



# A New Mechanism for Ribosome Rescue Can Recruit RF1 or RF2 to Nonstop Ribosomes

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**ABSTRACT** Bacterial ribosomes frequently translate to the 3' end of an mRNA without terminating at an in-frame stop codon. In all bacteria studied to date, these "nonstop" ribosomes are rescued using *trans*-translation. Genes required for *trans*-translation are essential in some species, but other species can survive without *trans*-translation because they express an alternative ribosome rescue factor, ArfA or ArfB. *Francisella tularensis* cells lacking *trans*-translation are viable, but *F. tularensis* does not encode ArfA or ArfB. Transposon mutagenesis followed by deep sequencing (Tn-seq) identified a new alternative ribosome rescue factor, now named ArfT. *arfT* can be deleted in wild-type (wt) cells but not in cells that lack *trans*-translation activity. Overexpression of ArfT suppresses the slow-growth phenotype in cells lacking *trans*-translation and counteracts growth arrest caused by *trans*-translation inhibitors, indicating that ArfT rescues nonstop ribosomes *in vivo*. Ribosome rescue assays *in vitro* show that ArfT promotes hydrolysis of peptidyl-tRNA on nonstop ribosomes in conjunction with *F. tularensis* release factors. Unlike ArfA, which requires RF2 for activity, ArfT can function with either RF1 or RF2. Overall, these results indicate that ArfT is a new alternative ribosome rescue factor with a distinct mechanism from ArfA and ArfB.

**IMPORTANCE** *Francisella tularensis* is a highly infectious intracellular pathogen that kills more than half of infected humans if left untreated. *F. tularensis* has also been classified as a potential bioterrorism agent with a great risk for deliberate misuse. Recently, compounds that inhibit ribosome rescue have been shown to have antibiotic activity against *F. tularensis* and other important pathogens. Like all bacteria that have been studied, *F. tularensis* uses *trans*-translation as the main pathway to rescue stalled ribosomes. However, unlike most bacteria, *F. tularensis* can survive without any of the known factors for ribosome rescue. Our work identified a *F. tularensis* protein, ArfT, that rescues stalled ribosomes in the absence of *trans*-translation using a new mechanism. These results indicate that ribosome rescue activity is essential in *F. tularensis* and suggest that ribosome rescue activity might be essential in all bacteria.

**KEYWORDS** *Francisella tularensis*, ribosomes, Tn-seq, *trans*-translation

Bacterial ribosomes frequently translate to the 3' end of an mRNA that does not have a stop codon (1–3). These "nonstop" ribosomes cannot terminate translation using one of the canonical termination factors, RF1 or RF2, because they require interactions with the stop codon to activate peptidyl-tRNA hydrolysis (4, 5). Data from *Escherichia coli* indicate that 5% to 10% of ribosomes that initiate translation do not terminate translation at a stop codon on the mRNA and instead have to be rescued (2, 3). The primary ribosome rescue pathway in all bacteria that have been investigated is

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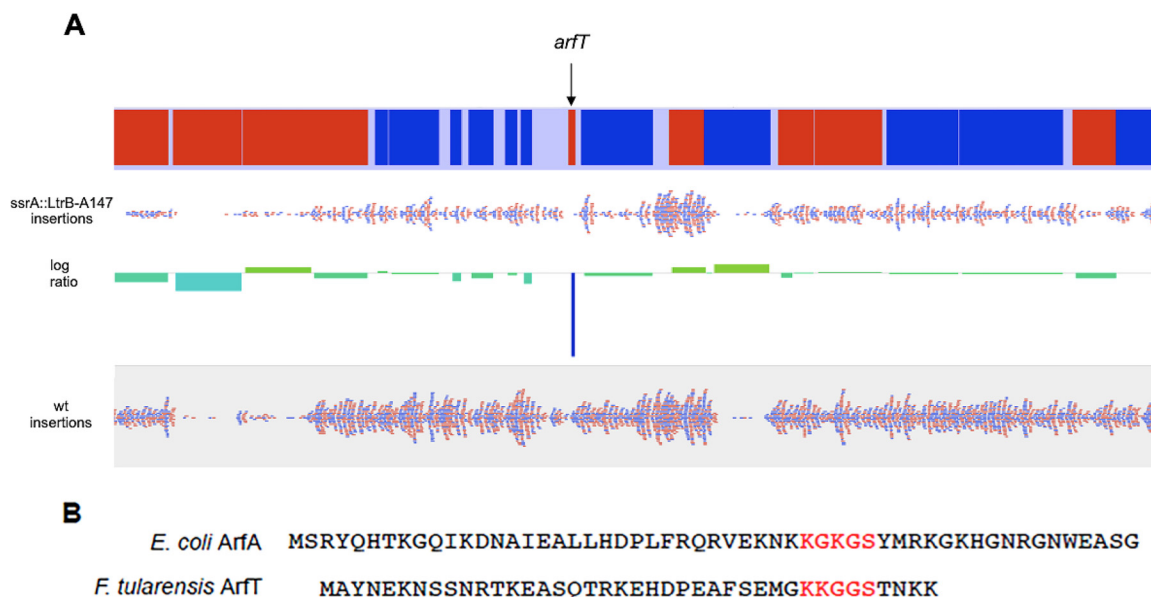
*trans*-translation (1, 2, 6). In this pathway, the transfer-messenger RNA (tmRNA)–SmpB complex recognizes a nonstop ribosome and uses a tRNA-like domain of tmRNA and a specialized reading frame within tmRNA to tag the nascent polypeptide for degradation and release the nonstop ribosome (1, 2, 6, 7). Genes encoding tmRNA (*ssrA*) and SmpB (*smpB*) have been identified in >99% of sequenced bacterial genomes, and in some species these genes are essential (1, 8). In other species, *trans*-translation is not essential due to the presence of an alternative ribosome rescue factor, ArfA or ArfB (9, 10). ArfA is a short protein that inserts its C-terminal tail into the mRNA channel of nonstop ribosomes and rescues them by activating RF2 to hydrolyze the peptidyl-tRNA (10–16). ArfA does not interact with the RF2 residues that recognize a stop codon but instead binds a different part of RF2 to stabilize the active conformation and promote peptidyl-tRNA hydrolysis (13–16). These interactions cannot be made with RF1, so ArfA functions only in conjunction with RF2 (11–16). ArfB also binds the empty mRNA channel of nonstop ribosomes with its C-terminal tail, but ArfB contains an RF1-like catalytic domain that can hydrolyze peptidyl-tRNA on nonstop ribosomes in the absence of RF1 or RF2 (17–20). In bacteria that have a functional ArfA or ArfB, deletions of *ssrA* and the gene encoding the alternative ribosome rescue factor are synthetically lethal, indicating that these species require at least one mechanism for rescuing nonstop ribosomes (9, 10).

Although *ssrA* has been deleted from the pathogen *F. tularensis* (21), no homologues of *arfA* or *arfB* have been found in sequenced *F. tularensis* genomes. *F. tularensis* has a reduced genome size and a life cycle that is different from that of many other bacteria, so it is possible that ribosome rescue is not essential. Alternatively, *F. tularensis* may have an alternative ribosome rescue system that is sufficiently different from ArfA and ArfB that it cannot be identified by homology searches. *F. tularensis* is a Gram-negative, facultative intracellular bacterium responsible for the vector-borne zoonosis tularemia (22–28). Pneumonic tularemia is infectious at  $\leq 10$  CFU (of aerosolized bacteria) and has a 60% mortality rate if left untreated (22–27). *F. tularensis* has been classified as a tier 1 select agent by the CDC because the bacteria can be easily propagated and disseminated as an aerosol, making the threat of a bioterrorist attack with an antibiotic-resistant strain of *F. tularensis* a significant concern (26, 27).

To determine if ribosome rescue is essential in *F. tularensis*, we screened for an alternative ribosome rescue factor using transposon mutagenesis followed by deep sequencing (Tn-seq) in the *F. tularensis* subsp. *holarctica* live vaccine strain (LVS). One gene, *F. tularensis* 0865 (*FTA\_0865*), renamed here as alternative ribosome rescue factor T (ArfT), was found to be essential in cells lacking *trans*-translation but not in wild-type *F. tularensis*. We show that ArfT can rescue nonstop ribosomes *in vivo* and *in vitro* and that ArfT can function in conjunction with either RF1 or RF2. These data indicate that ribosome rescue is essential in *F. tularensis* and that ArfT is the first representative of a new family of alternative ribosome rescue factors that can recruit either RF1 or RF2 to nonstop ribosomes.

## RESULTS

**Identification of an alternative rescue factor in *F. tularensis*.** A published report demonstrated that an *F. tularensis* strain in which *ssrA* was disrupted by insertion of an LtrB intron (*ssrA::LtrB-bp147*) was viable (21). We used reverse transcription-PCR (RT-PCR) to confirm that there was no detectable tmRNA in *ssrA::LtrB-bp147* cells (see Fig. S1 in the supplemental material), suggesting either that ribosome rescue is not essential in *F. tularensis* or that *F. tularensis* has another mechanism to rescue nonstop ribosomes. Homology searches of the *F. tularensis* genome using sequences or motifs from ArfA and ArfB did not identify any candidate alternative ribosome rescue factors. Therefore, we took a genetic approach to identify genes that might be involved in an alternative ribosome rescue pathway. If *F. tularensis* has an unknown alternative ribosome rescue pathway, genes required for the alternative pathway should be essential in *ssrA::LtrB-bp147* cells but not in wild-type cells. We used Tn-seq to identify genes that could be disrupted in each strain. Cells from each strain were mutagenized with a Himar1-based

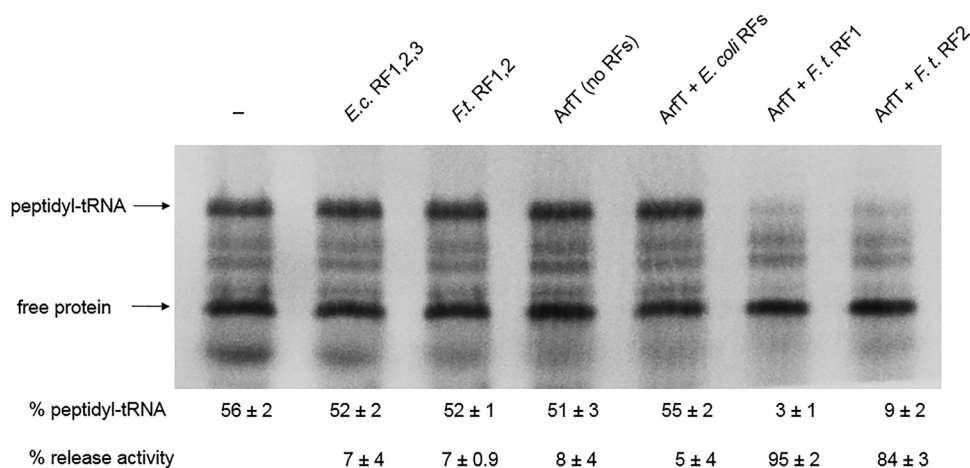


**FIG 1** Tn-seq identified ArfT as a candidate alternative ribosome rescue system. (A) Representation of Tn-seq data. The portion of the *F. tularensis* subsp. *holarctica* FTNF002-00 chromosome containing *arfT*, with genes transcribed to the right in red and those transcribed to the left in blue (top), is shown with mapped transposon insertion sites (red and blue dots) in strain *ssrA::LtrB-bp147* and wild-type *F. tularensis* (wt). The number of insertions per gene was normalized to the total number of reads, and the log ratio of the normalized number of insertions was plotted (center) to identify genes that can be deleted in the wild-type strain but not in strain *ssrA::LtrB-bp147*. (B) Alignment of *E. coli* ArfA and ArfT protein sequences. The KGKGS motif that is conserved in ArfA genes and that binds the empty mRNA channel of the ribosome is shown in red, as are the corresponding residues in ArfT.

transposon (29, 30) and the transposon insertion sites were sequenced. The ratio of the normalized number of insertions in *ssrA::LtrB-bp147* to the normalized number of insertions in wild-type was used to identify genes that were much less fit in strain *ssrA::LtrB-bp147* (see Table S1 in the supplemental material).

Among the genes with no insertions in strain *ssrA::LtrB-bp147* and typical insertion density in the wild-type strain, *arfT* was a candidate alternative ribosome rescue factor because it shared some characteristics with ArfA and had no annotated function (Fig. 1A). *arfT* encodes a protein of 40 amino acids, whereas mature ArfA has 52 to 55 amino acids, and ArfT contains a stretch of residues near the C terminus that are similar to a conserved KGKGS sequence found in ArfA (Fig. 1B). Structural studies of ArfA indicate the KGKGS sequence binds in the empty mRNA channel of nonstop ribosomes. A tblastn search (31) showed that ArfT homologues are found in other *F. tularensis* subspecies and in the closely related species *F. hispaniensis* but not in more distantly related species (see Table S2 in the supplemental material). *arfT* was not previously annotated as an open reading frame in *F. tularensis* LVS, the Schu S4 strain, or a number of other sequenced *F. tularensis* strains, but was annotated in *F. tularensis* subsp. *holarctica* FTNF002-00. For this reason, transposon insertions were mapped to this genome.

**Deletion of *arfT* is synthetically lethal with disruption of *ssrA*.** The Tn-seq data suggested that the absence of both *trans*-translation and ArfT is lethal to *F. tularensis* cells. This prediction was tested by attempting to produce markerless, in-frame deletions of *arfT* using a two-step recombination procedure (32) in the wild type, *ssrA::LtrB-bp147*, and *ssrA::LtrB-bp147* with a plasmid-borne copy of *ssrA* expressed from a strong, constitutive promoter (*ssrA::LtrB-bp147 pFtssrA*). In the first step of this procedure, a suicide plasmid containing a copy of the *arfT* locus with the *arfT* coding sequence deleted was recombined into the chromosome. The second recombination step eliminates one copy of the *arfT* locus, so cells can retain either the *arfT* deletion or the wild-type *arfT* gene (Fig. S2) (32). The first recombination step was successful in all strains. For the wild-type strain, 20% of the second-step recombinants had the *arfT*

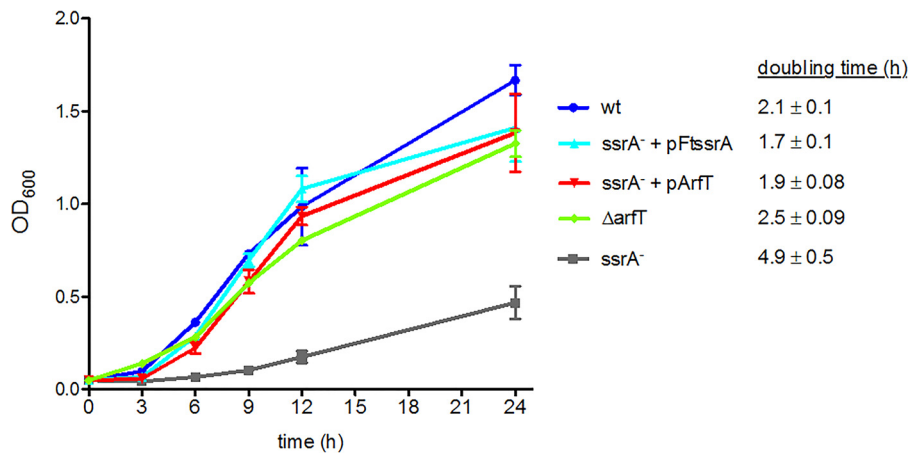


**FIG 2** ArfT promotes peptidyl-tRNA hydrolysis on nonstop ribosomes in conjunction with either RF1 or RF2. Gel image of *in vitro* ribosome rescue assays. *In vitro* transcription/translation assays were programmed with a nonstop DNA template and synthesized protein was labeled by incorporation of  $^{35}\text{S}$ -methionine. ArfT and release factors were added to individual reaction mixtures in the combinations indicated. Bands corresponding to peptidyl-tRNA and free protein were quantified. The percentage of protein in the peptidyl-tRNA band and the percentage of peptidyl-tRNA that was hydrolyzed compared to a reaction with no release factors or ArfT added (release activity) are shown ( $\pm$  standard deviation). The data represent averages of results from 3 biological replicates. *E.c.*, *E. coli*; *F.t.*, *F. tularensis*.

deletion, demonstrating that *arfT* is not essential. Deletion of *arfT* did not cause a large defect in growth or morphology (see Fig. 3). For the *ssrA::LtrB-bp147* strain, 100 second-step recombinants were screened and all had retained the wild-type copy of *arfT*, indicating that disruption of both *ssrA* and *arfT* was lethal. When a plasmid-borne copy of *ssrA* was present in *ssrA::LtrB-bp147* cells, 20% of the second-step recombinants had *arfT* deleted, demonstrating that the synthetic lethal phenotype can be complemented by an ectopic copy of *ssrA*. FTA\_0993, a gene that had transposon insertions in both the wild-type and *ssrA::LtrB-bp147* strains in the Tn-seq experiment, was successfully deleted from the *ssrA::LtrB-bp147* strain (Fig. S2), confirming that *ssrA::LtrB-bp147* cells are competent for recombination in the two-step procedure. Taken together, these data demonstrate that deletion of *arfT* is lethal to *F. tularensis* cells lacking *trans*-translation and indicate that ribosome rescue is required in *F. tularensis*.

**ArfT can recruit either RF1 or RF2 to hydrolyze peptidyl-tRNA on nonstop ribosomes *in vitro*.** *In vitro* ribosome rescue assays were performed to assess whether ArfT was capable of rescuing nonstop ribosomes. Nonstop ribosomes were generated by programming a transcription-translation reaction with a gene that does not have a stop codon (dihydrofolate reductase [DHFR]-NS) (Fig. 2) (9). In the absence of ribosome rescue, peptidyl-tRNA was stable on the ribosome and could be observed on protein gels. As expected for nonstop ribosomes, addition of RF1, RF2, and RF3 from *E. coli* or RF1 and RF2 from *F. tularensis* did not dramatically decrease the amount of peptidyl-tRNA. Addition of ArfT alone did not promote hydrolysis of the peptidyl-tRNA, indicating that ArfT does not have intrinsic hydrolytic activity to rescue nonstop ribosomes. Likewise, addition of ArfT in conjunction with RF1, RF2, and RF3 from *E. coli* did not promote peptidyl-tRNA hydrolysis. However, addition of ArfT with *F. tularensis* RF1 resulted in 95% peptidyl-tRNA hydrolysis and addition of ArfT with *F. tularensis* RF2 resulted in 84% peptidyl-tRNA hydrolysis (Fig. 2). These data suggest that ArfT can rescue ribosomes by recruiting either RF1 or RF2 to nonstop ribosomes.

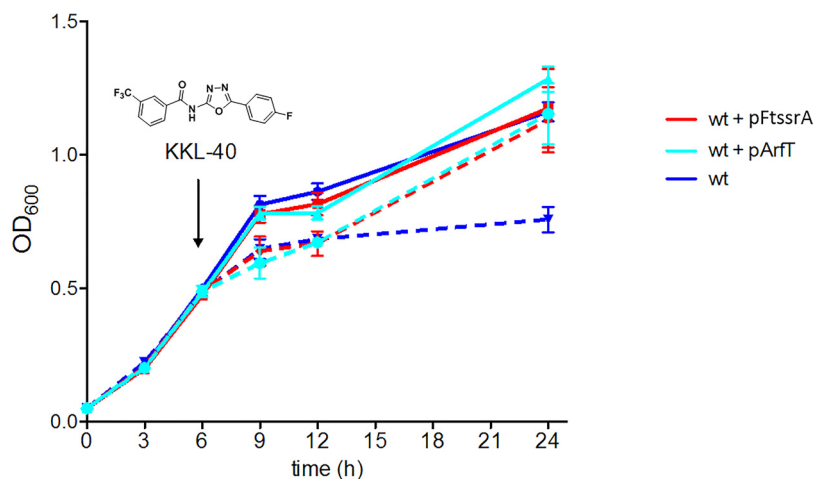
**Overexpression of *arfT* rescues the growth defect in cells lacking *trans*-translation.** It was previously reported that the *ssrA::LtrB-bp147* strain grows much more slowly than the wild type in liquid culture and that this growth defect could be complemented by expression of *ssrA* from a plasmid (21). To determine whether overexpression of *arfT* could restore normal growth to cells in the absence of *trans*-translation, we cloned *arfT* under the control of the strong, constitutive bacterioferritin



**FIG 3** Overexpression of ArfT rescues the growth defect in strain *ssrA::LtrB-bp147*. Growth curves of wild-type *F. tularensis* (wt), the  $\Delta arfT$  strain, and the *ssrA::LtrB-bp147* strain (*ssrA*<sup>-</sup>) with and without plasmids expressing *ssrA* (pFtssrA) or *arfT* (pArfT) are shown. Error bars indicate standard deviations. The doubling time for each strain ( $\pm$  standard deviation) is indicated. The data represent averages of results from 3 biological replicates.

(Bfr) promoter on a multicopy plasmid (pArfT) and tested its impact on growth rates. As expected, the *ssrA::LtrB-bp147* strain grew substantially more slowly than the wild-type strain, but the *ssrA::LtrB-bp147 pFtssrA* strain grew at the same rate as the wild-type strain (Fig. 3). *ssrA::LtrB-bp147* cells with pArfT also grew at the same rate as wild-type cells, indicating that multicopy *arfT* genes can suppress the *ssrA* phenotype. pArfT did not increase the growth rate of wild-type cells (Fig. 4). These results suggest that ArfT can rescue nonstop ribosomes in the absence of *trans*-translation.

**Overexpression of ArfT prevents growth arrest due to ribosome rescue inhibitors.** It has been shown that the members of a class of oxadiazole compounds such as KKL-40 inhibit ribosome rescue and arrest the growth of many bacterial species, including *F. tularensis* (33–35). Overexpression of *E. coli* ArfA prevents growth arrest by these oxadiazoles in *Shigella flexneri*, confirming that growth arrest is due to inhibition of ribosome rescue (33, 34). If ArfT had ribosome rescue activity similar to that seen with ArfA, overexpression of ArfT should inhibit growth arrest in *F. tularensis* mediated by



**FIG 4** Overexpression of ArfT prevents growth inhibition caused by ribosome rescue inhibitors. Growth curves of wild-type *F. tularensis* (wt) with and without plasmids expressing *ssrA* (pFtssrA) or *arfT* (pArfT). A ribosome rescue inhibitor, KKL-40 (structure shown), was added to half the cultures after 6 h (indicated by arrow) at  $10\times$  MIC. Cultures with KKL-40 are indicated with dotted lines, and cultures with no drug are indicated with solid lines. The data represent averages of results from 3 biological replicates, with error bars indicating the standard deviations.

KKL-40. To test this prediction, KKL-40 was added to growing cultures of *F. tularensis* strains and growth was monitored over 18 h (Fig. 4). As previously observed, addition of KKL-40 resulted in growth arrest of wild-type *F. tularensis* and the bacteria were unable to recover to normal levels. Addition of KKL-40 to *F. tularensis* carrying pFtssrA or pArfT caused an initial decrease in the growth rate, but after 18 h the cultures had reached the same density as the wild-type cultures. Because growth inhibition is suppressed by extra ribosome rescue activity in the form of either tmRNA-SmpB or ArfT activity, it is likely that KKL-40 inhibits growth through ribosome rescue and not through off-target effects. These results are consistent with a model in which KKL-40 arrests growth in *F. tularensis* by binding to nonstop ribosomes and tmRNA-SmpB or ArfT can counteract the effects of KKL-40 by rescuing the ribosomes before KKL-40 binds.

## DISCUSSION

The data described here answer two recently posed outstanding questions regarding ribosome rescue: are there other alternative rescue factor systems, and will ArfA-like systems emerge in bacteria where RF1 is recruited to nonstop ribosomes (36)? The answer to both questions is yes. The data presented here indicate that ArfT has all the characteristics of an alternative ribosome rescue factor in *F. tularensis*. ArfT has ribosome rescue activity *in vitro* because it can release nonstop ribosomes in conjunction with RF1 or RF2. *In vivo*, deletion of *arfT* is synthetically lethal with disruption of *ssrA*, consistent with ArfT providing essential ribosome rescue activity in the absence of *trans*-translation. Overexpression of ArfT suppresses the slow-growth phenotype in cells lacking *trans*-translation and counteracts growth arrest mediated by a ribosome rescue inhibitor in *F. tularensis*, indicating that ArfT can perform the same physiological role as *trans*-translation in *F. tularensis*. These results demonstrate that the presence of ArfT in *F. tularensis* makes *trans*-translation dispensable and that ribosome rescue activity is essential in *F. tularensis*.

ArfT has some similarities to ArfA, and the two factors may recognize nonstop ribosomes in the same manner. The C-terminal tail of ArfA binds in the empty mRNA channel of nonstop ribosomes through a number of lysine and arginine residues, including a conserved KGKGS motif (13–16). None of these residues are essential for ArfA activity (16, 37), but replacement of individual residues decreases ribosome rescue activity *in vitro* (16). The KKGSTNKK sequence near the C terminus of ArfT has an arrangement of positively charged residues that is similar to that in ArfA, suggesting that ArfT may use this sequence to bind the ribosome. SmpB and ArfB also bind in the empty mRNA channel of nonstop ribosomes using positively charged C-terminal tails, but ArfA, SmpB, and ArfB each make different interactions with the mRNA channel (7, 13–20, 37). Because of this variation in binding, structural studies will be required to define the interactions between ArfT and the ribosome.

Despite the similarities in protein size and C-terminal tail sequence between ArfT and ArfA, the observation that ArfT can activate RF1 or RF2 suggests that ArfT may not interact with release factors (RFs) in the same way as ArfA. Cryo-electron microscopy (cryo-EM) analyses of a nonstop ribosome bound to *E. coli* ArfA-RF2 showed that residues 15 to 31 of ArfA interact with RF2 to stabilize the active conformation of RF2 and promote hydrolysis of the peptidyl-tRNA (13–16). In a key feature of this interaction, ArfA forms a  $\beta$ -strand that extends the  $\beta$ -sheet formed by  $\beta$ 4- $\beta$ 5 of RF2, with F25 of ArfA binding in a hydrophobic pocket formed by V198 and F217 of RF2. Residues in RF2  $\beta$ 4- $\beta$ 5 and the SerProPhe (SPF) loop are highly conserved between *E. coli* RF2 and *F. tularensis* RF2 (see Fig. S3 in the supplemental material), raising the possibility that ArfT could bind in a manner similar to that seen with ArfA. However, ArfT does not have a hydrophobic residue at the position corresponding to F25 (Fig. 1B). The absence of the V198-F217 pocket in *E. coli* RF1 has been suggested to be the reason that ArfA does not activate *E. coli* RF1 (13–16). This region of *E. coli* RF1 is highly conserved in *F. tularensis* RF1, and yet ArfT activates *F. tularensis* RF1 but not *E. coli* RF1. Therefore, if the interaction between ArfT and RF2 were similar to the interaction between ArfA and RF1, ArfT would have to activate RF1 through a distinct mechanism. Alternatively, ArfT may

activate *F. tularensis* RF1 and RF2 in the same manner as but through a different mechanism than that used by ArfA. Little was known about the interactions among ArfA, RF2, and the ribosome before structural data of the complex were obtained, and similar studies will be required to understand how ArfT can activate both RF1 and RF2.

Another likely difference between ArfT and ArfA concerns regulation. The *arfA* gene includes a transcriptional terminator and RNase III cleavage site before the stop codon, such that ArfA protein is made from nonstop mRNA (38, 39). When *trans*-translation is active, the nascent ArfA peptide is tagged and degraded, but when *trans*-translation activity is not available, active ArfA is produced and accumulates in the cell. This genetic arrangement makes ArfA a true backup ribosome rescue system, functioning only when *trans*-translation activity is low or absent (38, 39). The *arfT* gene does not include a transcriptional terminator or an RNase III cleavage site before the stop codon. RT-PCR using a primer corresponding to the final 33 nucleotides of the *arfT* reading frame (including the stop codon) showed that *arfT* mRNA accumulated in wild-type *F. tularensis* and the *ssrA*-disrupted strain at similar levels (Fig. S4). Although these results do not exclude the possibility that *arfT* mRNA is truncated in the last few codons, the gene product does not appear to be controlled by transcriptional termination and RNase III cleavage in the same manner as ArfA.

The observations indicating that ArfT interacts with RF1 and is not regulated like ArfA and the overall low sequence similarity between ArfT and ArfA suggest that ArfT evolved independently from ArfA and represents a third different alternative ribosome rescue factor. Our sequence homology searches identified ArfT only in the closely related *F. tularensis* and *F. hispaniensis* strains, but the small size of ArfT makes more distant homologues difficult to identify with this method. Characterization of the ArfT residues required for interaction with RF1 and RF2 will allow more-specific searches for ArfT in other species. The number of different ribosome rescue mechanisms discovered to date suggests that the problem presented by nonstop ribosomes has been solved many times throughout evolution, and more alternative ribosome rescue factors may yet be discovered. It is not yet clear what conditions would limit *trans*-translation activity enough that an alternative ribosome rescue factor would be needed. However, such conditions must exist in a wide variety of environments. Alternative ribosome rescue factors have been selected for in enteric bacteria such as *E. coli*, which has ArfA; aquatic bacteria such as *C. crescentus*, which has ArfB; and intracellular pathogens such as *F. tularensis*, which has ArfT.

## MATERIALS AND METHODS

**Bacterial culture.** Bacterial strains are listed in Table S1 in the supplemental material. *E. coli* DH10B was used for routine cloning procedures and was grown in Luria-Bertani (LB) broth (10% Bacto tryptone, 5% yeast extract, 10% NaCl [pH 7.5]) or on LB agar supplemented with ampicillin (100  $\mu$ g/ml) or kanamycin (30  $\mu$ g/ml) where appropriate. *F. tularensis* was grown in Chamberlain's defined medium (CDM) (40) adjusted to pH 6.2 at 37°C with shaking or on chocolate agar plates (Mueller-Hinton agar supplemented with 1% bovine hemoglobin [Remel, USA] and 1% Isovitalex X Enrichment [Becton, Dickinson, France]) at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 48 to 72 h. Kanamycin (10  $\mu$ g/ml), tetracycline (10  $\mu$ g/ml), and sucrose (5%) were added to cultures and plates where appropriate. For growth curve experiments, *F. tularensis* cultures were grown in CDM overnight at 37°C and 200 rpm and back diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.05. Growth was monitored by performing OD<sub>600</sub> readings. When indicated, 1.4  $\mu$ g/ml KKL-40 was added 6 h postinoculation. (Supplemental details of the materials and methods used are presented in Text S1 in the supplemental material.)

**Plasmid construction.** Oligonucleotide sequences are listed in Table S3 in the supplemental material. To generate plasmids pMP812- $\Delta$ ArfT and pMP812- $\Delta$ O993, 600-bp PCR products flanking the gene to be deleted were amplified using primer pair ArfT\_UF and ArfT\_UR and primer pair ArfT\_DF and ArfT\_DR for pMP812- $\Delta$ ArfT and primer pair O993\_UF and O993\_UR and primer pair O993\_DF and O993\_DR for pMP812- $\Delta$ O993, digested with BamHI, and ligated together. The sequence was then reamplified as one unit with primer pair ArfT\_UF and ArfT\_DR and primer pair O993\_UF and O993\_DR and cloned into pMP812 using Sall and NotI restriction sites. Plasmids pArfT and pFtssrA were constructed by amplifying the coding sequences of each gene using primer pair ArfT\_CF and ArfT\_CR and primer pair FtssrA\_CF and FtssrA\_CR. The Bfr promoter (41) was amplified using primers Bfr\_F and Bfr\_R, ligated upstream of either the ArfT or SsrA PCR product using a BamHI restriction site, and reamplified as one unit with primer Bfr\_F and either primer ArfT\_CR or primer ssrA\_CR. The resulting PCR product was digested with EcoRI and ligated into the pKK214-MCS<sub>3</sub> plasmid (41). In order to construct plasmids pET28ArfT, pET28RF1, and pET28RF2, primer pair RF1\_PF and RF1\_PR, primer pair RF2\_PF and RF2\_PR, and primer pair ArfT\_PF

and ArfT<sub>PR</sub> were used to generate PCR products of the protein coding sequence of RF1, RF2, and ArfT from *F. tularensis*, respectively. The PCR products were then cloned into pET28a(+) using NdeI and XhoI restriction sites for protein expression of ArfT, as well as release factor 1 (RF1) and release factor 2 (RF2) from *F. tularensis*.

**Tn-seq.** Overnight cultures of wild-type *F. tularensis* and the *ssrA::LtrB-bp147* strain were grown to an OD<sub>600</sub> of 0.5, washed 3 times with 500 mM sucrose, and transformed with ~300 ng of plasmid pHimar H3. Over 50,000 colonies were pooled, and chromosomal DNA was extracted. The libraries were prepared and sequenced on an Illumina HiSeq 2000 instrument by Fasteris (Geneva, Switzerland). The data were mapped to the genome of *F. tularensis* subsp. *holarctica* FTNF002-00 and were analyzed in Geneious version 11.1.4 using parameters described previously (9). The frequency of transposition for each gene was quantified in both strain backgrounds. Additionally, the relative fitness of each gene in the two strains was quantified by looking at the ratio of the number of times that a sequence was recovered in the *ssrA* mutant to the number seen with the wt. Insertion ratio data were generated for each gene to determine if the genes were essential in the absence of *ssrA* (see Table S1 in the supplemental material).

**Purification of ArfT, *F. tularensis* RF1, and *F. tularensis* RF2.** Strains TG001, TG002, and TG003 were grown to an OD<sub>600</sub> of ~0.8, and the expression of ArfT, RF1, or RF2 was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. Cells were harvested by centrifugation, resuspended in native lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole [pH 8.0]), and sonicated or processed through a French press. The lysate was cleared by centrifugation at 14,000 × *g* for 10 min. Nickel-nitrilotriacetic acid (NTA) agarose (Qiagen) that had been equilibrated with lysis buffer was added to the cleared lysate, followed by incubation with gentle rocking at 4°C for 1 h. The slurry was packed in a column and washed with 10 volumes of native wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole [pH 8.0]). Bound protein was eluted with native elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole [pH 8.0]) and visualized by SDS-PAGE. Fractions containing 6×His-protein were dialyzed against RF or ArfT storage buffer (50 mM HEPES, 300 mM NaCl [pH 7.5] for FTA\_0865, 50 mM Tris-HCl, 300 mM NaCl [pH 7.0] for RF1 and RF2). The 6×His tag was removed from RF1 by use of a Thrombin CleanCleave kit (Sigma-Aldrich) following the manufacturer's instructions. The cleaved RF1 protein solution was loaded with NTA agarose, incubated with gentle rocking at 4°C for 1 h. The slurry was packed into a column, and the flowthrough containing RF1 was collected. RF2 was dialyzed against buffer A (50 mM Tris-HCl, 100 mM NaCl [pH 7.0]) and purified on a MonoQ column using an AKTA purifier (GE Healthcare Life Sciences). Proteins were visualized by SDS-PAGE and dialyzed into RF storage buffer.

**In vitro translation and peptidyl hydrolysis assays.** ArfT peptidyl hydrolysis activity was assessed using a previously described assay (9). Briefly, nonstop DHFR was PCR amplified with primers HAF\_T7 and UTR\_DHFR\_FL, added to the PURExpress ΔRF system (New England Biolabs) A and B reaction mixtures, and incubated for 1 h at 37°C. Where indicated, ArfT was added to a final concentration of 25 μg/ml and *E. coli* or *F. tularensis* LVS RFs were added to a final concentration of 500 μg/ml, and the reaction mixtures were incubated for 1 h at 37°C. Total protein was precipitated by addition of cold acetone, resuspended in sample loading buffer (5 mM sodium bisulfite, 50 mM MOPS [morpholinepropanesulfonic acid], 50 mM Tris base, 1 μM EDTA, 0.1% SDS, 5% glycerol, 0.01% xylene cyanol, 0.01% bromophenol blue), and resolved on a Bis-Tris gel using MOPS running buffer.

**Genetic deletions.** Targeted, markerless in-frame deletions were generated for both *FTA\_0865* and *FTA\_0993* with a two-step allelic exchange system designed for *F. tularensis* using the pMP812 *sacB* suicide vector (32). *F. tularensis* strains were transformed with either pMP812-ArfT or pMP812-0993, and primary recombinants were selected on kanamycin after incubation at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 48 to 72 h. Primary recombinants were grown overnight without selection and plated on 5% sucrose to select for secondary recombinants. Secondary recombinants were confirmed by replica plating on chocolate agar containing kanamycin and on chocolate agar without selection. Genetic deletions were confirmed via PCR using primers ArfT\_KOF and ArfT\_KOR and primers 0993\_KOF and 0993\_KOR.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02436-18>.

**TEXT S1**, PDF file, 0.1 MB.

**FIG S1**, TIF file, 69.4 MB.

**FIG S2**, TIF file, 90.2 MB.

**FIG S3**, TIF file, 18.7 MB.

**FIG S4**, TIF file, 67.9 MB.

**TABLE S1**, PDF file, 0.2 MB.

**TABLE S2**, PDF file, 0.1 MB.

**TABLE S3**, PDF file, 0.1 MB.

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