tmRNA Is Essential in *Shigella flexneri*

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Abstract

Nonstop mRNAs pose a challenge for bacteria, because translation cannot terminate efficiently without a stop codon. The trans-translation pathway resolves nonstop translation complexes by removing the nonstop mRNA, the incomplete protein, and the stalled ribosome. P1 co-transduction experiments demonstrated that tmRNA, a key component of the trans-translation pathway, is essential for viability in *Shigella flexneri*. tmRNA was previously shown to be dispensable in the closely related species *Escherichia coli*, because *E. coli* contains a backup system for trans-translation mediated by the alternative release factor ArfA. Genome sequence analysis showed that *S. flexneri* does not have a gene encoding ArfA. *E. coli* ArfA could suppress the requirement for tmRNA in *S. flexneri*, indicating that tmRNA is essential in *S. flexneri* because there is no functional backup system. These data suggest that resolution of nonstop translation complexes is required for most bacteria.

Introduction

mRNAs that lack a stop codon can originate from many events, including premature transcription termination, physical or chemical damage to a complete mRNA, or macleolytic activity. Translation of a nonstop mRNA is problematic, because termination requires a stop codon. Release factors specifically recognize a stop codon in the ribosomal A site and promote hydrolysis of the peptidyl-tRNA, releasing the newly-synthesized protein and the ribosome [1,2]. Eukaryotes have mRNA proofreading mechanisms to limit translation initiation on nonstop mRNAs [3]. However, bacteria lacks most mRNA proofreading mechanisms and ribosomes frequently translate to the end of a nonstop mRNA [4], generating a nonstop translation complex composed of a truncated mRNA, an incomplete nascent polypeptide, and a ribosome that cannot elongate or terminate translation by the canonical reactions. These nonstop translation complexes are resolved by trans-translation, a reaction mediated by tmRNA and a small protein, SmpB [5]. tmRNA contains a tRNA-like acceptor stem and a reading frame encoding a short peptide. SmpB binds tmRNA with high affinity [6]. During trans-translation, tmRNA-SmpB recognizes the nonstop translation complex and promotes translation of the tmRNA-encoded peptide onto the end of the nascent polypeptide [7,8]. This reaction releases the ribosome at a stop codon within tmRNA. The tmRNA-encoded peptide is recognized by several proteases, so the incomplete protein is rapidly degraded [9,10,11,12]. trans-Translation also stimulates degradation of the nonstop mRNA, so all components of the nonstop translation complex are efficiently removed [13,14].

trans-Translation occurs with high frequency in bacteria, and is found throughout the bacterial kingdom. Estimates from *E. coli* suggest that 2–4% of translation reactions end in trans-translation [4]. Genes encoding tmRNA (*ssrA*) and SmpB (*smpB*) have been identified in all sequenced bacterial genomes, indicating that trans-translation confers a selective advantage in all environments that can support bacterial life [15].

The abundance and ubiquity of trans-translation suggest that it is very important, and in some species *ssrA* and *smpB* are essential [16,17,18,19]. However, mutants of *E. coli K12* lacking trans-translation activity are viable and have only mild growth defects in typical culture conditions [20,21]. A screen for *E. coli* genes that cannot be deleted in *ArfA* cells identified *arfA*, which encodes an alternative release factor [22]. ArfA binds nonstop translation complexes and recruits RF-2 to hydrolyze the peptidyl-tRNA, releasing the nascent polypeptide and ribosome [23,24]. ArfA is a backup system for trans-translation, because it is only produced when trans-translation activity is limiting [25,26]. In *E. coli*, *arfA* mRNA contains an RNase III cleavage site 5′ of the stop codon, so expression of *arfA* will result in a nonstop complex [25]. When trans-translation is functional, ArfA will be tagged and degraded. However, if trans-translation is limiting, the truncated but active ArfA will be released [25]. *arfA* genes have been found in the genome sequences of many bacteria, including most enteric gamma-proteobacteria [22,26].

In this paper we show that *ssrA* is essential in *S. flexneri*, a human pathogen that causes acute dysentery. *S. flexneri* is closely related to *E. coli* [27]. In fact, the *Shigella* and *Escherichia* genera are phylogenetically indistinguishable [27]. *S. flexneri* lacks *arfA*, but when *E. coli* *arfA* is expressed in *S. flexneri*, *ssrA* can be deleted. These results suggest that trans-translation is essential in *S. flexneri* because it is the only available mechanism to resolve nonstop translation complexes.
Results and Discussion

**ssrA is essential in *S. flexneri***

Efforts to replace *ssrA* in the chromosome of *S. flexneri* 2a 2457T with a kanamycin-resistance gene using Red-mediated recombination were not successful in wild-type cells. However, when a second copy of *ssrA* was provided on a plasmid (pSsrA), kanamycin-resistant colonies were recovered. Diagnostic PCR reactions confirmed that *ssrA* was deleted in kanamycin-resistant cells containing pSsrA (Fig. 1). These results suggested that *ssrA* is essential in *S. flexneri*.

The requirement for *ssrA* was confirmed using a co-transduction experiment. A marker linked to the chromosomal *ssrA* locus was introduced into *S. flexneri* by transducing *zfg-2003::Tn10* from a donor *E. coli* strain. The *Tn10* insertion in *zfg-2003* confers tetracycline resistance, and is located ~0.25 minutes from *ssrA*. *S. flexneri* *zfg-2003::Tn10* was then transformed with pSsrA and the chromosomal copy of *ssrA* was replaced with a kanamycin-resistance gene to produce *S. flexneri* *zfg-2003::Tn10 ssrA::kan* pSsrA. P1 lysates were prepared from this strain and used to measure co-transduction of *ssrA::kan* and *zfg-2003::Tn10* into *S. flexneri* strains. The co-transduction frequencies were measured by selecting for tetracycline-resistant transductants and screening these transductants for kanamycin resistance. Based on the map distance between *zfg-2003* and *ssrA*, the tetracycline-resistance gene and kanamycin-resistance gene should be co-transduced with a frequency of ~70% if *ssrA* was not essential. When *S. flexneri* pSsrA was used as a recipient, the co-transduction frequency was 72±4%, close to the theoretical value. However, when wild-type *S. flexneri* with no additional copy of *ssrA* was used as a recipient, no kanamycin-resistant colonies were recovered from 550 tetracycline-resistant transductants. If *ssrA* were not essential, the probability of obtaining no co-transductants in these experiments would be (0.3)550, or ~10–23%. These results show that unlike *E. coli* K12, *S. flexneri* requires *ssrA* for viability.

*S. flexneri* strains do not have *arfA*

*E. coli* can survive without *trans-translation* activity because *arfA* is expressed in the absence of *trans-translation* and *ArfA* activity can resolve nonstop translation complexes [22,23,24,25]. Deletions of *ssrA* and *arfA* in *E. coli* are synthetically lethal [22]. Searches of genome sequences of *Shigella* species using BLAST [28] revealed that *arfA* homologs are present in *S. boydii*, *S. sonnei*, and *S. dysenteriae*, but not in *S. flexneri*. In *Escherichia* and *Shigella* species that have *arfA*, the gene is encoded between *mstA* and *zntA*, 3′ of *tbcL*. This chromosomal locus in *S. flexneri* strains contains an insertion element 3′ of *tbcL*, suggesting that *arfA* has been deleted by genetic rearrangement (Fig. 2A).

**E. coli ArfA can suppress the lethal phenotype of ssrA deletion in *S. flexneri***

Given the close phylogenetic relationship between *E. coli* and *S. flexneri*, it was surprising that the phenotypes caused by deleting *ssrA* were so different. However, the absence of *arfA* in *S. flexneri* suggested that the difference might be due to the absence of a backup mechanism for *trans-translation* in *S. flexneri*. To determine if *E. coli* ArfA could suppress the requirement for *ssrA* in *S. flexneri*, a plasmid encoding His6-ArfA from the ASKA collection (pCA24N-His6-ArfA) was transformed into *S. flexneri* and these cells were used as the recipient in a co-transduction experiment with P1 lysates from *S. flexneri* *zfg-2003::Tn10 ssrA::kan* pSsrA. ssrA::kan and *zfg-2003::Tn10* were co-transduced into *S. flexneri* pCA24N-His6-ArfA with a frequency of 69±9%, indicating that *ssrA* is not essential in cells with pCA24N-His6-ArfA. Diagnostic PCR reactions confirmed that *ssrA* was deleted in the kanamycin-resistant cells (Fig. 2B). These results indicated that ArfA can suppress the requirement for *ssrA* in *S. flexneri*.

*ssrA* could be deleted in *S. flexneri* pCA24N-His6-ArfA cells even when IPTG was not added to induce *arfA* expression. Western blotting revealed that amount of ArfA in uninduced *S. flexneri* **ssrA::kan** pCA24N was 15–20% the amount in cells that had been induced (Fig. 3A). When *S. flexneri* **ssrA::kan** pCA24N-His6-ArfA cells were inoculated into fresh medium containing IPTG, the lag phase of growth was shorter than when no IPTG was added, but the doubling time during logarithmic growth was similar.
The sequences of ssrA genes from E. coli and S. flexneri are identical. Plasmid pSsrA was made by amplifying ssrA from E. coli K-12 MG1655 using primers ssrAU_BamHI and ssrAL_HindIII, digesting the product with BamHI and HindIII, and ligating the resulting DNA into pJS14 cut with the same enzymes. Red-mediated recombination was performed using the Wanner method [33]. S. flexneri cells containing pK2D0 and pSsrA were transformed with a PCR product made using primers Shi_ssrA_del-F and Shi_ssrA_del-R with plasmid pKD4 as the template. E. coli strain BD167 was used as the donor strain to transduce zfg-2003::Tn10 mutation into S.flexneri. Plasmid pCA24N-His6-ArfA was a gift from the Ades lab, and the sequence of arfA on the plasmid was verified prior to use.

P1 transduction

P1 lysates were prepared from E. coli zfg-2003::Tn10 and S. flexneri zfg-2003::Tn10 ssrA::kan pSsrA according to published protocols [34]. For transductions, cells of the recipient strain were harvested from 1.5 ml saturated culture and resuspended in 0.75 ml P1 salts solution (10 mM CaCl₂, 5 mM MgSO₄), 0.1 ml cell suspension was incubated with 1, 10, or 100 μl P1 lysate for 30 min at 37°C. After incubation, 1 ml lysogeny broth and 0.2 ml 1 M sodium citrate were added and the samples grown 1 h at 37°C with aeration. Cells were harvested by centrifugation, resuspended in 50 μl lysogeny broth and grown on LB plates with the appropriate antibiotic at 37°C. The expected cotransduction frequency was calculated according to the formula 1-[1/(d/L)]² [35].

PCR to verify gene replacement

Replacement of ssrA in S. flexneri ssrA::kan pSsrA was verified by colony PCR using primers ssrA_KO_check-F and ssrA_KO_check-R, which flank the ssrA gene. As a control, colony PCR using the same primers was also performed on wild-type S. flexneri. To verify replacement of ssrA in S. flexneri ssrA::kan pCA24N-His6-ArfA, genomic DNA was prepared from the deletion strain [36], and used as template for PCR amplification using primers ssrA_KO_check-F and ssrA_KO_check-R. As a control, genomic DNA was prepared from wild-type S. flexneri and used as a template for PCR amplification using the same primers. The expected product size for wild-type was 681 bp, and for ssrA::kan the expected product size was 1724 bp.

ArfA expression and growth

Expression of ArfA in S. flexneri ssrA::kan pCA24N-His6-ArfA was examined under three different conditions. Saturated cultures of S. flexneri ssrA::kan pCA24N-His6-ArfA grown with or without IPTG were diluted 1:100 into growth medium with 1 mM final concentration of IPTG, or cells were grown without any exposure to the inducing agent. As a negative control, wild-type S. flexneri without plasmid was tested. Cultures were grown to OD₆₀₀ = 0.4 at 37°C, and cells were harvested by centrifugation and analyzed by Western blotting.

Growth curves were obtained by diluting saturated cultures of S. flexneri ssrA::kan pCA24N-His6-ArfA 1:100 in growth medium with or without 1 mM IPTG at 37°C with constant shaking, and sampling cultures every 20 min to measure OD₆₀₀. Points between 80 min and 160 min were fit to the single exponential function OD₆₀₀ = e^(bt), where t is time, and the value for b was used as the growth rate.

![Figure 3](https://www.plosone.org/article/fetchObject.action?uri=info:doi/10.1371/journal.pone.0057537.g003)

**Figure 3. ArfA is expressed in cells containing pCA24N-His6-ArfA.** (A) Western blots to determine the expression of ArfA in wild-type S. flexneri (lane 1), S. flexneri ssrA::kan pCA24N-His6-ArfA grown with IPTG at all times (lane 2), grown without IPTG and diluted into medium containing IPTG (lane 3), and grown without exposure to IPTG (lane 4). The amounts of ArfA relative to lane 3 are shown (n.d.: not detectable). (B) Growth of S. flexneri ssrA::kan pCA24N-His6-ArfA with IPTG (closed circles) and without IPTG (open circles) monitored by optical density at 600 nm. Doubling times during exponential growth (80-160 min) are indicated.

doi:10.1371/journal.pone.0057537.g003

(Fig. 3B). These data indicate that the amount of ArfA produced in uninduced cultures is sufficient for viability of S. flexneri in the absence of translation, but higher levels of ArfA are required for optimal growth in culture.

The results described here suggest that all species of the Escherichia/Shigella lineage require a mechanism to resolve nonstop translation complexes. For most species in this group ssrA is essential. In other proteobacteria, such as Caulobacter crescentus, ssrA is not essential [29], but there is no ArfA homolog. Perhaps nonstop translation complexes are not as severe a challenge in these species. Alternatively, these species may have a distinct mechanism for releasing nonstop translation complexes.

Like E. coli, S. flexneri has a gene encoding ArfB (YaeJ), a second alternative release factor. Purified ArfB can release nonstop translation complexes in vitro, and multicopy expression of arfB in E. coli can suppress the synthetic lethality of ssrA and arfA deletions [30,31]. However, endogenous arfB does not support deletion of ssrA in S. flexneri or simultaneous deletion of ssrA and arfA in E. coli [30], suggesting that it is not expressed under culture conditions even when nonstop translation complexes accumulate to lethal levels.

**Materials and Methods**

**Bacterial strains and plasmids**

All strains were grown at 37°C in lysogeny broth supplemented with 30 μg/ml kanamycin, 20 μg/ml chloramphenicol, or 12 μg/ml tetracycline as appropriate (Table 1). Transformation of plasmids into S. flexneri was performed by electroporation [32].
Western blotting

Cell pellets were lysed by boiling in SDS sample buffer (63 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% 2-mercaptoethanol, 0.005% bromophenol blue). The samples were resolved on a 15% SDS polyacrylamide gel, blotted to PVDF membrane, and probed with 1:5000 dilution anti-PentaHis antibody (Qiagen) [37]. Goat anti-mouse antibody (GE Healthcare) was added at 1:5000 dilution. The relative amounts of ArfA protein were determined by quantifying the bands using ImageQuant software (GE Healthcare). The samples were resolved on 15% SDS polyacrylamide gel, blotted to PVDF membrane, and probed with 1:5000 dilution anti-PentaHis antibody (Qiagen) [37]. Goat anti-mouse antibody (GE Healthcare) was added at 1:5000 dilution. The relative amounts of ArfA protein were determined by quantifying the bands using ImageQuant software (GE Healthcare).

Acknowledgments

We thank Sarah Ades at Penn State for gifts of E. coli K-12 MG1653 and pCA24N-His6-ArfA.

Author Contributions

Conceived and designed the experiments: NSR XZ KCK. Performed the experiments: NSR XZ. Analyzed the data: NSR XZ KCK. Wrote the paper: NSR XZ KCK.

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