnaC-tnaA spacer with a high affinity (Kd ~2.1 μM). (Ba) Altered mobility of the tnaC-tnaA spacer RNA was determined in a competition assay using L4 binding to an excess of cold RNA competitors. Competitive binding of L4 to specific targets S10-UTR RNA (lanes 3 to 6) or 23S rRNA-DI (lanes 7 to 10) or to the nonbinding target rpsO-UTR RNA (lanes 11 to 13). The cold excess of L4-binding RNA was 12, 24, 36, and 48 ng, and for the nonbinding RNA it was 12, 24, and 36 ng, while maintaining a constant amount of radiolabeled tnaC-tnaA spacer RNA (30,000 cpm; ~2 ng). The competitor RNAs were in excess of ~6- to 24-fold (for L4-binding targets) or ~6- to 18-fold (for the nonbinding target) over that of the radiolabeled tnaC-tnaA spacer RNA. (Bb) Graph representing the percentage of remaining L4-tnaC-tnaA complexes competing with excess (as indicated (Continued on next page)
to the tnaC-tnaA spacer with an affinity of dissociation constant ($K_d$) = 2.1 μM (Fig. 4Ab). To ascertain if L4 specifically binds the tnaC-tnaA transcribed spacer RNA, we used the known L4-specific RNA targets S10-UTR (10) and 23S rRNA-DI (11) as positive competitors. Stelzl et al. (26) found that addition of an rpsO-UTR 218-nt RNA, which contains the interaction site for r-protein S15 (RpsO) that regulates translation of the rpsO operon, did not affect complex formation between the known L4-specific RNA target S10-UTR and r-protein L4 (RplD). Therefore, in this study, we used the rpsO-UTR as a nonspecific competitor and compared their competitive binding with that of L4 to the α-$^{32}$P-labeled tnaC-tnaA spacer RNA complex. In this competition experiment, increasing doses of cold S10-UTR RNA or 23S rRNA-DI successfully competed with L4 for binding of labeled tnaC-tnaA spacer RNA, leading to an increase in free α-$^{32}$P-labeled tnaC-tnaA spacer RNA fragments released from the bound complex (Fig. 4Ba, lanes 3 to 6 versus lane 2 for S10-UTR RNA and lanes 7 to 10 versus lane 2 for 23S rRNA-DI RNA). In contrast, addition of an increased concentration of cold nonspecific competitor rpsO-UTR did not significantly alter amounts of the labeled L4-tnaC-tnaA spacer RNA complex (Fig. 4Ba, lanes 11 to 13). Therefore, the nonspecific competitor (rpsO-UTR) did not successfully compete with the tnaC-tnaA spacer RNA for L4 binding. This competitive binding assay also shows that S10-UTR and 23S rRNA-DI are strong competitors in terms of their affinity for L4 (Fig. 4Bb). Our results experimentally demonstrate that L4 specifically binds within the tnaC-tnaA transcribed spacer region.

We performed an L4 coimmunoprecipitation (co-IP) assay (using specific antibodies against L4 protein) on N3433 cells at different growth stages and then performed reverse transcriptase PCR (RT-PCR) assays to demonstrate binding of L4 to the tnaC-tnaA spacer in E. coli cells. Using specific forward and reverse primers to detect the tnaC-tnaA spacer, we observed the expected 261-bp fragment in the L4 pulldown portion, and amounts of the 261-bp fragment were significantly increased in cells in stationary-phase growth (at optical density at 600 nm [OD$_{600}$] values of 0.4, 1.4, and 1.8, where the levels were highest at 1.8) (Fig. 4C, left panel). As a positive control, we generated the expected 177-bp fragment (Fig. 4C, middle panel) in the co-IP assay using specific forward and reverse primers to detect S10-UTR. In contrast, specific forward and reverse primers for the rpsO-UTR region did not generate any amplified fragments in the L4 pulldown portion upon RT-PCR (Fig. 4C, right panel), which is consistent with lack of interaction between L4 and rpsO-UTR. Collectively, our results suggest a possible mechanism by which L4 regulates TnaA translation by specifically binding to the tnaC-tnaA spacer RNA region.

**L4 differentially downregulates tnaA translation rather than tnaC translation in a cell-free transcription/translation system.** To investigate the possible role of L4 in downregulating translation of the tna operon through its binding to the tnaC-tnaA transcribed spacer region, we compared TnaA immunoblot signals after protein synthesis from two plasmids (a positive and negative control) to determine signal specificity for TnaA in a cell-free transcription/translation system. We first confirmed that TnaA protein synthesis occurs from the plasmid carrying the synthetic tna operon (positive control) and not from a negative-control plasmid encoding E. coli dihydrofolate reductase (DHFR) (Fig. S3Aa, lower panel) in which no TnaA was generated (Fig. S3Ab, lane 2 versus 1). We found that TnaA protein synthesis decreased in the presence of increased purified FLAG-L4 abundance (Fig. 5Ba, lanes 2 to 6; quantification data in Fig. 5Bb).

**FIG 4 Legend (Continued)** by the x axis) cold individual competitor RNA. (C) Polyclonal L4 antibody coimmunoprecipitation (co-IP) assay confirming complex formation of the tnaC-tnaA spacer RNA with L4 in E. coli N3433 cells. Cell cultures for L4 co-IP using L4 polyclonal antibody were collected at different cell growth stages (OD$_{600}$ values = 0.4, 1.4, and 1.8), the RNA was isolated, and then reverse transcription and PCR were performed as described in the Materials and Methods using specific primers for the tnaC-tnaA spacer region, S10-UTR (as an L4-binding positive control), and rpsO-UTR (a nonbinding control). The expected sizes of the PCR products for the tnaC-tnaA spacer (261 bp [lanes 2 to 3 of L4Ab in the left panel]) or S10-UTR (177 bp [lane 3 of L4Ab in the middle panel]) are shown by arrows, or there was no amplified product for rpsO-UTR (right panel). Each product was resolved on a 1.4% agarose gel and visualized after staining with ethidium bromide dye. The same experiment was performed on each RNA species but with preimmune (PI) serum (lanes 1 to 3), which resulted in no detectable signal. The protein A-Sepharose beads incubated with total lysate but without L4 antibody also showed no detectable signals (shown as –ve). The DNA size marker is indicated (Mbp).
FIG 5 L4 downregulates TnaA but not TnaC protein synthesis in an in vitro transcription/translation assay. (A) Schematic representation of a synthetic tna operon for in vitro transcription and translation with the PURExpress in vitro protein synthesis kit (New England BioLabs, UK). The upstream T7 promoter (P), ribosome binding site (RBS), NdeI and BamHI cloning sites, and the T7 terminator (TERM) downstream of the stop codon are indicated, respectively. Internal deletion variants (indicated by Δspacer, Δ5’-end, and Δ3’-end) of the tnaC-tnaA spacer in the synthetic tna operon were generated as described in the Materials and Methods and further used for in vitro transcription/translation in the absence and presence of FLAG-L4. (B) L4 downregulates TnaA protein synthesis. Shown are Western blot analysis (a) and quantitative data (b) for TnaA protein produced from the wild-type (wt) synthetic tna operon and FLAG-L4 upon detection by TnaA polyclonal antibody (H9251-TnaA) or FLAG peptide monoclonal antibody (H9251-FLAG) on 10% SDS-PAGE. (a) Increasing concentrations of FLAG-L4 (0.75, 1.5, 2.25, 3, and 3.75 μM, lanes 2 to 6) inhibited TnaA translation. Lane 1 shows the reaction control (without FLAG-L4). (C) Comparison of RNA sequences of tnaC-tnaA spacer (220nt) and S10 upstream regions (172nt) by means of a CLUSTAL O (1.2.4) multiple sequence alignment. Solid and dotted lines box nucleotides involved in RNA binding of L4 with S10 UTR to regulate transcription and both transcription and translation, respectively (11, 53, 57). Asterisks indicate identical nucleotides. The Shine-Dalgarno (SD) sequence of TnaA is shown in red. (D to F) The inhibitory effect of L4 on the synthesis of TnaA but not TnaC by the synthetic tna operon. Shown are Western blots of TnaA (Da) and a 15% Bis-Tris polyacrylamide gel (Ea) of TnaC produced from the wt and various deletion constructs (see panel A) of the synthetic tna operon in the absence of L4 (lanes 1, 3, 5, and 7) compared with a positive-control plasmid in the presence of L4 (lanes 2, 4, 6, and 8). The reaction was stopped using 2X SDS loading dye 60 min after adding template DNA, and we then conducted 10% SDS-PAGE and detection with antibodies against FLAG peptide and TnaA or a 15% Bis-Tris polyacrylamide gel to resolve the very small molecular weight TnaC protein (~3 kDa). The concentration of FLAG-L4 was 1.5 μM. The TnaC protein was further confirmed by peptide sequencing. (Db and Eb) Quantification of the data obtained in panels Da and Ea is presented, and TnaA and TnaC protein levels were compared from different spacer deletion constructs as indicated. n = 3; (Continued on next page)
Because r-protein L4 regulates translation by binding to an mRNA sequence/structure in the S10 operon that mimics the binding sites for the r-protein on rRNA, we investigated whether some kind of similar molecular mimicry also existed in the tnaCA spacer RNA. We compared the RNA sequences of the tnaC-tnaA spacer (220 nt) and the S10 upstream region (172 nt) by means of CLUSTAL O (1.2.4) multiple sequence alignment (27), and found that the tnaC-tnaA spacer and S10 upstream regions exhibit >50% identity (Fig. 5C), suggesting that molecular mimicry may be the mechanistic basis for L4 binding to the tnaC-tnaA spacer region.

We constructed different deletion mutants of the tnaC-tnaA spacer region (but with retention of the ribosomal binding sequences upstream of tnaA) and performed in vitro translation studies. The resulting Δspacer (as a negative control), Δ5′-end (deletion of the 5′ half of the spacer region, from nt 1 to 110), and Δ3′-end (deletion of the 3′ half of the spacer region, from nt 111 to 207) mutants (Fig. 5A) allowed us to determine the RNA-interacting region of L4 protein that regulates TnaA translation. Our results show that the wild type and the Δ5′-end and Δ3′-end mutants exhibited similarly reduced TnaA translation in the presence of L4 (Fig. 5Da, compare lanes 2, 6, and 8 with lanes 1, 5, and 7; quantification data in Fig. 5Db). As expected, TnaA translation was not reduced in the presence of L4 for the Δspacer mutant (Fig. 5Da, compare lanes 3 and 4 with lanes 1 and 2; quantification data in Fig. 5Db). We confirmed the reduction of TnaA synthesis by means of time-dependent experiments (Fig. 5Fa, compare lanes 1 to 5 with lanes 6 to 10; quantification data in Fig. 5Fb).

We hypothesized that if L4 binds within the tnaC-tnaA transcribed spacer region to inhibit TnaA translation, L4 should not affect translation of the leader peptide TnaC that lies upstream and proximal to the transcribed spacer. Indeed, in vitro translation of TnaC occurred for all constructs, both in the presence and absence of FLAG-L4 (detected in a Bis-Tris acrylamide gel [Fig. 5E]). The Coomassie blue-stained gel revealed migration of a protein of molecular weight similar to TnaC (2.894 kDa) under both conditions, which we subsequently confirmed as TnaC by tandem mass spectrometry (MS-MS) protein sequencing. Additionally, we assessed translation of DHFR from a control plasmid containing the DHFR template in the presence of the highest concentration of L4 (3.75 μM) and found that L4 does not have any effect on DHFR translation (Fig. S3B). Thus, translational inhibition of TnaA by L4 in the tna operon is very specific and does not affect translation of the TnaC leader peptide.

**Binding of L4 in the tnaC-tnaA transcribed spacer results in structural alteration of the RNA.** Our in vitro RNA binding and in vitro translation assays demonstrated that L4 binds to the tnaC-tnaA transcribed spacer region in the tnaCA operon to regulate TnaA translation. Regulatory regions of bacterial RNA undergo conformational changes in response to RNA-protein interactions (28).

We therefore investigated whether there were conformational changes to the tnaC-tnaA spacer RNA induced by RNA-protein interactions in the presence of L4 (Fig. 6A). Secondary structure predictions of the tnaC-tnaA spacer reveal highly paired regions and a helical structure (Fig. 6B and C). We used RNase V1 (which preferentially cleaves paired regions of structured double-stranded RNA) to probe a fragment of the tnaC-tnaA spacer (220 nt) spanning the 35th nucleotide of the 5′ end of the spacer RNA and the 33rd nucleotide of the tnaA coding region, thereby retaining the ribosomal binding sequences upstream of tnaA. Transcribed RNA was labeled at the 5′ end and then probed with RNase V1 in the absence (Fig. 6B, lanes 4, 12, and 19) or presence (Fig. 6B, lanes 5 to 7, 13 to 15, and 20 to 22) of increasing concentrations of FLAG-L4.

**FIG 5 Legend (Continued)**

mean ± SEM; ****, P < 0.0001; n.s. represents no significance after one-way ANOVA and Tukey’s post hoc test. (F) Time-dependent inhibition of TnaA protein synthesis by L4. Shown are Western blot analysis (a) and quantitative data (b) of TnaA protein produced from the WT and various deletion constructs (see panel A) of the synthetic tna operon in the absence of L4 (lanes 1 to 5) compared with a positive-control plasmid in the presence of L4 (lanes 6 to 10). The concentration of FLAG-L4 was 1.5 μM. Aliquots for SDS-PAGE were withdrawn every 10 min for 60 min after addition of template DNA, and then we conducted 10% SDS-PAGE and detection with antibodies against TnaA. The faint bands in lane 1 are from nonspecific binding by use of polyclonal anti-TnaA. Error bars indicate ±SEM.
FIG 6 Structural probing of the 5'-end-labeled tnaC-tnaA transcribed spacer in the absence and presence of FLAG-L4. (A) DNA template (239 bp) containing the tnaC-tnaA spacer was transcribed under the T7 promoter to generate a 220 nt final RNA product. The sequence of the final 220-nt RNA product (with addition of a G at the 5' end by RNA polymerase during in vitro transcription) is shown. The Shine-Dalgarno (SD) sequence of TnaA is underlined. Affected regions I, II, and III in the footprint determination are indicated in the primary sequence. The numbers at the top of the primary sequence represent the RNase T1 cleavage sites between guanosine 3'-phosphate residues and the 5'-OH residues of adjacent nucleotides, as shown in panel B (lanes 3, 11 and 18). The gray font in the primary sequence from the 3' end shows the beginning of the coding sequence for TnaA. (B) The RNA was further 5'-end labeled with [γ-32P]ATP and purified from the denatured acrylamide gel. The 5'-end-labeled RNA (30,000 cpm, 11011 2 ng) was then incubated at room temperature in the absence (un) or presence (L4) of L4 (5.5 μM) for binding, before conducting RNase V1 digestion (double strand-specific cleavage; lanes 5 to 7, 13 to 15, and 20 to 22) and separation on an 8% polyacrylamide–8 M urea gel. The 5'-end-labeled RNA fragments generated by alkaline and RNase T1 hydrolysis (OH and T1, respectively) are shown. The experiments were carried out on untreated RNA (un) or in the presence of increasing concentrations of purified FLAG-L4. Regions I, II, and III affected by L4 in the footprint determination are indicated (also in panel C). Regions a, b, and c used for quantification are indicated with colored lines. Bands (N) used for normalization of the phosphor image signals of regions a, b, and c are indicated. (C) The 220 nt RNA secondary structure predicted by RNAfold (56) (dG = -44.05). (D) Quantification of regions a, b, and c affected by L4 in the footprint determination.
Denaturing polyacrylamide gel electrophoresis revealed that the region between nucleotides G17 to A41 of the \textit{tnaC-tnaA} spacer fragment is not accessible for RNase V1 cleavage because of L4 binding (Fig. 6B, marked as region III; quantification data in Fig. 6D). Regions corresponding to nucleotides U72 to U79 and G99 to U108 (stem-loop regions) were highly prone to RNase V1 cleavage in the presence of L4 (Fig. 6B, marked as regions I and II, respectively; quantification data in Fig. 6D), perhaps because conformational alteration of region III rendered regions I and II more accessible to RNase VI cleavage (Fig. 6C). Together, these data show that structural changes induced by L4 binding in the \textit{tnaC-tnaA} transcribed spacer region may result in decreased TnaA synthesis/translation levels.

\textbf{E. coli fine-tunes posttranscriptional expression of its TnaA protein during early stationary growth phase.} We assessed \textit{in vivo} expression of TnaA protein during two stages of \textit{E. coli} N3433 growth (log and early stationary phase) (Fig. 7A and B). We showed that \textit{tnaA} mRNA and TnaA protein levels do not vary in a coordinated way. During log phase growth, \textit{tnaA} mRNA expression was much lower than for early stationary phase (Fig. 7C, compare \textit{tnaA} mRNA levels for OD$_{600}$ of $-0.4$ at t2 and OD$_{600}$ of $-1.1$ at t5). Quantitative results showed that levels of the \textit{tnaA} 1.9- and 3.1-kb mRNA species in early stationary growth phase were increased by 3.1- and 6.2-fold (Fig. 7D), respectively, compared to log phase. However, corresponding TnaA protein levels did not increase concomitantly by 9-fold (only by 1.2-fold [Fig. 7D and Ab]) during early stationary growth phase compared to log phase. These data provide evidence that posttranscriptional regulation can fine-tune TnaA protein expression in response to cell growth.

\section*{DISCUSSION}

Certain r-proteins have been reported as having extraribosomal functions relating to various biological processes or functions in mammals (29, 30), yeast (31–33), and bacteria (3, 4, 34). However, there are some functions of r-proteins unrelated to ribosome function that remain to be determined (2, 3, 35). r-proteins are an integral part of the translation machinery and are responsible for stabilizing rRNA structure (36, 37). However, some r-proteins in their free form exert extraribosomal functions (such as in translational repression, transcriptional regulation, mRNA stability, DNA repair and replication, and phage RNA replication) by binding RNA and interacting with specific cellular proteins (3). In this study, we have identified a novel extraribosomal function for the r-protein L4. We have shown that ectopically expressed L4 binds to the \textit{tnaC-tnaA} transcribed spacer region, induces conformational changes in the spacer RNA, and affects adjacent \textit{tnaA} translation, resulting in decreased levels of TnaA protein. Our data suggest that L4 tends to bind to \textit{tnaC-tnaA} mRNA by means of molecular mimicry (Fig. 5). We have also shown that endogenous L4 binds to the \textit{tnaC-tnaA} spacer RNA transcripts in early stationary phase but not in exponentially growing \textit{E. coli} cells and that \textit{tnaA} mRNA abundance and TnaA protein levels are not positively correlated. The increase in early stationary-phase \textit{tnaA} mRNA levels were 6-fold higher in early stationary phase than in log phase, but TnaA protein levels did not change in parallel. Our study reveals a possible mechanism by which binding of L4 to the \textit{tnaC-tnaA} spacer mRNA may interfere with translation, thereby inhibiting TnaA protein synthesis throughout cell growth. Our study also suggests that L4, which is known to regulate its own operon, is a multifaceted protein that can precisely control the levels of various proteins through its binding affinity for RNA or protein, allowing \textit{E. coli} to survive diverse physiological conditions.

L4 regulates RNase E activity and stabilizes transcripts to increase their protein levels (4). It also binds to cellular RNAs such as S10-UTR to repress translation by interfering with translation initiation (7, 11). It was not known previously that the \textit{tnaA} transcript is regulated by L4. The half-life of the 3.1-kb or 1.9-kb \textit{tnaA} transcript increased by only 25% to 50% upon ectopic L4 expression (Fig. 2C). Since the N3433 strain we used in this study contained \textit{relA1} and \textit{spoT1} mutations that would tend to reduce ppGpp levels, it is possible that greater regulation would occur in a wild-type strain able to produce...
Fig 7 Posttranscriptional regulation of the tna operon during early stationary-phase growth can fine-tune TnaA levels. (Aa) TnaA protein expression levels. N3433 bacterial cells were grown in LB medium at 32°C (with 180 rpm) and 1 ml of culture was harvested for Western blot analysis (10% SDS-PAGE) at the indicated OD$_{600}$ (represented at the corresponding time points in the parentheses starting from t2 to t5) using anti-TnaA, -L4, and -Eno antibodies. (Ab) Quantitative data for TnaA, L4 and enolase (Eno) expression levels shown in the Western blot of panel Aa. Western blots of endogenous TnaA, L4, and Eno were detected from cultured cells at the indicated t2 (OD$_{600}$ = 0.40) to t5 (OD$_{600}$ = 1.1) growth phase. The integrated band density of individual proteins obtained from the Western blot signals for antibody detection against TnaA, L4, and Eno were plotted for each time point (t2 to t5) as shown. (B) Semilogarithmic plot of E. coli strain N3433 as observed for more than 6 h after culture inoculation (t0, OD$_{600}$ value of 0.02). The time point t1 indicates a culture sample collected after 2 h of inoculation (OD$_{600}$ of 0.2), and culture samples were collected (Continued on next page)
high levels of ppGpp. However, despite the increased abundance of tna transcripts in our system, TnaA protein levels were reduced in the presence of ectopically expressed L4. We show that free L4 downregulates translation but not transcription of TnaA (Fig. 3), with the former function dependent on interactions of L4 with tnaC-tnaA spacer RNA. We detected an increase in the stability of tna transcripts (the 3.1-kb and 1.9-kb RNA species) when temperature-sensitive rne [rne(Ts)] N3431 cells were shifted to a nonpermissive temperature (44°C) compared to cells maintained at a permissive temperature (32°C) (Fig. 2D and E). Moreover, TnaA protein levels increased (Fig. 1C) with an increase in tna transcript abundance under those conditions (Fig. 2D and E). These results indicate that E. coli uses the extraribosomal function of L4 to respond to certain growth conditions. In this study, we have demonstrated that endogenous L4 binds to the tnaC-tnaA spacer during early stationary growth of E. coli (Fig. 4).

We generated constructs with various deletions in the tnaC-tnaA spacer region and used them to establish the L4-RNA interaction region by in vitro transcription/translation (Fig. 5). We found that deletion of either the 5' or 3' half of the spacer region between tnaC and tnaA resulted in a similar reduction in TnaA translation in the presence of L4 compared to WT, suggesting that a L4 binding site(s) is located within both halves of the spacer region. The process by which L4 binding to the tnaC-tnaA spacer impacts downstream tnaA mRNA translation, ribosome occupancy, and ribosome density is still unknown.

Our results highlight the importance of the extraribosomal function of L4 in translational regulation of TnaA, which may be involved in the physiological transition of E. coli cells into early stationary phase. TnaA primarily degrades l-tryptophan to indole, pyruvate, and ammonia (38). It has also been shown to exhibit l-cysteine desulfhydrase (CD) activity to catalyze degradation of l-cysteine to pyruvate, ammonia, and sulfide (39, 40). During the exponential/early stationary phase of growth, E. coli may preferentially maintain low levels of TnaA protein, despite there being high levels of corresponding mRNA, since amino acids like tryptophan and cysteine are needed to sustain cell growth during the stationary or late stationary phases of E. coli cell growth. During the early stationary phase of bacterial cell growth, free-form L4 may bind to the tnaC-tnaA transcribed spacer region to negatively control subsequent TnaA protein translation, thereby maintaining low levels of TnaA protein and preventing rapid degradation of tryptophan/cysteine. This layer of regulation may be necessary since tryptophan levels in the cell are known to control transcription of the trp operon for tryptophan synthesis via a negative feedback loop (41, 42). Furthermore, ribosomes undergo degradation during the stationary phase of cell growth (43, 44), which can generate free-form L4 to fine-tune TnaA protein levels.

RNA-binding proteins are important regulators of gene expression at the posttranscriptional level. The RNA-binding activity of r-proteins and their ability to exist in a free-form state render them suitable for performing extraribosomal functions. Here, we present a previously undocumented extraribosomal function for the r-protein L4 that is important for responses to physiological growth conditions (Fig. 8A). Our study further confirms the multifaceted nature of L4, as summarized in Fig. 8B, wherein L4 can interact with rRNA (ribosome-bound form) (11), bind to its own S10-UTR to control transcription and translation (free-form) (10), and bind to the tnaC-tnaA transcribed spacer region to inhibit TnaA translation (this study). L4 also interacts with specific cellular proteins, including RNase E (14). Thus, L4 can distinguish its specific transcripts in a cellular pool of mRNAs through one or more of its diverse functional domains and

**FIG 7** Legend (Continued)

thereafter at different time points (t2 to t10). (C) Aliquots (50 ml) of bacterial cells from log phase (t2, OD600 = ~0.4) to early stationary phase (t5, OD600 = ~1.1) were collected and their tna transcripts were examined by Northern blotting analysis. The 3.1-kb and 1.9-kb tna RNA transcripts, as well as 16S rRNA, are shown. The OD value of each sample is shown above the gel. (D) Fold change in abundances of tna mRNA and TnaA protein. To calculate fold change, the band intensity of an individual tna transcript detected in panel C, i.e., 3.1 kb or 1.9 kb, and the TnaA protein detected in panel A were quantitated using VisionWorks LS software.
subsequently execute its unique extraribosomal roles either alone or in a complex cellular machinery.

Tryptophan-induced transcription antitermination is known to regulate the tna operon in *E. coli* (45, 46). This mechanism requires synthesis of the tryptophan-containing leader peptide TnaC, TnaC-tRNA(Pro), and an inducing (excess) level of tryptophan. The nascent TnaC-peptidyl-tRNA(Pro) is retained in the ribosome, resists peptidyltransferase cleavage, stalls at the TnaC stop codon, and prevents Rho factor from entering the *rut* binding site on the *tnaC* transcript, thereby preventing transcription termination (47). The downstream-paused RNA polymerase restores transcription of the *tna* operon genes (*tnaA* and *tnaB*). The *tnaC-tnaA* spacer region, which separates the *tnaC* stop codon from the *tnaA* start codon, contains several transcriptional pause sites that can serve as regulated sites of Rho-dependent transcription termination (46). In the present study, we show that the *tnaC-tnaA* transcribed spacer RNA is the binding site of the *r*-protein L4 responsible for regulating translational levels of TnaA. (B) The multifaceted nature of the *r*-protein L4 as represented by L4 protein-protein or L4 protein-RNA interactions. References for the various functions of L4 are indicated below.

Our discovery, together with the previously known regulatory mechanisms of the *tna* operon, provide multiple strategies for bacterial cells to prevent accumulation of high levels of tryptophanase. High levels of tryptophanase would degrade cell-synthesized tryptophan, resulting in a tryptophan deficiency that would induce the
tryptophan operon, which might inhibit cell survival under various physiological conditions.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and antibodies.** *E. coli* K-12 strain N3433 (45) encoding full-length RNase E was used to determine RNA stability, RNase E-dependent regulation of protein expression by L4, and detection of endogenous RNase E and Flag-L4. *E. coli* strain BL21(DE3) (pET15b) (46) encoding a truncated version of RNase E (amino acids 1 to 585) was used to purify Flag-L4. RNase E temperature-sensitive [rne(Ts)]N3431 strain (47, 48) at the permissive (32°C) and nonpermissive (44°C) temperatures was used for the analysis of transcripts by Northern blotting.

The plasmid pPWflagL4 was constructed as described previously (4) and was used to express Flag-L4. *E. coli* ASKA JW3686 clone (49) encoding tnaA tagged with histidine at its N terminus and having the IPTG-inducible promoter pT5/lac was used for TnaA protein purification to generate polyclonal rabbit antibody. RNase E polyclonal antibody was generated previously in our laboratory (50). Monoclonal antibodies for FLAG-M2 (Sigma, USA) and RpoS (Abcam, USA) were purchased commercially. Antibodies for Lon and CspE were a generous gift from Susan Gottesman (NIH, USA) and Narendra Jawal (BARC, India), respectively.

**Northern blot analysis.** Bacterial cell cultures were grown at 32°C to an OD_{600} of ~0.3 and total RNA was isolated from the steady-state cell cultures after ectopic expression of L4 (induced with 0.5 mM IPTG) for 30 min. To analyze the effect of L4 expression on tnaA mRNA stability, cell cultures were collected at different time points after the addition of rifampin (0.5 mg/ml) as described previously (4). The temperature was increased from 37°C to 44°C for 30 min to inactivate RNase E in the rne(Ts) mutant before rifampin treatment. Total RNAs were fractionated on 0.8% agarose-formaldehyde gels. The fractionated RNA was further transferred to Zeta-Plate blotting membranes (Bio-Rad) and separately hybridized with RNA probes designed from all regions of the tnaA operon (Fig. 2A). Probe labeling with α[^32]P probe synthesis using an *in vitro* transcription kit (MAXIscript, Ambion). Northern signal visualization, and quantification were conducted as described previously (4) and according to the manufacturer’s instructions. RNA half-lives were determined by nonlinear regression curve fitting (one phase decay) using GraphPad Prism. We used following the parameters: (i) least-squares (ordinary fit); (ii) confidence level of 95%; (iii) asymmetrical (likelihood) CI; (iv) goodness of fit quantified with R square; and (v) convergence criteria, medium. DNA templates for transcription of individual RNA probes were generated by PCR using gene-specific primers (see Table S2 in the supplemental material).

**SDS-PAGE and Western blot analysis.** Total lysates were prepared from the control plasmid (pPWflagL4) only and from the N3433 strain after L4 expression induced with IPTG (0.5 mM) for 30 min. Electrophoresis of protein samples was carried out as described previously (51). For Western blot analysis, proteins were separated in 10% SDS polyacrylamide gels and transferred onto PVDF membranes using a semidyam apparatus (ATTO Corp.). After blocking, the membranes were cut into smaller pieces and then probed with specific antibodies for TnaA, RNase E, Flag-L4, and TolB (as a loading control). Incubation of secondary anti-IgG antibodies, as well as detection and visualization of protein bands, was conducted as described previously (4). Assays were performed with primary rabbit (anti-L4, anti-RNase E, anti-TnaA, anti-CspE, anti-Lon, and anti-TolB), and mouse (anti-M2 for FLAG-L4 and anti-RpoS) antibodies.

**Protein purification of Flag-L4 and His-TnaA.** Protein purification of Flag-L4 (~23 kDa) was performed as described previously (4). Briefly, clear lysate from *E. coli* strain BL21(DE3) (pET15b) containing Flag-tagged protein was loaded on an anti-M2 affinity column (Sigma) preequilibrated in buffer A (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl). Protein was purified using 1 ml of a solution of synthetic FLAG oligopeptide (1 mg/ml) (PAN Laboratory, Stanford University). To generate tryptophanase (TnaA, 52.7 kDa) polyclonal antibody, we used the ASKA JW3686 clone (49) for TnaA protein purification. Bacterial culture, induction with IPTG (0.1 mM), and His-tagged protein purification was performed as per the ASKA clone description (49). Briefly, clear lysates obtained after cell lysis were loaded onto a Ni-NTA affinity chromatography column (Thermo Fisher Scientific, USA) and washed with washing buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 5 mM imidazole) until the OD_{280} of flow-thru reached ~0.5. Finally, proteins were eluted with buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 250 mM imidazole). The highly purified protein was dialyzed and concentrated using an Amicon ultra column (Millipore). Purified TnaA protein quality (22.7 kDa) was checked on 10% SDS-PAGE. Purified TnaA protein was then used for rabbit immunization to generate TnaA polyclonal antibody under the supervision of the IMB Animal Facility (Academia Sinica) using a standard procedure.

**Mobility gel shift assay.** To examine RNA-protein interactions between the tnaC-tnaA transcribed spacer region and the r-protein L4, we prepared DNA template of this transcribed spacer region using specific PCR primers (Table S2), with a T7 promoter incorporated into the reverse primer. RNA template was in *vitro* transcribed (261 nt comprised of 20 nt upstream and 20 nt downstream of the spacer [220 nt] plus one “G” added by RNA polymerase from the 5’ end) and internally labeled with [α-32P]UTP in an *in vitro* transcription assay as per the vendor’s instructions (MAXIscript T7 transcription kit; Ambion). RNA templates for S10-UTR, 23S rRNA domain I and L4 nontarget rpsO-UTR were also prepared using gene-specific primers (Table S2) before undergoing *in vitro* transcription and internal labeling to generate final products of 172, 182, and 164 nt, respectively. Mobility gel shift assays were performed as described previously (52). Briefly, full-length radioisotope-labeled tnaC-tnaA transcript was purified from the denaturing gel and incubated in the absence of or increasing concentrations of Flag-L4. Reaction mixtures were incubated at 30°C for 20 min and were stopped by adding nondenaturing sample loading buffer (1 mM EDTA, 50% [vol/vol] glycerol, 0.025% xylene cyanol, and 0.025% bromophenol blue).
Reaction products were analyzed by electrophoresis on nondenaturing 5% polyacrylamide gels followed by exposure to X-ray film (Kodak, Thermo Fisher Scientific, USA).

Binding affinity analysis of the L4-tnaC-tnaA complex was performed with a fixed concentration of 32P-labeled tnaC-tnaA spacer RNA (30,000 cpm, 2 ng) and increasing concentrations of L4 protein (0.7, 1.4, 2.1, 2.7, 4.1, and 5.5 μM). The RNA bands in the gel were detected using a Typhoon FLA 9000 Biomolecular Imager (GE Healthcare). The integrated band intensity for L4-tnaC-tnaA complex was quantitated by VisionWorks LS software based on the intensity of signals obtained by scanning the gel. For the \( K_d \) calculation, we used nonlinear regression performed in GraphPad Prism software to fit the data to a simple binding equation.

β-Galactosidase activity assay. To create the transcriptional fusion construct of the tna promoter (Ptna-gal-\( \alpha \)-lacZ), we used pMU575 vector (single copy number lacI Replicon with a promoterless lacZ gene and trimethoprim resistance) (22); pMU575 is a galK-lacZ‘YA’ fusion vector in which the amino terminus of the galK gene, containing all the translational start signals but no promoters, is fused in phase with the eighth codon of the lacZ gene. The tna promoter sequence (123 nt upstream from the transcriptional start site in the tna operon) was amplified using specific forward (including a BamHI site) and reverse (including a HindIII site) primers, generating a PCR product of ~150 bp (Table S2). PCR product and the vector pMU575 were then digested with BamHI and HindIII restriction enzymes. Reaction mixtures after restriction digestion were cleaned, ligated, and transformed into DH5α.

To create the transcriptional fusion constructs of the tna promoter (Ptna-tnaC-lacZ′) or with the tnaC-tnaA spacer (Ptna-tnaC-tnaA-lacZ′), we used pMU2386 derived from pMU525 that lacks the appropriate translation start signal (23). The tna promoter sequence of the tna operon (nt −123 to +36 for Ptna-tnaC-lacZ′ or −123 to +288 for Ptna-tnaC-tnaA-lacZ′) was amplified using specific forward (including a HindIII site) and reverse (including a BamHI site) primers (Table S2), generating PCR products of ~180 bp. These were then digested with BamHI and HindIII restriction enzymes and cloned into pMU2386. Positive transformants for the tna transcriptional and translational fusion constructs were screened against trimethoprim (40 μg/ml) and positive sequences were confirmed by sequencing using the LacZ-Seq primer (Table S2) designed from the β-galactosidase gene. The tna transcriptional and translational fusion constructs were then separately cotransformed with either pBAD-Flag only (control plasmid) or pBAD-Flag-L4 plasmids in strain N3433, and transformants were selected against ampicillin (50 μg/ml) and trimethoprim (40 μg/ml). The respective positively transformed bacterial cells were first grown overnight and then subcultured in LB medium to grow to OD600 of 0.3 to 0.4 before being induced with 0.2% arabinose for L4 expression for 30 min at 32°C × 180 rpm. For the control experiment, we used N3433 cells without vectors, which contain a Δ55-DHFR DNA with appropriate restriction enzymes (NdeI and BamHI). Internal deletion variants (Δspacer, ΔS10-UTR, ΔtnaC-tnaA transcribed spacer RNA) of the tna operon (comprising 1,243 bp harboring tnaC, the transcribed spacer region, and the initial 750 bp of tnaA) was constructed using specific primers (Table S2) after excising the DHFR DNA with appropriate restriction enzymes (Ndel and BamHI). Internal deletion variants (Δspacer, ΔS5′-end, and ΔS3′-end) of the tnaC-tnaA spacer were generated by inverse PCR (55) using specific primers (Table S2). L4-mediated inhibition of TnaA translation was achieved using plasmid DNA (containing the synthetic tna operon) as the template and according to the manufacturer’s instructions (PUREexpress in vitro protein synthesis kit; New England Biolabs, UK). Fixed (1.5 μM) or increasing concentrations (from 0.75 to 3.75 μM) of FLAG-L4 were added to the in vitro translation assay. Reactions were stopped by adding SDS-PAGE sample loading dye and further fractionated on a 10% SDS denaturing polyacrylamide gel. The levels of TnaA and L4 in the in vitro translation assays were detected by Western blotting using the specific antibodies described above in “SDS-PAGE and Western blot analysis.”

Structural probing of tnaC-tnaA transcribed spacer RNA. DNA template (239 bp), corresponding to 186 bp of the transcribed spacer region between tnaC and tnaA and 33 bp of the coding region of tnaA, was amplified using specific primers (Table S2). The DNA template was transcribed under the T7 promoter (20 bp) to generate RNA transcripts of 220 nt in length (with an additional “G” added by RNA polymerase) (Ambion). The transcript was further purified to remove unincorporated nucleotides using G50 spin columns (GE Healthcare). We dephosphorylated the 5′ end of the RNA using alkaline phosphatase and 5′ end labeled it with γ-32P-ATP before purifying it from a denaturing acrylamide gel. The
5’-end-labeled inoC-tnaA RNA was incubated in the absence or presence of L4 at room temperature for r-protein binding, before being subjected to enzymatic digestion. RNase T1 (5 U/µl; Ambion), which cleaves single-stranded regions after G, was titrated to obtain a 1:800 dilution and incubated at 50°C for 15 min in 1X sequencing buffer (20 mM sodium citrate [pH 5], 1.0 mM EDTA, 7 M urea). RNase V1 (0.1 U/µl; Ambion), which cleaves paired regions, was titrated to obtain a 1:100 dilution and incubated at room temperature (25°C) in 1X RNA structural buffer (100 mM Tris [pH 7], 1.0 M KCl, 100 mM MgCl2; Ambion). The 5’-end-labeled RNA was treated with alkaline hydrolysis buffer (50 mM sodium carbonate [pH 9.2], 1.0 mM EDTA; Ambion) at 95°C for 3 min to generate an alkaline ladder. Reactions were stopped with an equal volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and further fractionated on 8% polyacrylamide–8 M urea gels, run at 26 W for various time frames, and visualized by PhosphorImager (FLA-5000; FUJIFILM). The secondary structure of RNA used for structural probing was predicted using the Mfold web server (http://mfold.rna.albany.edu/frames, and visualized by PhosphorImager (FLA-5000; FUJIFILM). These secondary structures of RNA were predicted using the Mfold web server (http://mfold.rna.albany.edu/frames) and visualized by PhosphorImager (FLA-5000; FUJIFILM).

Quantification and statistical analysis. Integrated band intensity was quantitated using VisionWorks LS software based on the intensity of signals obtained by scanning the gels, membranes, or phosphor image plates. Optical density (OD) for β-galactosidase activity assays was obtained using a Prema PRO-739 visible spectrophotometer. Statistical significance was determined using one-way ANOVA and Tukey’s post hoc tests. All statistical tests were performed using GraphPad Prism version 8.0.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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