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Diverse effects of residues 74–78 in ribosomal protein S12 on decoding and antibiotic sensitivity

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ABSTRACT

Ribosomal protein S12 plays key roles in the ribosome's response to the error-promoting antibiotic streptomycin and in modulating the accuracy of translation. The discovery that substitutions at His76 in S12, distant from the streptomycin binding site, conferred streptomycin resistance in the thermophilic bacterium *Thermus thermophilus* prompted us to make similar alterations in the S12 protein of *Escherichia coli*. While, none of the *E. coli* S12 mutations confers streptomycin resistance, they all have distinct effects on the accuracy of translation. In addition, a subset of the S12 alterations renders the cells hypersensitive to fusidic acid, an inhibitor of the translocation step of translation. These results indicate that the His 76 region of ribosomal protein S12 plays key roles in tRNA selection and translocation steps of protein synthesis, consistent with its interaction with elongation factors EF-Tu and EF-G, as deduced from structural studies of ribosomal complexes.

1. Introduction

Ribosomal protein S12 of bacterial ribosomes has long been linked with resistance to the antibiotic streptomycin and the fidelity of decoding. Streptomycin induces misreading of the genetic code and resistance can be conferred by substitutions at distinct residues in S12. Some of the streptomycin resistant ribosomes carrying altered S12 proteins also display an increased accuracy of decoding. More recently, our laboratory has generated mutations in the *rpsL* gene encoding S12 that either increase or decrease the accuracy of decoding, without altering streptomycin sensitivity [1]. Structural analyses of ribosomes suggest that S12 has the potential to affect decoding through its roles in organizing the decoding center, in promoting conformational transitions of the 30S subunit and/or through its contacts with ternary complexes of aminoacyl-tRNA–EF-Tu·GTP, during delivery of aminoacyl tRNAs to the ribosomal A site [2–4]. In addition to its effects on decoding, biochemical experiments have established a role for S12 in the translocation step of translation [5]. More recently, X-ray crystallography of ribosomal complexes have uncovered contacts between elongation EF-G and protein S12, providing a structural basis for linking this protein with both tRNA selection and translocation activities [6–8].

While much of the genetic and biochemical work on bacterial ribosomes has been carried out in *Escherichia coli*, the success in crystallizing ribosomes from the thermophilic bacterium, *Thermus thermophilus* has spurred genetic analyses of translation components in that organism. These efforts have resulted in the generation of *T. thermophilus* ribosomal mutants similar to those previously isolated in other bacteria as well as some novel mutants. Among the latter class are alterations at position His76 in protein S12, that confer resistance to streptomycin in *T. thermophilus*. In contrast to other S12 alterations conferring streptomycin resistance, His76 is distant from the streptomycin binding site and the H76R mutant ribosomes do not interfere with binding of the drug to ribosomes [9]. These considerations led to the proposal that alterations at His76 in S12 confer streptomycin resistance by altering the conformational changes that occur during binding of aminoacyl-tRNA–EF-Tu·GTP to the ribosome and which are required for subsequent triggering of GTP hydrolysis. These findings prompted us to construct a range of mutations at H76 in protein S12 in *E. coli*. While none of the *E. coli* S12 mutants is resistant to streptomycin, they displayed altered responses to a range of antibiotics targeting decoding and translocation and also are affected in the accuracy of translation. These results are consistent with a critical role for the H76 region of S12 in mediating the ribosome's interaction with both aminoacyl-tRNA–EF-Tu·GTP ternary complexes and EF-G during the sequential steps of translation.

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2. Materials and methods

2.1. Bacterial strains and plasmids

An *E. coli* strain carrying a chloramphenicol resistance cassette, inserted 114 bases upstream of the S12 AUG initiation codon [1] was used for recombineering-based mutagenesis of *rpsL*. Strain MC323 (*F*$^-$ *lacZ521* (UGA) Tn10) carrying a leaky UGA mutation in the *lacZ* coding region was used to screen *rpsL* mutations for effects on decoding fidelity, on X-gal and IPTG plates. Wild type MC323 forms pale blue colonies on X-gal medium, due to low-levels of readthrough of the UGA codon. Error-restrictive and error-promoting (*ram*) mutations form white and dark blue colonies, respectively, on X-gal plates. Following identification by DNA sequencing, the desired *rpsL* mutations were transferred into strain MC41 (*F*$^-$ Δ(*lac-pro*) *thi*$^-$) by P1-mediated transduction [10], using the linked chloramphenicol resistance (*cat*) cassette.

The previously described tetracycline resistant *lacZ* plasmids [11] pSG12-6 and pSG3/4 UGA carry a UAG and a UGA stop codon, respectively, in the 5′ end of the *lacZ* coding region and were used to monitor stop codon readthrough. Plasmid pSG12DP is a −1 frameshift reporter. Synthesis of full length β-galactosidase encoded by these plasmids requires decoding of a stop codon by a near-cognate tRNA, or ribosomal frameshifting, respectively. Plasmid pSG25 carries a wild type *lacZ* gene.

2.2. β-Galactosidase assays, growth rate and minimum inhibitory concentration (MIC) determinations

β-galactosidase activities were determined from cells grown in minimal E medium with glucose and required nutritional supplements and tetracycline (25 mg/l) as described previously [11]. Doubling times of MC41-derived strains were determined from logarithmically-growing cultures in LB medium at 37 °C. Minimal inhibitory concentrations (MIC) were determined in 96 well plates, using twofold dilution series of antibiotics [12]. Antibiotic sensitivity was also monitored by plating 3 μl of serially diluted cultures on LB plates containing sub-inhibitory concentrations of antibiotics.

2.3. Mutagenesis of rpsL by recombineering

Chromosomal DNA from a bacterial strain containing a *cat* cassette upstream of the *rpsL* gene was used as a template in an extension overlap PCR, to introduce mutations into the *rpsL* coding region. The entire 5-codon region spanning codons 74–78, as well as positions 74–76 and 77–78 were targeted for mutagenesis in separate experiments, using 'spiked' oligonucleotides. The 'spiked' primers were designed so that each codon position targeted for mutagenesis contained 91% of the wild type nucleotide and 3% of the 3 mutant nucleotides. In addition, positions H76 and V78 were separately mutated using oligonucleotides carrying all four bases at these codon positions. In a first set of reactions, a forward primer rpsLup1 (5′GAAGACCTTATTGCCCGCGG3′) and a reverse primer carrying the mutation(s) of interest were used to amplify the *cat* cassette and 5′ part of the *rpsL* gene. In parallel, a forward primer carrying the desired mutation(s) and a reverse primer, rpsLdown2 (5′CGCGACGACGTGGCATGGAAA3′) were used to amplify the 3′ part of the *rpsL* gene. These (overlapping) fragments were purified, combined and allowed to extend for 10 cycles, after which, primers rpslup1 and rpsLdown2 were added and amplification continued for 25 more cycles. The purified fragment, from this second PCR, containing the *cat* cassette and mutated *rpsL* gene, was used to electroporate MC323 cells expressing the λ red recombinase from plasmid pKD46 [13]. Electroporated cells were plated on LB medium containing chloramphenicol (25 mg/l), IPTG (1 mM) and X-gal. Mutations were identified by sequencing the *rpsL* coding region on a fragment amplified from individual MC323 transformants. Following transfer of the *cat* cassette and linked *rpsL* mutation into MC41, the *cat* cassette was removed by transient expression of the Flp recombinase from plasmid pCP20 [13]. The entire *rpsL* gene and flanking regions that had been amplified during the recombineering process were then sequenced from MC41 strains, to ensure that no unanticipated mutations were present.

3. Results

3.1. Mutagenesis of residues 74–78 (QEHSV) of ribosomal protein S12

The *rpsL* fragments carrying mutations at desired codons were introduced into a strain (MC323) carrying a leaky UGA mutation in *lacZ* and screened for accuracy-altering mutations. From the two individual codon mutageneses, as well as the three multi-codon mutagenesis experiments, a total of 2701 transformants were obtained on X-gal plates. Of these, 67 displayed a white (error-restrictive) phenotype, while 35 transformants displayed a deep blue (error-promoting, *ram*) phenotype. From this collection, the *rpsL* genes were sequenced from 14 putative *ram* mutants, 23 restrictive mutants and 5 transformants with wild type phenotypes The *rpsL* genes from the 5 isolates with wild type levels of UGA readthrough did not contain any sequence changes. The S12 alterations in isolates with error-restrictive phenotypes were: H76C, H76G, H76I, H76K, H76L, H76M, H76P, H76R, H76S, H76V, V78F, V78L, S77L and a multi-codon change Q74E/E76Q/H76R. The S12 alterations in the *ram* mutants were Q74H, V78D V78E, V78G and a double alteration, Q74H/H76W. Several of the same S12 alterations were encountered in multiple instances, while some isolates with distinct *ram* or restrictive phenotypes had wild type *rpsL* sequences. Some of the S12 alterations isolated here were also recovered and analyzed in a previous study [1] and were thus not pursued further. From the collection of mutants isolated above, 13 single-substitution mutants were chosen for further study (Table 1). On solid media, all mutants grew at wild type rates at 37 °C and 42 °C, while the mutants showed slightly decreased growth at 20 °C. In liquid rich broth medium at 37 °C, a majority of the 13 mutants grew at rates close to the wild type strain, only the H76I and H76V mutants had substantially longer doubling times (42 and 45 min., respectively), suggesting that most of the mutant ribosomes function robustly in protein synthesis.

3.2. Response of the mutant ribosomes to streptomycin

None of the 13 mutants tested was streptomycin resistant, as judged by their inability to grow on media containing at least 50 mg/l of streptomycin. This is surprising, given that the H76K and H76R substitutions constructed here have previously been shown to confer streptomycin resistance in *T. thermophilus* [9]. A standard MIC determination, using a series of twofold dilutions of streptomycin showed that mutants differed by at most, twofold, from the 12 mg/l MIC of the wild type strain (Table 1). H76I and H76V displayed an MIC of 25 mg/l, while V78D and V78G mutants had an MIC of 6 mg/l. The response of the mutants to streptomycin was also monitored by spotting dilutions of overnight cultures onto plates containing low concentrations (5 mg/l) of streptomycin (Fig. 1). From these experiments, the Q74H and V78D mutants appeared to be hypersensitive to this concentration of streptomycin, while H76C, H76I, H76K, H76L, H76R, H76V, S77l, V78F and V78G all grew better than the wild type strain. These results suggest that while none of the mutants is streptomycin resistant, the substitutions have subtle effects on the ribosomal response

Table 1
Readthrough of the *lacZ* UGA mutation in strain MC323 was used to score error-restrictive and error-prone (*ram*) ribosomal accuracy phenotypes. Doubling times were determined in rich LB medium at 37 °C. Minimum inhibitory concentrations (MICs; μg/ml) were determined using a series of twofold dilutions of the indicated antibiotics. WT = wild type; N.A. not applicable.

S12 alteration	Accuracy phenotype	Doubling time (min)	Paromomycin MIC	Capreomycin MIC	Fusidic acid MIC	Spectinomycin MIC	Streptomycin MIC
WT	N.A.	30 ± 1	6	125	500	25	13
Q74H	*ram*	32 ± 1	6	63	500	25	13
H76C	restrictive	33 ± 3	13	125	500	25	13
H76G	restrictive	39 ± 1	6	125	500	13	13
H76I	restrictive	42 ± 1	13	125	63	13	25
H76K	restrictive	39 ± 1	13	125	250	25	13
H76L	restrictive	38 ± 3	13	125	63	13	13
H76R	restrictive	39 ± 1	13	250	500	25	13
H76V	restrictive	45 ± 1	13	250	63	13	25
S77L	restrictive	30 ± 1	6	125	250	25	13
V78D	*ram*	38 ± 1	3	125	1000	13	6
V78F	restrictive	30 ± 1	6	125	250	25	13
V78G	*ram*	31 ± 3	6	125	500	13	6
V78L	restrictive	29 ± 3	6	125	125	13	13

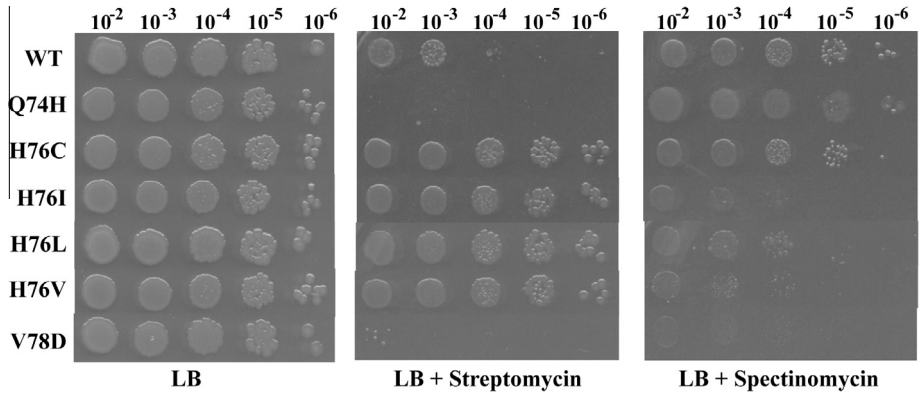

Fig. 1. Effects of S12 substitutions on sensitivity to streptomycin and spectinomycin. 3 μl aliquots of serially diluted overnight cultures of the indicated S12 mutants were spotted onto antibiotic-free LB plates (left panel), LB plates containing 5 μg/ml of streptomycin (middle panel) and 10 μg/ml of spectinomycin (right panel) and incubated overnight at 37 °C. WT refers to the parental MC41 strain expressing a wild type S12 protein.

to the antibiotic. The difference between these two assay results may simply reflect the fact that the spotting assay more sensitively monitors growth at a single 5 mg/l concentration, which is close to the highest concentration of streptomycin (6 mg/l) that still allows growth in an MIC assay. The response of the S12 mutants to another miscoding-inducing agent, paromomycin, was also assessed. The paromomycin MIC was twofold higher than wild type in a H76C, H76I, H76K, H76L, H76R and H76V mutants, while the V78D mutant had a twofold lower MIC (Table 1).

The H76R *E. coli* mutant is streptomycin sensitive while the same alteration confers streptomycin resistance in *T. thermophilus*. Conceivably, differences in the sequences of one or more ribosomal components between the two bacteria may be responsible for this phenotypic difference. To address this discrepancy, we asked if streptomycin resistant isolates could be derived from the H76R *E. coli* strain by single-step mutations. Aliquots of overnight cultures of MC323 carrying the H76R S12 mutation were plated on LB agar plates and subsequently exposed to streptomycin by the underlay technique [14]. 24 streptomycin resistant colonies were recovered and the *rpsL* gene from 10 of these was sequenced. All 10 *rpsL* sequences carried the original H76R mutation along with additional secondary mutations. These were P90Q, P90L (×5), G91D (×3) and K42T. Numbers in parentheses represent the multiple isolations of identical changes. All of these secondary mutations have previously been shown to confer streptomycin dependence or resistance. As expected, the strains carrying the H76R alteration in S12 along with P90Q, P90L or G91D substitutions were streptomycin dependent, while the H76R/K42T double mutant was streptomycin resistant. Thus, the H76R alteration does not appear to confer resistance, or alter the streptomycin phenotypes of other alterations in S12.

The H76R alteration in S12 has been proposed to confer streptomycin resistance in *T. thermophilus* by altering a signal relay pathway, leading to activation of GTP hydrolysis by EF-Tu. We next considered the possibility that differences in the sequences of the EF-Tu proteins in *E. coli* and *T. thermophilus* might be responsible for the differing phenotypes of the H76R S12 protein in the two bacteria. This idea was pursued by recovering streptomycin resistant derivatives of the H76R mutant in a strain where one of the two *tuf* genes had been inactivated by a *cat* cassette (*tufB*::Cat) to facilitate the recovery of otherwise recessive EF-Tu mutants. Exposure of this strain to streptomycin yielded 4 streptomycin dependent and 23 streptomycin resistant colonies from 12 independent platings. The *rpsL* gene was sequenced from 11 streptomycin resistant colonies. All 11 sequences carried the original H76R mutation along with an additional secondary mutation in S12 gene. These alterations included K42T (×5), K42N (×4) and K42R (×2). Again, all of these single substitutions at K42 have previously been shown to confer streptomycin resistance. Similarly, attempts to generate streptomycin resistance by targeted mutagenesis of the *tufA* gene in a *tufB*::Mu strain carrying the H76R substitution in S12, did not yield any alterations in *tufA*. Together these results suggest that multiple sequence differences in the translation systems, or potentially, the divergent growth temperatures of *T. thermophilus* and *E. coli* may underlie the differing streptomycin phenotypes of the H76R substitution in these two bacteria.

3.3. Altered S12 affects the ribosomal response to translocation inhibitors

Since protein S12 has also been linked to translocation functions on the ribosome, the response of the mutants to antibiotics that target this step in translation was also assessed. Fusidic acid inhibits translocation by stabilizing the binding of EF-G·GDP on the ribosome, following GTP hydrolysis. MIC assays showed that H76I, H76L, H76V and V78L mutants are hypersensitive to fusidic acid, having an eightfold lower MIC, relative to wild type. Less dramatic hypersensitivities were seen with H76K, S77L and V78F mutants, while the V78D had a twofold increased MIC (Table 1). Two other antibiotics that target translocation, capreomycin and spectinomycin, were also investigated. Capreomycin and the related antibiotic, viomycin, bind between the ribosomal subunits, at intersubunit bridge B2a and affect both decoding fidelity and translocation [15]. A majority of the mutants were unaffected in their response to capreomycin; only the Q74H displayed an increased sensitivity, while the H76R and H76V mutants were twofold more resistant. Spectinomycin is believed to inhibit translocation by preventing swiveling of the head domain of the small ribosomal subunit [16]. In MIC assays, several of the mutants H76G, H76I, H76L, H76V and V78D were twofold more sensitive than wild type to spectinomycin. The MIC assays were substantiated by growth of diluted cultures on plates containing 10 mg/l of spectinomycin. This concentration of spectinomycin almost completely inhibited growth of the V78D mutant, and to a lesser extent, growth of H76I, H76L and H76V mutants (Fig. 1). These results suggest that alterations in the H76 region of S12 affect the translocation step of protein synthesis, consistent with the known interactions of EF-G with this ribosomal region [6–8].

3.4. Substitutions at residues 74–78 of S12 alter decoding fidelity

The region 74–78 mutants constructed here were tested for their effects on decoding accuracy, using a series of *lacZ* plasmids carrying nonsense or frameshift mutations in the β-galactosidase coding region (Fig. 2). When transformed with the *lacZ*$^{+}$ plasmid, pSG25, the wild type strain and all of the S12 mutants supported equivalent levels of β-galactosidase activity (not shown). β-galactosidase assays on strains carrying the UAG and UGA stop codon reporter plasmids showed that all of the H76 mutants and two of the four V78 mutants decreased readthrough of both stop codons. In the V78L mutant, UGA readthrough was decreased significantly, while UAG readthrough was close to wild type levels. Similarly, in the S77L mutant, only UAG readthrough was decreased. Three mutants, Q74H, V78D and V78G, which were classified as error-prone in our initial screen for decoding accuracy in strain MC323, supported increased levels of UGA readthrough. Of these, only V78D showed increased UAG readthrough levels. In the Q74H and V78G mutants, the UAG readthrough level was at, or barely above that seen in the wild type strain. These assay data show that substitutions in the 74–78 region of S12 can produce both error prone and error-restrictive ribosomes and that different substitutions at Val78 can affect decoding in diverse ways.

A more complex pattern was observed when the mutant strains were assayed for effects on −1 frameshifting. While a majority of the mutants that restricted UAG and/or UGA readthrough also restricted −1 frameshifting, there were some exceptions. Frameshifting in the H76R and S77L mutants was close to wild type levels. More surprisingly, in the H76G and H76K mutants, frameshifting increased 2.0- and 2.7-fold, respectively. Of the three *ram* mutants that supported increased UGA readthrough, only V78G displayed increased frameshifting levels. In the Q74H mutant, frameshifting was close to wild type levels, while in the V78D mutant, frameshifting was decreased. The lack of correspondence between the effects of particular mutants on stop codon readthrough and frameshifting is noteworthy and suggests that alterations in the 74–78 region of S12 may affect multiple aspects of the translation cycle. In the −1 frameshift reporter construct used here, frameshifting occurs at the sequence UU**G *GG*A** UAA and likely involves (i) decoding of the in-frame (*in italics*) GGA codon by the near-cognate GGG-decoding tRNA^{Gly-1}, followed by (ii) backward slippage of the P-site-bound tRNA^{Gly-1} onto the overlapping GGG codon (in bold). Previous studies have shown that tRNA slippage in the P-site is in competition with A-site occupancy [17]. Frameshifting is thus governed both by the acceptance of near-cognate tRNAs at the A site, as well as the occupancy of the A site in the next cycle of translation, when the near cognate tRNA has moved to the P site. Previous biochemical experiments with error-restrictive S12 mutant ribosomes have demonstrated that they increase rejection rates of near-cognate aminoacyl-tRNA–EF-Tu·GTP ternary complexes [18,19]. In the context of the frameshifting event used here, decreased rates of near-cognate tRNA binding at the A site are expected to decrease subsequent slippage events in the P site. Conversely, *ram* mutants are expected to increase the availability of slippage-prone, near-cognate tRNAs in the P site, as is seen in at least some instances (Fig. 2 and [20]). However, it has also been shown that at least some error-restrictive ribosomes can increase frameshifting, possibly by limiting A site occupancy and thereby increasing the opportunity for slippage by the P site tRNA [21]. Thus, the influence of ribosomal mutations on binding and slippage by near-cognate tRNAs can be modulated by their additional effects on A site occupancy. The complex pattern of effects of S12

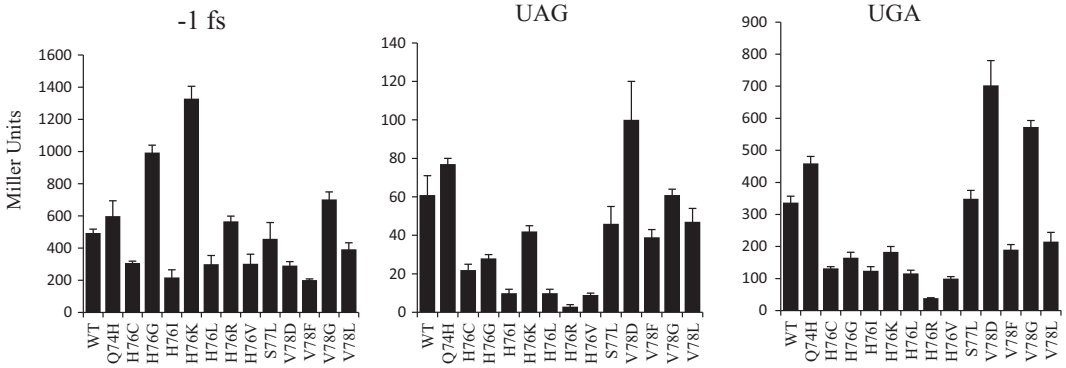

Fig. 2. Substitutions in the aa 74–78 region of ribosomal protein S12 affect the accuracy of decoding. Strains expressing wild type (WT) S12 protein or the indicated substitutions were transformed with the *lacZ* reporter plasmids pSG12DP, pSG12-6 or pSG3/4UGA and assayed for β-galactosidase activity. The levels of −1 frameshifting (left panel), UAG (middle panel) or UGA readthrough (right panel) supported by the S12 mutants, are represented as Miller units of β-galactosidase activity. Each bar represents the mean of at least three independent determinations ± SD.

mutations on stop codon reading and frameshifting reflects the interplay between these two processes.

4. Discussion

Activation of GTP hydrolysis on ribosome-bound ternary complexes of aminoacyl-tRNA–EF-Tu·GTP is a critical step in the selection of correct tRNAs by the ribosome. This activation follows inspection of tRNA–mRNA codon–anticodon pairings in the decoding center of the small ribosomal subunit and allows dissociation of EF-Tu·GDP, as well as engagement of the aminoacyl end of the tRNA by the peptidyltransferase center of the large subunit. GTPase activation is coupled to codon recognition and thus cognate tRNAs are far more effective than near-cognate tRNAs in their ability to activate GTP hydrolysis. Biochemical experiments suggest that ribosomal protein S12 aids in the activation of GTP hydrolysis and this activity is perturbed by streptomycin or error-restrictive, streptomycin resistant alleles of S12 [22,23]. In crystal structures of ternary complexes bound to ribosomes, Q74 and H76 of S12 contact A67 and U68, respectively, of the tRNA and E249 in EF-Tu forms a salt bridge with K119 of S12 [4]. Genetic analysis of streptomycin resistant *T. thermophilus* mutants carrying alterations at H76 in S12 suggested a model in which resistance is conferred by altering the signaling between the ribosomal decoding center and EF-Tu, leading to GTP hydrolysis. Reconstruction of the H76 mutants in *E. coli* show that while substitutions at H76 and adjacent residues have some subtle effects on the cell's response to streptomycin, none confers substantial resistance. The lack of streptomycin-resistance phenotypes in the *E. coli* mutants is likely to be due to the evolutionary distance separating *E. coli* and *T thermophilus*. Our attempts to generate second-site mutations that allow H76R substitution in S12 to confer resistance suggest that either multiple changes in the *E. coli* S12 and EF-Tu proteins are required to recapitulate H76R-mediated resistance, or that additional differences in the ribosomal components between the two bacteria contribute to streptomycin resistance. In addition, *T. thermophilus* has an optimal growth temperature of 72 °C, *vs.* the 37 °C temperature used to cultivate *E. coli*. The structural dynamics of the 30S subunit are likely to be distinct under these differing temperatures and thus similar mutations may exert differential effects on ribosome function as a consequence of these diverging growth conditions. The *E. coli* S12 substitutions do, however, have prominent effects on the fidelity of decoding. This likely occurs by altering the coupling between codon recognition and activation of ribosome-bound EF-Tu for GTP hydrolysis. Our finding that different substitutions in the H76 region can either increase or decrease the fidelity of translation is consistent with the previously proposed role for the H76 region in S12–EF-Tu signaling [9].

In addition to its roles in decoding, S12 also contributes to the translocation step of translation. Removal, or chemical modification of S12 (and S13) permits translocation in the absence of EF-G, suggesting that S12 and S13 serve as control elements to regulate mRNA–tRNA movement. More recently, X-ray crystallography of EF-G-ribosome complexes show that domain III of EF-G contacts the QEH tripeptide of S12 [6–8]. The hypersensitivity of H76I, H76L and H76V mutants to the translocation inhibitor, fusidic acid described here, as well as the recent demonstration that the antibiotic dityromycin inhibits translocation by binding to the H76 region of S12 [24] are all consistent with the functional importance of this S12–EF-G contact during translocation.

In summary, mutagenesis of the conserved QEHSV peptapeptide of S12 shows that these residues contribute to both decoding and translocation functions on the ribosome, consistent with the sequential interaction of this region of S12 with aminoacyl-tRNA–EF-Tu·GTP and EF-G, as established by structural studies.

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