Translational pausing in *Escherichia coli* can lead to mRNA cleavage within the ribosomal A site. A-site mRNA cleavage is thought to facilitate tmRNA•SmpB-mediated recycling of stalled ribosome complexes. Here, we demonstrate that the aminoglycosides paromomycin and streptomycin inhibit A-site cleavage of stop codons during inefficient translation termination. Aminoglycosides also induced stop codon read-through, suggesting that these antibiotics alleviate ribosome pausing during termination. Streptomycin did not inhibit A-site cleavage in *rpsL* mutants, which express streptomycin resistant variants of ribosomal protein S12. However, *rpsL* strains exhibited reduced A-site mRNA cleavage compared to *rpsL*+ cells. Additionally, tmRNA•SmpB-mediated SsrA-peptide tagging was significantly reduced in several *rpsL* strains, but could be fully restored in a subset of mutants when treated with streptomycin. The streptomycin-dependent *rpsL*(P90K) mutant also showed significantly lower levels of A-site cleavage and tmRNA•SmpB activity. Mutations in *rpsD* (encoding ribosomal protein S4), which suppressed streptomycin dependence, were able to partially restore A-site cleavage to *rpsL*(P90K) cells, but failed to increase tmRNA•SmpB activity. Taken together, these results show that perturbations to A-site structure and function modulate A-site mRNA cleavage and tmRNA•SmpB activity. We propose that tmRNA•SmpB binds to streptomycin resistant *rpsL* ribosomes less efficiently, leading to a partial loss of ribosome rescue function in these mutants.

Translational pausing in *Escherichia coli* elicits a unique RNase activity that cleaves the A-site codon within paused ribosomes (1,2). A-site mRNA cleavage results in a ribosome that is arrested at the 3` end of a truncated transcript. In eubacteria, these stalled ribosomes are “rescued” by the tmRNA•SmpB quality control system. tmRNA is a specialized RNA that functions as both a transfer RNA and a messenger RNA to remove stalled ribosomes from truncated messages. tmRNA acts first as a tRNA to bind the ribosomal A site and add its charged Ala residue to the nascent peptide chain (3,4). The truncated message is then released, and the ribosome resumes translation using a small open reading frame within tmRNA to add the SsrA peptide tag to the nascent chain (3,4). SmpB is a tmRNA-binding protein that is required for both the delivery of tmRNA to the ribosome and translation of the SsrA peptide tag (5,6). The tmRNA system performs at least three distinct quality control functions. Firstly, the SsrA peptide tag targets proteins for rapid degradation by ClpXP and other ATP-dependent proteases (7-9), ensuring that incomplete polypeptides do not accumulate in the cell. Secondly, tmRNA•SmpB mediates the recycling of stalled ribosomes into 50S and 30S subunits, which are then able to reinitiate protein synthesis on other messages. Finally, tmRNA•SmpB facilitates truncated mRNA turnover by delivering RNase R to the released transcripts during ribosome rescue (10). Because tmRNA•SmpB is not recruited to ribosomes that are paused on full-length mRNAs (11), A-site cleavage is thought to produce the truncated transcripts required for tmRNA•SmpB-mediated ribosome rescue (1,2). In this manner, A-site mRNA cleavage and tmRNA•SmpB are proposed
to collaborate in the rescue of distressed ribosomes.

A-site mRNA cleavage was first identified as an in vitro activity of the *E. coli* RelE protein (12). Subsequently, translational pauses were found to induce A-site cleavage in cells that lack RelE and all of its known homologs and paralogs (1,2,13,14). We have recently discovered that RelE-independent A-site cleavage requires at least two distinct RNase activities. RNase II is an exoribonuclease that degrades mRNA in a 3′→5′ direction until it encounters the leading edge of the paused ribosome (14). This ribosome-border degradation is presumably required for subsequent A-site nuclease activity, because A-site cleavage does not occur in ΔRNAse II cells (14). None of the known *E. coli* RNases catalyze A-site cleavage during translational pausing, and the ribosome itself has been proposed to play a catalytic role (1,2,14). The 30S ribosome subunit binds the A-site codon, and is therefore appropriately positioned to catalyze A-site cleavage. However, the A site is dynamic and could recruit an unknown RNase in the same manner it binds translation factors. Regardless of the catalytic scenario, the 30S A site defines the mRNA substrate, and the ribosome must, at a minimum, act as a scaffold for the A-site nuclease.

The A-site codon is held within the decoding center of the 30S subunit, where base-pairing interactions between the codon and the incoming tRNA are monitored (15,16). Three 16S rRNA residues, G530, A1492 and A1493 (Fig. 1), make direct contact with the A-site codon. A1492 and A1493 bind in the minor groove of the codon-anticodon helix, ensuring that only Watson-Crick base pairs are allowed at the first two positions (15,16). Although 16S rRNA is critical for the differentiation of cognate from near-cognate tRNA, ribosomal proteins S12, S4, and S5 also play important roles in decoding. The *ram*, or ribosome ambiguity, mutations in *rpsD* and *rpsE* (encoding S4 and S5, respectively) are thought to stabilize a closed A-site conformation characterized by high affinity for aminocyl-tRNA (16-18). The *ram* conformation stabilizes the binding of near-cognate tRNA, and thereby increases the frequency of decoding errors. The aminoglycoside antibiotic streptomycin binds to the 30S A site (Fig. 1), and induces decoding errors, presumably by stabilizing the *ram* conformation (16,19). Streptomycin resistance is conferred by a variety of mutations in *rpsL*, which encodes ribosomal protein S12. Streptomycin-resistant S12 variants are predicted stabilize an open A-site conformation, and thereby counteract the closed conformation induced by streptomycin binding (16,18). In general, streptomycin-resistant *rpsL* alleles also confer an ‘error-restrictive’ phenotype characterized by reduced A-site affinity for near-cognate tRNA and hyper-accurate decoding (20). Neamine-containing aminoglycosides, such as paromomycin, also stabilize the closed A-site conformation, but have ribosome binding sites that are distinct from that of streptomycin (Fig. 1) (18,19).

In this report, we ask whether structural perturbations to the ribosomal A site influence A-site mRNA cleavage and tmRNA•SmpB activities. We show that two aminoglycoside antibiotics, streptomycin and paromomycin, inhibit A-site cleavage of stop codons during inefficient translation termination. Aminoglycosides also induced stop codon read-through, suggesting that these antibiotics reduce A-site cleavage by alleviating the underlying translational pause. A-site cleavage and tmRNA•SmpB-mediated SsrA-peptide tagging activities were significantly reduced in several streptomycin-resistant *rpsL* mutants. In general, SsrA-peptide tagging was reduced in cells containing error-restrictive ribosomes. However, streptomycin was able to fully restore peptide-tagging activity in a subset of *rpsL* strains, without significantly affecting error-restriction in these mutants. It appears that tmRNA may be more sensitive than tRNA to structural changes in the A site, perhaps reflecting the unique manner in which tmRNA•SmpB binds the ribosome. Based on these results, we propose that *rpsL* mutations specifically interfere with the recruitment of tmRNA•SmpB to the ribosome.

**Experimental Procedures**

**Bacterial strains and plasmids**

Table 1 lists the bacterial strains and plasmids used in this study. All bacterial strains were derivatives of *E. coli* strain X90. Streptomycin resistant alleles of *rpsL* (encoding ribosomal
protein S12) were generated by phage λ Red-mediated recombination using random oligonucleotide libraries as described previously (21). The codons for S12 residues lysine-42 (K42) and proline-90 (P90) were mutagenized using oligonucleotides, rpsL-K42X, (5′- CAT ACT TTA CGC AGC GCG GAG TTC GGT TTN NNA GGA GTG GTA GTA TAT ACA CGA GTA CAT), and rpsL-P90X, (5′- GCA CCA CGT ACG GTG TGG TAA CGA ACA CCN NNG AGG TCT TTA ACA CGA CCG CCA CGG ATC), respectively; and streptomycin resistant mutants were selected as described (21). The rpsL gene was amplified from streptomycin resistant mutants using oligonucleotides, rpsL-Nco, (5′- ACC CAT GGT TAA GCA CCC CAG CGC), and rpsL-Bam, (5′- TCT GGA TCC GGC AGA ATT TTA CGC); and sequenced using rpsL-seq, (5′- CTC CTC GAG TTT AGT TTG ACA TTT AAG ACC CAT), and rpsE-rev, (5′- CAA GCA GCG TCT AAC TAT GTT CCT TGT TCG G). The rpsD and rpsE genes (encoding ribosomal proteins S4 and S5, respectively) were amplified from streptomycin independent cells on antibiotic free plates, and selecting for streptomycin independent mutants. The rpsD and rpsE genes encoding ribosomal proteins S4 and S5, respectively) were amplified from streptomycin independent cells using oligonucleotides, rpsD-for, (5′- GCT CTG AAC GCC GCA GGT TTC CGC), rpsD-rev, (5′- TTT CTC GAT ATC AAC CAG GCG CGG), rpsE-for, (5′- CGT TCC GGG TTC CAA TAT CAT GGT CGC), and rpsE-rev, (5′- CAA GCA GCG TTG CCT TGT TCG G). The rpsD and rpsE PCR products were sequenced using oligonucleotides, rpsD-seq, (5′- GAT CCC TCA TAA CGG TTG TCG TTC), and rpsE-seq, (5′- GGC AGA TGC TGC CCG TGA AGC TGG CC). The details of all strain constructions are available upon request.

Plasmids pKW1, pKW11, pKW23, pPW500, pCH201, and pAD8 have been described previously (8,22-24). DNA fragments encoding the trc promoter and the N-terminal domain of phage λ Ci repressor (λN) were PCR-amplified from plasmid pWP500 using oligonucleotide primers containing restriction sites (underlined bases). The λN-flag-his6(PP) construct was generated with oligonucleotides, lacI-Hpal (5′- TAT CCC GCC GTT AAC TAG TAT CAA ACA GGA TTT TCG C), and His6(PP)-SacI, (5′- AAT GAG CTC AAT TAG GCC GGA TGA TGC GGA TGA TGG TGC). The λN-flag-his6(PP) fragment was generated with oligonucleotides, His6(LA)-SacI (5′- ACC GAG CTC AAT TAT GCC AGA TGG TGA TGA TGG), and lacI-Hpal. The resulting PCR fragments were digested with Hpal and Sacl and ligated to plasmid pTrc99A (GE Healthcare). The λN expression constructs were generated by PCR using oligonucleotide λN-Ndel, (5′- CAA TTT CAC ACA GGA AAC AGC ATA TGG GCA AAA AGA AAC G), in conjunction with: λN(PP)-SacI, (5′- GCG GAG CTC TCG AAT TAG GCC GGA GAC ATG CTA ACC GCT TCA TAC); λN(LA)-SacI, (5′- TTT AGC TCT CGA ATG CCA GAG ACA TGC TAA CC); and λN(PP)-SacI, (5′- GCG GAG CTC TCG AAT TAG GCC GGA GAC ATG CTA ACC GCT TCA TAC). The resulting PCR products were digested with Ndel and Sacl, and ligated to plasmid pFG501. Plasmid pFG501 is a modified version of pTrc99A (GE Healthcare) encoding the FLAG epitope between Neol and Ndel restriction sites, facilitating the construction of N-terminal FLAG fusion proteins (25).

mRNA expression and analysis

E. coli strains were grown overnight at 37 °C in LB medium supplemented with the appropriate antibiotics (150 µg/mL of ampicillin, 25 µg/mL of tetracycline, or 50 µM of streptomycin). The following day, cells were resuspended at an optical density at 600 nm (OD600) of 0.05 in 15 mL of fresh medium and grown at 37 °C with aeration. Once cultures reached OD600 ~0.5, mRNA expression was induced with 2 mM isopropyl β-D-thiogalactopyranoside (IPTG), and simultaneously treated with paromomycin or streptomycin at the indicated concentrations. After further incubation for 15 min, the cultures were poured into 15 mL of ice-cold methanol, collected by centrifugation, and cell pellets frozen at -80 °C. Total RNA was extracted from cell pellets using 1.0 mL of a solution containing 0.6 M ammonium isoosothiocyanate – 2 M guanidinium isothiocyanate – 0.1 M sodium acetate (pH 4.0) – 5% glycerol – 40% phenol. The disrupted cell suspension was extracted with 0.2 mL of chloroform, the aqueous phase removed and added to an equal volume of isopropyl alcohol to precipitate total RNA. RNA
pellets were washed once with ice-cold 75% ethanol and dissolved in 10 mM sodium acetate (pH 5.2) – 1 mM EDTA.

Northern blot and S1 nuclease protection analyses of all mRNAs were performed as described (1). Oligonucleotide 
\textbf{Trc-RBS} (5’ - CAT GGT CTG TTT CCT GTG TGA AAT TG) was 5’-end labeled with \[^{32}P\]-\(\gamma\)-ATP and used as a probe for Northern blot hybridizations. Oligonucleotides \textbf{cI(PP) S1}, (5’ - GCA GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA GTT AGG GCG GAG ACA TGC TAA CCG CTT CAT ACA TCT CGT AG); and \textbf{cI-His\(_6\)(PP) S1}, (5’ - GGT CGA CTC TAG AGG ATC CCC GGG TAC CGA GCT CGA GTT AGG GCG GAG ACA TGC TAA CCG CTT CAT ACA TCT CGT AG) were used as probes for nuclease S1 protection assays. Northern blots were visualized by phosphorimaging, and A-site mRNA cleavage was quantified using Quantity One software (Bio-Rad). A-site cleavage efficiency was determined as a percentage of total transcripts, defined as full-length transcripts plus A-site truncated transcripts.

\textbf{Protein expression and Western blot analysis}

Strains were cultured as described above for RNA analysis. Total protein was extracted from frozen cells in 8 M urea – 10 mM Tris-HCl (pH 8.0) – 150 mM NaCl, and His\(_6\)-tagged proteins purified by Ni\(^{2+}\)-NTA (nitrilotriacetic acid) affinity chromatography as described previously (23). SsrA(His\(_6\))-tagged protein was further purified by reverse phase-HPLC as described (26), for electrospray-ionization mass spectrometry analysis. Ni\(^{2+}\)-NTA purified proteins were resolved on SDS-polyacrylamide gels followed by staining with Coomassie blue. Stained gels were scanned using the LI-COR\textsuperscript{®} Odyssey infrared imaging system, and the percentage of SsrA(DD)-tagged chains was quantified as described (13). Reported tagging efficiencies are the mean ± standard error for three independent experiments.

Western blot analysis was performed using the LI-COR\textsuperscript{®} Odyssey infrared imaging system according to the manufacturer’s instructions with minor modifications. Briefly, 10 \(\mu\)g of total urea-soluble protein was resolved by SDS-PAGE followed by electrotransfer to nitrocellulose membranes. Membranes were blocked with 2% (w/v) BSA in phosphate buffered saline [2.7 mM KCl – 1.8 mM KH\(_2\)PO\(_4\) – 137 mM NaCl – 10.1 mM Na\(_2\)HPO\(_4\) (pH 7.4)], followed by incubation overnight with anti-SsrA(DD) polyclonal and anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich). IRDye\textsuperscript{™} 800-conjugated anti-mouse (Rockland Immunochemicals) and Alexafluor 680-conjugated anti-rabbit (Invitrogen) secondary antibodies were used for fluorescence detection.

\textbf{Dual luciferase assay}

\textit{E. coli} strains carrying plasmid pAD8 (24) were grown overnight in LB media supplemented with 150 \(\mu\)g/mL ampicillin. The following day, cells were diluted 1:200 into fresh LB containing 150 \(\mu\)g/mL ampicillin, and 50 \(\mu\)M streptomycin as indicated. Cultures were grown at 37 °C to mid-logarithmic phase, collected by centrifugation, and resuspended in 200 \(\mu\)L of lysis buffer [1 mg/mL lysozyme – 10 mM Tris-HCl (pH 8.0) – 1 mM EDTA]. Cell suspensions were incubated on ice for 10 min, and then frozen in a dry ice/ethanol bath. Samples were then thawed on ice, and 5 \(\mu\)L of each lysate was assayed for firefly (F-luc) and Renilla (R-luc) luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). For each reaction, luminescence (expressed as counts per second) was collected over a 10 s interval using a model 1420 Victor\textsuperscript{®} V plate reader with injectors (PerkinElmer Life Sciences). Each lysate was assayed in triplicate, and all rpsL mutants were independently tested at least three times.

\section*{RESULTS}

\textbf{Aminoglycoside antibiotics inhibit A-site mRNA cleavage}

We examined the effect of streptomycin on A-site mRNA cleavage using a plasmid-borne construct that expresses the N-terminal domain of \(\lambda\) phage cI repressor containing a C-terminal Pro-Pro sequence (Fig. 2A). The C-terminal Pro-Pro motif interferes with normal translation termination and is sufficient to elicit A-site cleavage at stop codons (1,27). This construct also encodes an N-terminal FLAG epitope to facilitate tracking of the reporter protein by Western blot (Fig. 2A). Expression of FLAG-2AN(PP) resulted in the accumulation of truncated mRNA in cells
lacking tmRNA (ΔtmRNA) but not in tmRNA* cells (Fig. 2B). A-site cleaved transcripts do not typically accumulate in tmRNA* cells, presumably because tmRNA•SmpB releases the truncated messages from stalled ribosomes, thereby facilitating their rapid degradation (1,10,28). S1 nuclease protection analysis confirmed that these transcripts were truncated in the stop codon (data not shown), consistent with A-site mRNA cleavage during inefficient translation termination (1,2). Truncated mRNA was not detected in cells expressing λN with a C-terminal Leu-Ala sequence (Figs. 1A, and data not shown), in agreement with previous studies showing that this C-terminal peptide sequence does not induce ribosome pausing during translation termination (14,27). Treatment of ΔtmRNA cells with streptomycin led to a dose-dependent decrease in A-site truncated mRNA, without affecting the levels of full-length transcript (Fig. 2B). These results suggest that streptomycin inhibits A-site mRNA cleavage during inefficient translation termination.

Translation initiation and ribosome recycling after termination are both inhibited at high aminoglycoside concentrations (29,30). Ribosomes must translate to the stop codon in order to elicit A-site cleavage in our system, and therefore it is possible that streptomycin reduced A-site cleavage indirectly by shutting down protein synthesis. To test whether streptomycin inhibited protein synthesis under these conditions, we measured the accumulation of FLAG-λN(PP) as a function of time in the absence and presence of streptomycin (Fig. 2C). FLAG-λN(PP) synthesis was not inhibited by 7.5 μM streptomycin during the first 20 min of treatment, and continued over the entire time course (Fig. 2C). Streptomycin significantly inhibited protein synthesis after 40 min of treatment (data not shown). However, we note that A-site cleavage was assessed after 15 min of streptomycin treatment, a point at which there was no decrease in reporter protein synthesis. Although protein synthesis was not inhibited, a prominent FLAG-reactive alternative translation product was observed in cells treated with streptomycin (Fig. 2C). Streptomycin increases the frequency of miscoding events, including stop codon read-through (31,32), which could account for this product. To ascertain whether streptomycin induced read-through, we mutated the flag-λN(PP) stop codon to a Gln codon to mimic the read-through product, and found that the resulting protein co-migrated with the streptomycin induced product on SDS-polyacrylamide gels (Figs. 1A & 1C). Moreover, the alternative product was not detected in streptomycin treated cells expressing the control FLAG-λN(LA) protein (Fig. 2C). Taken together, these results suggest that streptomycin induced miscoding occurs specifically when ribosomes pause during translation termination.

To determine whether neamine-containing aminoglycosides also influence A-site mRNA cleavage, we examined the effects of paromomycin using an additional λN reporter construct. The λN-flag-his6(PP) construct is derived from the previously characterized pPW500 plasmid (8), which encodes internal FLAG and His6 peptide epitopes that allow for immunodetection and affinity purification (Fig. 3A). Like the flag-λN(PP) construct described above, the λN-flag-his6(PP) message also undergoes A-site cleavage in ΔtmRNA cells (Fig. 3B, and data not shown). A-site mRNA cleavage was significantly inhibited in ΔtmRNA cells treated with increasing concentrations of paromomycin (Fig. 3B). Western blot analysis showed that λN-FLAG-His6(PP) synthesis was not dramatically reduced in ΔtmRNA cells treated with up to 30 μM paromomycin (Fig. 3C). Moreover, time course analysis showed that λN-FLAG-His6(PP) accumulated at similar rates in either the absence or presence of 20 μM paromomycin (Fig. 3C). Although λN-FLAG-His6(PP) synthesis was not inhibited, paromomycin induced significant stop codon read-through (Fig. 3C). Similar to the findings with streptomycin, paromomycin induced read-through was not observed during expression of the control λN-FLAG-His6(LA) protein, which undergoes efficient translation termination (Figs. 2A & 2C). These results show that aminoglycosides inhibit A-site mRNA cleavage at concentrations that still permit efficient protein synthesis in ΔtmRNA cells. The accompanying increase in stop codon read-through suggests that aminoglycosides may
inhibit A-site cleavage by relieving the underlying translational pause during termination.

**Aminoglycosides and SsrA-peptide tagging activity**

A-site cleavage is thought to be required for tmRNA•SmpB recruitment to ribosomes during inefficient translation termination (1,2). We reasoned that if aminoglycosides alleviate ribosome pausing via stop codon read-through, then they should also reduce the attendant SsrA-peptide tagging activity. We first confirmed peptide tagging at the C terminus of FLAG-\(\lambda\)N(PP) using tmRNA(\(\text{His}_6\)), which encodes the protease resistant SsrA(\(\text{His}_6\)) tag (23,33). FLAG-\(\lambda\)N(PP) was expressed in tmRNA(\(\text{His}_6\)) cells, and SsrA(\(\text{His}_6\))-tagged proteins were purified for mass spectrometry analysis. The major tagged product had a mass of 13,365 Da, corresponding to SsrA(\(\text{His}_6\))tag addition after the C-terminal proline residue of FLAG-\(\lambda\)N(PP) (Fig. 4A). We also used mass spectrometry to confirm C-terminal tagging of the \(\lambda\)N-FLAG-\(\text{His}_6\)(PP) reporter protein with the stable SsrA(DD) tag (data not shown). These results confirm previous work showing that A-site cleavage at stop codons in \(\Delta\)tmRNA cells is correlated with SsrA-peptide tagging of full-length proteins in tmRNA\(^+\) cells (1).

We next tested whether aminoglycosides inhibited SsrA(DD)-peptide tagging at the C terminus of the reporter proteins. We note that tmRNA\(^-\) cells are more resistant to streptomycin than \(\Delta\)tmRNA cells (34), and therefore higher streptomycin concentrations were used in these tagging experiments (compared to the data shown in Fig. 2B). Western blot analysis showed that streptomycin and paromomycin both inhibited SsrA(DD)-peptide tagging, and concomitantly increased stop codon read-through (Fig. 4B). However, total \(\lambda\)N reporter protein synthesis also appeared to be inhibited by the aminoglycosides, particularly in paromomycin treated cells (Fig. 4B). The inhibition of \(\lambda\)N expression in these experiments was perplexing, because both aminoglycosides had little effect on protein synthesis in \(\Delta\)tmRNA cells (see Figs. 1C & 2C). To determine whether SsrA(DD)-peptide tagging was inhibited to a greater extent than \(\lambda\)N synthesis, we quantified and compared the relative decreases in SsrA(DD)- and FLAG-dependent fluorescence from Western blots. We found that streptomycin inhibited SsrA(DD)-tagging to a greater extent than it inhibited FLAG-\(\lambda\)N(PP) synthesis (Fig. 4B, and data not shown). However, SsrA(DD)-peptide tagging and \(\lambda\)N-FLAG-\(\text{His}_6\)(PP) synthesis were inhibited to the same extent by paromomycin treatment (Fig. 4B, and data not shown). Therefore, though these results suggest that streptomycin inhibits SsrA tagging via stop codon read-through, tmRNA•SmpB activity was also inhibited due to a general decrease in protein synthesis in paromomycin treated cells.

In principle, it is possible that aminoglycosides directly inhibit tmRNA•SmpB activity. Indeed, paromomycin has been reported to interfere with distinct stages of the tmRNA•SmpB activity cycle (35,36). To determine whether aminoglycosides inhibit SsrA-peptide tagging independent of stop codon read-through, we examined tmRNA•SmpB activity with ribosomes stalled on nonstop mRNA. Nonstop messages lack in-frame stop codons. Therefore, ribosomes translate to the 3’ end of these “truncated” transcripts, where they stall with no codon in the A site (Fig. 5A). In tmRNA\(^-\) cells, these stalled ribosomes are rapidly rescued by tmRNA•SmpB, and a large proportion of the protein chains are SsrA-tagged (3,22). In \(\Delta\)tmRNA cells, the untagged nascent chains are released from the stalled ribosome by an uncharacterized process (Fig. 5A). Thus, tmRNA•SmpB activity can be assessed by measuring the ratio of tagged to untagged protein synthesized from nonstop mRNA (Fig. 5A). Expression of \(\lambda\)N-FLAG-\(\text{His}_6\) from nonstop mRNA in \(\Delta\)tmRNA cells resulted in the accumulation of untagged protein, whereas almost all of the \(\lambda\)N-FLAG-\(\text{His}_6\) chains were tagged in tmRNA(DD) cells (Fig. 5B). When tmRNA(DD) cells were treated with increasing concentrations of streptomycin or paromomycin, we again saw that reporter protein synthesis was somewhat inhibited (Fig. 5B). However, aminoglycoside treatment did not increase the proportion of untagged \(\lambda\)N-FLAG-\(\text{His}_6\) chains (Fig. 5B). These results indicate that under these experimental conditions, aminoglycosides do not specifically inhibit the tmRNA•SmpB system.
A-site mRNA cleavage in streptomycin-resistant rpsL mutants

In addition to their well-described effects on the ribosome, aminoglycosides also bind and modulate the activity of other catalytic RNAs (37-39). To determine whether aminoglycosides influence A-site cleavage due to their effects on translation, we examined streptomycin-resistant rpsL mutants. The ribosomes from rpsL mutants are altered in ribosomal protein S12, and typically have much lower affinity for streptomycin (40-42). To generate a variety of rpsL mutations, we used phage λ. Red-mediated recombination and oligonucleotide libraries to randomize codons corresponding to S12 residues lysine-42 (K42) and proline-90 (P90) (see Fig. 1), and then selected the mutagenized cells for streptomycin resistant mutants. We focused on K42 and P90 because changes in these residues are most commonly associated with streptomycin resistance (20,43). We sequenced the rpsL gene from 100 streptomycin resistant mutants, and identified 16 different missense mutations: nine K42 alleles and seven P90 alleles (Table 2). Because oligonucleotide recombineering allows the routine isolation of unusual missense mutations (21), many of these mutations have not been previously identified in E. coli. Examination of rpsL mutant growth rates in the presence and absence of streptomycin led to the identification of two streptomycin dependent mutants, rpsL(P90K) and rpsL(P90R); and one streptomycin pseudodependent mutant, rpsL(P90Q), which grew almost two-fold faster in the presence of streptomycin (Table 2).

Initially, we examined A-site mRNA cleavage in three previously characterized mutants: rpsL(K42T), rpsL(K42R), and rpsL(K42A). The rpsL(K42T) mutation is a classical streptomycin resistance allele that produces error-restrictive ribosomes. In contrast, rpsL(K42R) is unique amongst known streptomycin resistance alleles in that its phenotype is non-restrictive (20). Green and colleagues recently described the rpsL(K42A) mutation, reporting that it conferred a streptomycin pseudodependent phenotype (44). However, we observed no change in the growth rate of rpsL(K42A) cells when cultured in media containing streptomycin (Table 2). A-site mRNA cleavage was reduced in all three of these mutants compared to rpsL+ cells (Fig. 6). Growth in the presence of 50 µM streptomycin had little effect on A-site mRNA cleavage in rpsL(K42A) and rpsL(K42T) cells, but decreased cleavage efficiency in the rpsL(K42R) mutant (Fig. 6). We also examined the novel rpsL(P90F) mutant, which was the most frequently identified mutation in the selection (Table 2). A-site mRNA cleavage was reduced in rpsL(P90F) cells, but could be restored when the mutant was grown with streptomycin (Fig. 6). Taken together, these results strongly suggest that streptomycin modulates A-site cleavage by virtue of its effects on the ribosome and translation.

We also examined the two streptomycin-dependent rpsL(P90R) and rpsL(P90K) alleles. Because streptomycin is presumably bound to streptomycin dependent ribosomes, we used these mutants to examine whether ribosome bound aminoglycoside influences A-site mRNA cleavage. Both streptomycin dependent mutants exhibited lower A-site cleavage levels compared to rpsL+ cells (Fig. 6). We next asked whether mutations that suppress streptomycin dependence could restore A-site cleavage to rpsL(P90K) cells. These suppressor mutations typically occur in the rpsD and rpsE genes, which encode ribosomal proteins S4 and S5, respectively (20). We isolated and identified four rpsD mutations that allowed rpsL(P90K) cells to grow in the absence of streptomycin. The rpsD-1, rpsD-2, and rpsD-3 alleles encode Q53K, N85Y, and Y203amber missense mutations, respectively (Table 1). The rpsD-4 mutation is a single-nucleotide deletion in codon 193, resulting in a frameshift that replaces the C-terminal D193 – K205 residues of S4 with the TLNTT pentapeptide (Table 1). Each of the rpsD mutants was streptomycin sensitive, but still contained the original rpsL(P90K) mutation. In addition to conferring streptomycin sensitivity, the rpsD mutations also appeared to restore some A-site cleavage activity compared to the parental rpsL(P90K) strain (Fig. 6). These results demonstrate that structural perturbations to the A site can significantly modulate A-site cleavage activity.

SsrA-peptide tagging activity in streptomycin-resistant rpsL mutants

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Given the effects of rpsL mutations on A-site cleavage, we next asked whether these alleles also influence tmRNA•SmpB activity. We expressed λN-FLAG-His$_6$(PP) in rpsL mutants containing tmRNA(DD), then purified the reporter protein by Ni$^2^+$-affinity chromatography to determine the percentage of SsrA(DD)-tagged chains. SsrA(DD)-tagging was largely unaffected in most of the rpsL(K42) mutants, although rpsL(K42V) cells showed significantly less tagging (28 ± 1.2% tagged) than rpsL$^\ast$ cells (43 ± 0.6% tagged) (Fig. 7). In contrast, most of the rpsL(P90) mutants displayed significant SsrA(DD)-peptide tagging defects. The greatest effect was observed in rpsL(P90Q) cells, in which only 19 ± 1.1% of the λN-FLAG-His$_6$(PP) chains were tagged (Fig. 7). Notably, SsrA(DD)-tagging was increased when the rpsL(P90N), rpsL(P90Y), rpsL(P90F), and rpsL(P90Q) mutants were grown in 50 μM streptomycin (Fig. 7). Surprisingly, there was little correlation between SsrA-tagging efficiency and A-site mRNA cleavage in the rpsL mutants. For example, the rpsL(K42A) mutation had no effect on peptide-tagging activity in streptomycin treated tmRNA(DD) cells (Fig. 7), yet reduced A-site cleavage in the ΔtmRNA background (Fig. 6). A similar disparity between A-site cleavage and peptide tagging was observed with the rpsL(P90R) streptomycin dependent mutation.

SsrA-tagging of full-length λN-FLAG-His$_6$(PP) chains results from inefficient translation termination. Therefore, tagging is inversely related to the efficiency of stop codon decoding by release factors (23,27). To assess tmRNA•SmpB activity without competition from release factors, we examined SsrA(DD) tagging of λN-FLAG-His$_6$ expressed from nonstop mRNA. As outlined above, decreased tmRNA•SmpB activity leads to the accumulation of untagged products, which are readily distinguished from tagged protein by gel electrophoresis. We focused on the rpsL(P90) mutants because these cells exhibited the most pronounced decrease in SsrA(DD)-tagging during inefficient termination (Fig. 7). In addition, the previously characterized rpsL(K42R), rpsL(K42T), and rpsL(K42A) mutants were also analyzed. All of the examined strains had significant tagging defects, except for the rpsL(K42R) and rpsL(P90R) mutants in which SsrA(DD) tagging was actually more efficient than rpsL$^\ast$ cells (Fig. 8). Streptomycin treatment increased tmRNA•SmpB activity in all of the non-dependent rpsL(P90) mutants. In fact, the rpsL(P90H), rpsL(P90F), and rpsL(P90Y) mutants regained wild-type tagging efficiency, or better, when treated with streptomycin (Fig. 8). In contrast, the rpsL(K42A) and rpsL(K42T) mutants showed no change in peptide tagging in response to streptomycin (Fig. 8). We also examined the effects of the four rpsD mutations, and found that the rpsD-1, -2, and -4 alleles decreased SsrA(DD)-tagging efficiency compared to the parental rpsL(P90K) strain (Fig. 8).

The results shown in Fig. 8 suggest that SsrA-tagging in rpsL mutants may be related to the error-restrictive phenotype. For example, the restrictive rpsL(K42T) mutation decreased SsrA tagging, whereas the non-restrictive rpsL(K42R) mutation allowed efficient tagging (Fig. 8). Additionally, streptomycin counteracts the restrictive phenotype (20), which could conceivably lead to more efficient tagging in the streptomycin treated rpsL(P90) mutants. Therefore, we assessed miscoding in several of the rpsL mutants to determine whether a correlation exists between error-restriction and reduced tmRNA•SmpB activity. Stop codon read-through was measured using a Renilla luciferase (R-Luc)-firefly luciferase (F-Luc) fusion construct, in which the firefly luc gene contains an in-frame UGA stop codon at position 417 (24). This construct only produces F-Luc activity when the UGA codon is inappropriately decoded as a sense codon. In the absence of streptomycin, most of the rpsL mutants had lower F-Luc/R-Luc activity ratios than rpsL$^\ast$ cells, indicative of reduced read-through and the error-restrictive phenotype (Fig. 9). As expected, the rpsL(K42R) mutant was non-restrictive, but surprisingly became more restrictive when grown in media supplemented with streptomycin (Fig. 9). Streptomycin slightly increased read-through in the rpsL(K42T) and rpsL(P90Q) mutants, but F-Luc/R-Luc ratios were unaffect ed by streptomycin in the other mutants (Fig. 9). Streptomycin-dependent rpsL(P90R) cells were moderately restrictive, whereas the rpsL(P90K) mutant was more restrictive similar to the other rpsL mutants we examined (Fig. 9). The rpsD mutations had little effect on miscoding in
the \textit{rpsL(P90K)} background, although we note that it was necessary to test these strains under different conditions due to their respective streptomycin dependent and streptomycin sensitive phenotypes (Fig. 9). Taken together, we found no direct correlation between error-restriction and efficiency of SsrA-peptide tagging (compare Figs. 7 & 8). These results indicate that some other property of \textit{rpsL} ribosomes influences tmRNA•SmpB activity.

\section*{DISCUSSION}

The results presented here show that alterations in ribosomal A-site structure and function have significant effects on A-site mRNA cleavage and tmRNA•SmpB activity. Streptomycin and paromomycin both inhibited A-site cleavage during inefficient translation termination. A-site cleavage during translational pauses is a complex process that requires at least two RNase activities. RNase II processively degrades downstream mRNA in a 3'→5' direction until it encounters the leading edge of the paused ribosome (14). mRNA degradation to the ribosome border appears to be required for subsequent cleavage in the A-site codon. The A-site nuclease has yet to be identified, and could be an activity of the ribosome itself. Because streptomycin and paromomycin bind very near the A-site codon and dramatically alter A-site structure and function (15,16,19,20,45,46), these aminoglycosides could directly inhibit A-site nuclease activity. However, aminoglycosides induced stop codon read-through, which occurred concomitantly with the decrease in A-site mRNA cleavage. Though correlative, this finding suggests that aminoglycoside induced miscoding alleviates the translational pause required for A-site cleavage. Other indirect evidence also suggests that aminoglycosides influence ribosome pausing in this system. (31,32,47). When A-site cleavage is inhibited, downstream mRNA is still degraded to the ribosome border, resulting in a slightly larger truncated transcript that indicates the ‘toe-print’ of the paused ribosome (14,25). The lack of A-site and ribosome-border truncated transcripts in aminoglycoside treated cells suggests that ribosomes no longer pause during translation termination. We note that paromomycin has the potential to actually \textit{increase} A-site cleavage during translation termination, because it inhibits release factor binding to A-site stop codons (48). However, paromomycin also stabilizes near cognate tRNA in the A site (19), providing an outlet for arrested ribosomes via stop codon read-through. Taken together, it appears that aminoglycoside induced miscoding accounts for the observed decrease in A-site mRNA cleavage.

Aminoglycosides also inhibited the SsrA-peptide tagging during inefficient translation termination. In our system, this inhibitory effect appears to be the result of increased stop codon read-through and decreased protein synthesis. Aiba and colleagues have previously shown that aminoglycoside induced stop codon read-through can result in translation through the 3'-UTR to the end of the message, and thereby increase SsrA-peptide tagging (49). Here, we see that the same miscoding event can actually prevent peptide tagging associated with inefficient translation termination. We note the constructs used in our study have additional in-frame stop codons in the 3'-UTR, which prevent ribosomes from reaching the 3' end of the transcript. Paromomycin has also been reported to inhibit the aminocacylation of tmRNA, and to shift the tmRNA reading frame into the -1 frame \textit{in vitro} (35,36). In those studies, tmRNA aminoacylation was inhibited at ~225 \( \mu \text{M} \) paromomycin, and the -1 frameshift effect became apparent at 55 \( \mu \text{M} \) paromomycin. We do not know the intracellular concentration of aminoglycosides in our experiments, but there was no evidence of -1 frame translation by Western blot analysis or mass spectrometry\(^3\). Moreover, there was no indication that tmRNA aminoacylation was specifically inhibited by either paromomycin or streptomycin. A lack of tmRNA aminoacylation would presumably be manifested as a \textit{ΔtmRNA} phenotype, yet we observed no increase in untagged chains during aminoglycoside treatment. Therefore, though aminoglycosides can clearly inhibit distinct stages of the tmRNA activity cycle, these effects appear to require higher concentrations than those used in our study. It is still unclear why the synthesis of our reporter proteins was more sensitive to aminoglycoside treatment in tmRNA(DD) cells compared to \textit{ΔtmRNA} cells, particularly as \textit{ΔtmRNA} cells have been shown to be more sensitive to aminoglycoside antibiotics (34). However, we...
note that protein synthesis was also inhibited in ΔtmRNA cells after about 40 min of aminoglycoside treatment. Thus, it appears that the inhibition of protein synthesis is merely delayed in ΔtmRNA cells, perhaps reflecting differences in the rate of aminoglycoside uptake between the two genetic backgrounds.

A-site mRNA cleavage was significantly decreased in several streptomycin-resistant rpsL mutants, yet there was no correlation between error-restriction and A-site cleavage in these strains. The non-restrictive rpsL(K42R) mutation, and the weakly restrictive rpsL(P90R) mutation both reduced A-site cleavage to the same extent as restrictive rpsL alleles. Streptomycin resistant ribosomes tend to suppress stop codon read-through, suggesting that translation termination may occur more efficiently in these mutants. Of course, increased fidelity during termination does not necessarily indicate more rapid stop codon decoding. Indeed, SsrA(DD) tagging of full-length FLAG-λN(PP) in the rpsL(K42A) and rpsL(P90R) mutants was essentially identical to rpsL+ cells, suggesting comparable translational pausing during termination in these backgrounds. It seems more likely that reduced read-through is due to low A-site affinity for suppressor tRNA, rather than an increase in the rate of termination (50-52).

Alternatively, reduced A-site cleavage may result from processivity errors, which are characteristic of both streptomycin resistant and dependent ribosomes (45,46,53). Processivity errors occur when translating ribosomes fail to reach the stop codon, and instead produce truncated peptide chains. Most of these errors have been proposed to be the result of “drop-off” (20), in which peptidyl-tRNA dissociates from the ribosome. Although the restrictive P site has high affinity for peptidyl-tRNA, the restrictive A-site has a corresponding low affinity for peptidyl-tRNA, and drop-off is hypothesized to occur from the A/P hybrid state prior to translocation (17,46). We did not detect incomplete FLAG-λN(PP) chains in rpsL mutants, but perhaps drop-off occurred while ribosomes paused during termination. Non-canonical release of full-length nascent chains in rpsL mutants would be indistinguishable from normal termination, and therefore very difficult to study in vivo. We are currently examining the kinetics of ribosome pausing in various rpsL mutants to determine whether pre-termination rpsL mutants to determine whether pre-termination ribosomes are recycled more rapidly in these cells.

Finally, our data show that ribosomal protein S12 plays an important role in tmRNA•SmpB-mediated ribosome rescue. This effect was most apparent during ribosome arrest on nonstop mRNA, in which a significant proportion of chains were not tagged in tmRNA(DD) cells. One possible explanation for these findings is that rpsL ribosomes do not support efficient tmRNA•SmpB activity. In principle, a defect in any stage of the tmRNA activity cycle could produce the partial loss of function observed in these mutants. But given the well-characterized role of S12 in tRNA selection, a defect in the initial binding of tmRNA to the A site seems most likely. A-site binding of tmRNA differs from that of canonical tRNAs in several respects. tmRNA lacks an anticodon, and readily binds ribosomes that contain no A-site codon. Additionally, SmpB appears to mimic the missing A-site codon-anticodon helix, and has recently been shown to make contacts with 16S rRNA residues G530, A1492, and A1493 in the decoding center (54-56). Perhaps these atypical interactions render tmRNA•SmpB more sensitive to perturbations in the A site. Although rpsL ribosomes tend to have lower A-site affinity for canonical tRNA, this phenomenon cannot completely account for our data. SsrA-peptide tagging was significantly reduced in several error-restrictive rpsL(P90) mutants. However, streptomycin was able to fully restore tagging in several of these mutants, while having no effect on error-restriction. These results suggest that the rpsL mutations have specific effects on tmRNA•SmpB recruitment, distinct from the effects on tRNA binding. Although we favor a model in which rpsL mutations interfere with tmRNA•SmpB binding to the A site, we recognize that there is a tmRNA-independent pathway that releases nascent chains from nonstop-arrested ribosomes. It is possible that rpsL mutations accelerate this tmRNA-independent ribosome-recycling pathway, thereby giving the appearance of defective tmRNA•SmpB function. These two models can be distinguished by measuring the rates of peptidyl-tRNA turnover from nonstop mRNA-arrested ribosomes in tmRNA+ and ΔtmRNA cells. Peptidyl-tRNA accumulates on
nonstop-stalled ribosomes in ΔtmRNA cells, but not in tmRNA− cells, presumably due to rapid ribosome rescue2. If rpsL mutations interfere with tmRNA•SmpB recruitment to paused ribosomes, then peptidyl-tRNA should be detectable in these mutants. Alternatively, if the rpsL mutations facilitate tmRNA-independent ribosome recycling, then peptidyl-tRNA should turn over more rapidly in ΔtmRNA cells carrying these mutations. We are currently measuring the kinetics of peptidyl-tRNA turnover in rpsL mutants to uncover the basis of this ribosome rescue phenotype.

REFERENCES


FOOTNOTES

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1L. E. Holberger and C. S. Hayes, unpublished results.

2B. D. Janssen and C. S. Hayes, manuscript in press.

FIGURE LEGENDS

Figure 1. Aminoglycoside binding sites in the ribosomal A site. The decoding center of the 30S subunit is depicted with helix 44 (h44) of 16S rRNA and ribosomal protein S12. 16S rRNA residues G530, A1492, and A1493 are indicated along with the ribosome binding sites of streptomycin (red) and paromomycin (green). Mutations that change S12 residues Lys42 and Pro90 are able to confer both streptomycin resistant and streptomycin dependent phenotypes. These data were taken from PDB accession number 1FJG (19), and rendered with PyMol.
Figure 2. Streptomycin inhibits A-site mRNA cleavage during inefficient translation termination. (A) FLAG-λN expression constructs. The generalized flag-λN transcript is shown schematically along with the Northern probe-binding site. The 3’ coding sequences of the flag-λN(PP), flag-λN(LA), and flag-λN(UAA→Gln) transcripts are shown. The UAA→Gln mutation changes the UAA stop codon to a glutamine codon, mimicking stop codon read-through. The boxed P and A indicate the positions of the ribosomal P and A sites during translation termination. (B) Northern blot analysis of flag-λN(PP) mRNA. The flag-λN(PP) transcript was expressed in tmRNA+ and ΔtmRNA cells as indicated. ΔtmRNA cells were treated with increasing concentrations of streptomycin for 15 min, total RNA was isolated, and the effects on A-site cleavage assessed by Northern blot. The 3’ end of the truncated transcript was mapped to the stop codon by S1 nuclease protection analysis (data not shown). The positions of full-length and A-site truncated transcripts are indicated. (C) Western blot analysis of protein synthesis in streptomycin treated cells. Expression of FLAG-λN(PP) was induced in ΔtmRNA cells concomitantly with the addition of 7.5 µM streptomycin, and samples were taken at the indicated times for Western blot using FLAG-specific antibodies. Streptomycin induced the production of an alternative translation product that co-migrated with protein expressed from flag-λN(UAA→Gln), consistent with stop codon read-through. Streptomycin did not induce stop codon read-through during FLAG-λN(LA) synthesis.

Figure 3. Paromomycin inhibits A-site mRNA cleavage during inefficient translation termination. (A) λN-FLAG-His6 expression constructs. The generalized λN-flag-his6(PP) and λN-flag-his6(LA) messages are shown. The boxed P and A indicate the positions of the ribosomal P and A sites during translation termination. (B) Northern blot analysis of λN-flag-his6(PP) mRNA. The λN-flag-his6(PP) message was expressed in tmRNA+ and ΔtmRNA cells as indicated. ΔtmRNA cells were treated with increasing concentrations of paromomycin, and the effects on A-site cleavage assessed by Northern blot. The 3’ end of the truncated transcript was mapped to the stop codon by S1 nuclease protection analysis (data not shown). The positions of full-length and A-site truncated transcripts are indicated. (C) Western blot analysis of protein synthesis in paromomycin treated cells. Expression of λN-FLAG-His6(PP) was induced in ΔtmRNA cells concomitantly with the addition of 3.75 µM paromomycin, and samples were taken at the indicated times for Western blot using FLAG-specific antibodies. The rate of λN-FLAG-His6(PP) synthesis in ΔtmRNA cells treated with 20 µM paromomycin was also compared to untreated cells. Finally, paromomycin induced stop codon read-through during the synthesis of λN-FLAG-His6(PP), but not λN-FLAG-His6(LA).

Figure 4. SsrA-peptide tagging during aminoglycoside treatment. (A) Mass spectrometry of SsrA(His6)-tagged FLAG-λN(PP). FLAG-λN(PP) was expressed in tmRNA(His6) cells, and SsrA(His6)-tagged protein was purified by Ni2+-affinity chromatography for mass spectrometry. The major species had a mass of 13,365 Da, corresponding to SsrA(His6) tag addition after the C-terminal Pro residue (calculated mass, 13,364.9 Da). (B) Western blot analysis of SsrA(DD)-peptide tagging during aminoglycoside treatment. FLAG-λN(PP) and λN-FLAG-His6(PP) were expressed in tmRNA(DD) cells treated with increasing concentrations of aminoglycosides for 20 min. Total urea-soluble protein was analyzed by Western blot using antibodies specific for the FLAG and SsrA(DD) epitopes. Individual fluorescence channels and the merged signals are presented.

Figure 5. tmRNA•SmpB-mediated ribosome rescue from nonstop mRNA during aminoglycoside treatment. (A) Schematic of tmRNA-mediated and tmRNA-independent ribosome recycling. The λN-flag-his6(trpL) nonstop message is depicted, and the positions of the encoded flag and his6 peptide epitopes indicated. The λN-flag-his6 open reading frame was fused to the intrinsic transcription start.
terminator from the *E. coli* trp leader to create a transcript lacking in-frame stop codons. Ribosomes arrested on nonstop messages are efficiently rescued by the tmRNA•SmpB system. In ΔtmRNA cells, the nascent chains are released from these stalled ribosomes by an uncharacterized pathway, and untagged protein accumulates. (B) Western blot analysis of SsrA(DD)-peptide tagging during nonstop mRNA expression. The λN-flag-his₆(trpL) transcript was expressed in ΔtmRNA and tmRNA(DD) cells as indicated. tmRNA(DD) cells were treated with increasing concentrations of streptomycin and paromomycin for 20 min. Total urea-soluble protein was analyzed by Western blot using antibodies specific for the FLAG and SsrA(DD) epitopes. Individual fluorescence channels and the merged signals are presented.

**Figure 6. Northern analysis of A-site mRNA cleavage in streptomycin-resistant rpsL mutants.** The flag-λN(PP) transcript was expressed in ΔtmRNA cells containing rpsL mutations that encode the indicated streptomycin-resistant S12 variants. Total RNA was isolated from cells grown in the absence and presence of streptomycin (50 µM) as indicated. Samples from tmRNA⁺ and ΔtmRNA cells containing wild-type S12 (rpsL⁺) were included in each blot as a reference control for A-site cleavage. The rpsL(P90K) and rpsL(P90R) mutants are streptomycin dependent, and therefore were not tested in the absence of streptomycin. Similarly, the rpsD mutations confer streptomycin sensitivity to the parental rpsL(P90K) strain, and therefore these mutants were not tested with streptomycin. The positions of full-length and A-site truncated transcripts are indicated. The percentage of A-site truncated transcripts, with respect to total transcript (full-length + truncated), was determined from phosphorimaging data as described in Experimental Procedures.

**Figure 7. Quantification of SsrA(DD)-peptide tagging in streptomycin-resistant rpsL mutants.** The efficiency of SsrA(DD)-peptide tagging during inefficient translation termination was determined using λN-FLAG-His₆(PP) as the reporter protein. λN-FLAG-His₆(PP) was synthesized in rpsL mutants expressing tmRNA(DD), in the absence or presence of streptomycin (50 µM) as indicated. Total λN-FLAG-His₆(PP) protein was purified by Ni²⁺-affinity chromatography, the SsrA(DD)-tagged and untagged chains resolved by SDS-PAGE, and quantified using LI-COR® Odyssey software. The percentage of tagged chains in each background is reported as the average ± standard error from three independently conducted experiments.

**Figure 8. Ribosome rescue activity in streptomycin-resistant rpsL mutants.** The efficiency of tmRNA•SmpB-mediated ribosome rescue from nonstop mRNA was assessed by SsrA(DD)-peptide tagging. λN-FLAG-His₆ was synthesized from a nonstop message (shown in Fig. 5A) in tmRNA(DD) cells containing the indicated rpsL mutations. Cells were grown in the absence or presence of streptomycin (50 µM) as indicated. Total λN-FLAG-His₆(PP) protein was purified by Ni²⁺-affinity chromatography, and the SsrA(DD)-tagged and untagged chains resolved by SDS-PAGE followed by Coomassie blue staining.

**Figure 9. Decoding fidelity of streptomycin-resistant cells.** The pAD8 plasmid encoding a Renilla-firefly luciferase fusion interrupted by a UGA stop codon at position 417 of *fluc* was introduced into ΔtmRNA cells containing the indicated rpsL mutations. Increased stop codon read-through results in a higher ratio of firefly to Renilla luciferase activity (F-Luc/R-Luc). F-Luc/R-Luc ratios were determined from lysates of cells grown in the absence and presence of streptomycin (50 µM) as indicated. Reported values are the average ± standard error for at least three independently conducted experiments.
## Table 1. Bacterial strains and plasmids

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<td>pKW1-derived plasmid expressing tmRNA(His&lt;sub&gt;6&lt;/sub&gt;), Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(23)</td>
</tr>
<tr>
<td>pAD8</td>
<td>Encodes Renilla-firefly luciferase fusion interrupted by UGA stop codon, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(24)</td>
</tr>
</tbody>
</table>

*Abbreviations used: ampicillin resistant, Amp<sup>R</sup>; chloramphenicol resistant, Cm<sup>R</sup>; streptomycin resistant, Str<sup>R</sup>; streptomycin dependent, Str<sup>D</sup>; streptomycin pseudodependent, Str<sup>P</sup>; streptomycin sensitive, Str<sup>S</sup>; tetracycline resistant, Tet<sup>R</sup>.*
Table 2. Selection of streptomycin-resistance rpsL mutations$^a$

<table>
<thead>
<tr>
<th>Mutagenized position</th>
<th>AA change</th>
<th>Phenotype$^b$</th>
<th>Isolation frequency</th>
<th>Doubling time (min)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No strept</td>
<td>50 µM strept</td>
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<tr>
<td><em>wild-type</em></td>
<td>--</td>
<td>Str$^S$</td>
<td>--</td>
<td>27</td>
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<tr>
<td><em>Lys42</em></td>
<td>Ala</td>
<td>Str$^R$</td>
<td>1/43</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Arg</td>
<td>Str$^R$</td>
<td>21/43</td>
<td>29</td>
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<td></td>
<td>Asn</td>
<td>Str$^R$</td>
<td>1/43</td>
<td>46</td>
</tr>
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<td></td>
<td>Cys</td>
<td>Str$^R$</td>
<td>5/43</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Ile</td>
<td>Str$^R$</td>
<td>1/43</td>
<td>44</td>
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<td>Ser</td>
<td>Str$^R$</td>
<td>6/43</td>
<td>48</td>
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<td>Thr</td>
<td>Str$^R$</td>
<td>2/43</td>
<td>31</td>
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<tr>
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<td>Tyr</td>
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<tr>
<td></td>
<td>Val</td>
<td>Str$^R$</td>
<td>5/43</td>
<td>40</td>
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<tr>
<td><em>Pro90</em></td>
<td>Arg</td>
<td>Str$^D$</td>
<td>1/57</td>
<td>ND$^c$</td>
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<td>Asn</td>
<td>Str$^R$</td>
<td>6/57</td>
<td>36</td>
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<td>Gln</td>
<td>Str$^P$</td>
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<td>76</td>
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<td>His</td>
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<td>36</td>
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<tr>
<td></td>
<td>Lys</td>
<td>Str$^D$</td>
<td>1/57</td>
<td>ND$^c$</td>
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<td></td>
<td>Phe</td>
<td>Str$^R$</td>
<td>34/57</td>
<td>50</td>
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<td>Tyr</td>
<td>Str$^R$</td>
<td>6/57</td>
<td>46</td>
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</table>

$^a$Streptomycin resistant mutants were produced by targeted mutagenesis of the chromosomal rpsL gene as described in Experimental Procedures. Amino acid residues are given in three-letter code.

$^b$Abbreviations: Str$^S$, streptomycin sensitive; Str$^R$, streptomycin resistant; Str$^D$, streptomycin dependent; Str$^P$, streptomycin pseudodependent.

$^c$Not determined. These strains would not grow in either the presence (or absence) of streptomycin.
Figure 2

A

flag

Northern probe

Pro-Pro
-AUG-UCU-CCG-CCC-UAA-UUC-
Met Ser Pro Pro stop

UAA → Gin -AUG-UCU-CCG-CCC-CAA-UUC-
Met Ser Pro Gln Phe

Leu-Ala -AUG-UCU-CUG-GCA-UAA-UUC-

B

Northern blot

<table>
<thead>
<tr>
<th>streptomycin (µM)</th>
<th>0</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tr>
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<td></td>
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<tr>
<td>ΔtmRNA</td>
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</table>

C

Western blots

<table>
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<tr>
<th>time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
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<tr>
<td>7.5 µM streptomycin</td>
<td>read-through</td>
<td>full-length</td>
<td></td>
<td></td>
<td>read-through</td>
<td>full-length</td>
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<tr>
<td>no drug</td>
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</tbody>
</table>

UAA → Gin

Pro-Pro

Leu-Ala

strept
Figure 3

A. **λN-flag-his$_6$ mRNA**

- Northern probe
- His  His  Pro  Pro  stop

**Pro-Pro**
- CAU-CAU-CCG-CCC-UAA-UUG-

**Leu-Ala**
- CAU-CAU-CUG-GCA-UAA-UUG-

B. **Northern blot**

<table>
<thead>
<tr>
<th>paromomycin (µM)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
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<tbody>
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<td>full-length</td>
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<td>A-site</td>
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</tr>
</tbody>
</table>

C. **Western blots**

- **λN-FLAG-His$_6$(PP)**
- paromomycin (µM) 0 5 10 15 20 25 30
- read-through — — — — — — —
- full-length — — — — — — —

- **no drug**
- 0 10 20 30
- read-through — — — —
- full-length — — — —

- **20 µM paromomycin**
- 0 10 20 30
- read-through — — — —
- full-length — — — —

- **Pro-Pro**
- paromomycin (µM) 0 10 20 30
- read-through — — — —
- full-length — — — —

- **Leu-Ala**
- paromomycin (µM) 0 10 20 30
- read-through — — — —
- full-length — — — —
A  

**Mass spectrometry**

- **FLAG-\(\lambda\)N(PP)-SsrA(His\(_6\))**
- 13,365 Da (calc. 13,364.9 Da)

B  

**Western blots**

**FLAG-\(\lambda\)N(PP)**

- Streptomycin (\(\mu\)M)
  - 0
  - 5
  - 10
  - 15
  - 20
  - 25

**\(\lambda\)N-FLAG-His\(_6\)(PP)**

- Paromomycin (\(\mu\)M)
  - 0
  - 5
  - 10
  - 15
  - 20
  - 25
  - 30
Figure 5

A

\[ \text{\text{\(\lambda\)N-flag-his\(_6\)(trpL)}\text{\ non-stop mRNA}} \]

\[ \text{\(\Delta\text{tmRNA} \rightarrow \text{untagged}\)} \]

\[ \text{\(\text{tmRNA(DD)} \rightarrow \text{SsrA(DD) tagged}\)} \]

B

**Western blots**

<table>
<thead>
<tr>
<th>Streptomycin ((\mu\text{M}))</th>
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<th>0</th>
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Ribosomal protein S12 and aminoglycoside antibiotics modulate A-site mRNA cleavage and transfer-messenger RNA (tmRNA) activity in Escherichia coli

Laura E. Holberger and Christopher S. Hayes

J. Biol. Chem. published online September 23, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.062745

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