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Alterations in the ribosomal protein bL12 of *E. coli* affecting the initiation, elongation and termination of protein synthesis.

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Abstract

In bacteria, ribosomal protein bL12 forms the prominent stalk structure on the ribosome and binds to multiple, distinct translational GTPase factors during the sequential steps of translation. Using a genetic selection in *E. coli* for altered readthrough of UGA stop codons, we have isolated seven different mutations affecting the C-terminal domain of the protein that forms the interaction surface with translation factors. Analysis of these altered proteins, along with four additional alterations previously shown to affect IF2-ribosome interactions, indicates that multiple steps of translation are affected, consistent with bL12's interaction with multiple factors. Surprisingly, deletion of the release factor GTPase, RF3, has relatively little effect on bL12-promoted stop codon readthrough, suggesting that other steps in termination are also influenced by bL12.

Keywords

Translational GTPase; Ribosomal protein bL12; Readthrough; Translation Initiation; Release Factor RF3; Initiation Factor IF2

1. Introduction

The initiation, elongation, termination and recycling phases of translation all involve the binding of translational GTPase factors to the ribosome. In bacteria, these GTPases include IF2, EF-Tu, EF-G and RF3. Other GTPases including LepA, BipA and Tet O/M also bind to the ribosome, but their roles in translation are dispensable and less well understood [1, 2].

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Author Contributions

Conceived and designed the experiments: Adam Younkin & Michael O'Connor.
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Ribosomal recruitment of translational GTPases involves interaction with a prominent stalk-like structure on the large ribosomal subunit. In bacterial ribosomes, this stalk is composed of multiple dimers of protein bL12, bound to protein bL10. In *E. coli*, bL12 can be acetylated on its N-terminus to generate a modified bL12 called L7 [3]. Varying proportions of the modified and unmodified proteins (previously referred to as L7/L12) can be present, depending on the physiological state of the cell. Both forms of the protein are denoted bL12 hereafter. The isolated bL12 protein consists of distinct N- and C-terminal domains, connected by a hinge region (Fig.1) [4]. The N-terminal domain is involved in dimerization and subsequent assembly into ribosomes, while the C-terminal domain interacts with the translation factors. In assembled ribosomes, the flexibility of the hinge regions of bL12 has precluded a full structural description of factor-ribosome interactions [3].

Protein bL12 is essential for viability and for ribosome-factor interactions *in vitro*. Removal of bL12 from ribosomes by salt/ethanol treatment has shown that it is required for IF2, EF-Tu, EF-G and RF3 binding and subsequent GTP hydrolysis and other functions [5, 6]. X-ray crystallography, cryo-electron microscopy and NMR studies have identified some of the translation factor and bL12 residues involved in their interactions. These studies highlight the importance of helices 4 and 5 of bL12 for factor binding and the contributions of some of these bL12 residues to translation factor functions have been tested by mutagenesis [7, 8]. Genetic selections in *E. coli* for accuracy-altering mutations have also uncovered a limited number of alterations in the hinge and C-terminal domain that decrease the accuracy of translation [9, 10]. In this study, we have targeted the *E. coli rplL* gene encoding bL12 for mutagenesis and identified bL12 residues in the C-terminal domain that affect the fidelity of termination. The seven mutations recovered from this selection were then tested for their effects on the fidelity of the initiation and elongation steps of translation. In addition we have tested the effects on fidelity of four bL12 mutations previously shown to alter IF2 functions *in vitro*. Several of the same alterations in bL12 affect initiation, elongation and termination phases of translation, consistent with effects of bL12 on interactions with multiple translation factors. The altered proteins have diverging effects on initiation from leaderless mRNA and from non-AUG codons and at least some of the effects on termination are independent of RF3 functions.

2. Methods

2.1. Bacterial strains and plasmids

Accuracy-altering *rplL* mutations were isolated in strain MC323 (F^-, λ^-, *lacZ521* (Tn*10* linked), *rph-1*), which carries a leaky UGA in *lacZ*. MC323 forms pale blue colonies on X-gal medium. Ribosomal mutations that increase or decrease accuracy give rise to white or darker blue colonies, respectively. All *rplL* mutations were reconstructed in strain MC361 (F^{--}, *ara*$^-$ Δ(*gpt-lac*)*5 thi*$^-$ *prfB* [*E. coli* B]), a K-12 strain that expresses the fully active Ala246 RF2 protein [11]. A subset of the mutations were reconstructed in strains MC323 or CSH142 (F^{--}, *ara*$^-$ Δ(*gptlac*)*5 thi*$^-$), both of which express the K-12-specific RF2 (Thr246) allele. P1 transduction was used to introduce a kanamycin-marked deletion of the *prfC* gene (encoding RF3) into MC361 strains, using phage prepared on JC5873–2 (F-, *Δ*(*araD-araB*)*567*, *ΔlacZ4787*(*::rrnB-3*), λ^-, *rph-1*, *Δ*(*rhaD-rhaB*)*568*, *hsdR514*, *ΔprfC770::kan*).

CSH102 (F'128 *lacZ572*; *ara-600*, *Δ*(*gptlac*)*5*, *λ*$^{-}$, *relA1*, *spoT1*, *thiE1*) carries a GAG→GGG transition mutation in *lacZ*, resulting in substitution of glycine for glutamine at position 461 [12]. A *prfA1* strain [13], encoding a temperature sensitive RF1 protein was obtained from Dr. Leif Isaksson, Stockholm University, Sweden.

Plasmids pKD46, pKD4 and pCP20 were used in recombineering protocols [14]. A series of *lacZ* reporter plasmids were used to monitor errors at initiation, elongation and termination. In plasmids pSG413 and pSG415, the AUG start codon is replaced by CUG and AUC, respectively [15]. The pDB plasmid expresses a λ c*I-lacZ* fusion from a leaderless mRNA [16]. Plasmids pSG3/4 UGA and p34–11 carry UGA mutations while p12–6 carries a UAG mutation in the 5' end of the coding region and pSG12DP is a −1 frameshift reporter [17]. Plasmid pAC102 is a missense reporter, constructed by amplifying the entire *lacZ* gene and upstream promoter from CSH102 and inserting the amplified fragment into the *EagI* site of pACYC184. Plasmid pLG25 carries a wild-type *lacZ* gene.

2.2. Mutagenesis of rplL

To facilitate manipulation of the *rplL* gene, a kanamycin-resistance cassette was inserted immediately following the UAA stop codon, using standard recombineering protocols and pKD4 as a template. Insertions of antibiotic-resistance cassettes at this position in the operon have previously been found not to impede ribosome assembly or function [18]. Chromosomal DNA from one such insertion was verified by sequencing and was then used as a template in PCR-based mutagenesis experiments. The 1.9 kb fragment encoding both bL12 and the phosphotransferase conferring kanamycin resistance was amplified with the error-prone Mutazyme II DNA polymerase (Agilent Technologies, Santa Clara, CA). The amplification conditions were adjusted to allow for 1–2 errors per molecule. The amplified fragment was introduced into electrocompetent MC323 cells expressing the λ Red recombinase from plasmid pKD46 and transformants were plated in LB medium containing X-gal, kanamycin and IPTG. Plates were incubated for 3 days at 37 °C and colonies that were paler or darker blue than unmutagenized MC323 were recovered, purified and the *rplL* gene was amplified and sequenced.

Specific mutations in *rplL* were created by a PCR method, using a mutagenic oligonucleotide carrying the desired mutation as a forward primer and a reverse primer that annealed 100 bp downstream of the *rplL* stop codon. Chromosomal DNA from a strain with a kanamycin-resistance cassette inserted immediately after the wild type *rplL* coding region was used as a template. The *rplL*-Kan^{R} fragments were amplified with high fidelity Phusion polymerase and electroporated into MC323 cells expressing the λ Red recombinase. Kanamycin-resistant transformants carrying the desired mutations were verified by sequencing.

All *rplL* mutations were reconstructed and analyzed in MC361. This was accomplished by amplifying the *rplL* gene and linked Kan^{R} fragment from MC323 strains with the high-fidelity Phusion polymerase (New England Biolabs) and electroporating electrocompetent cells expressing the λ Red recombinase with these *rplL*-Kan^{R} fragments. The kanamycin-resistance element was then removed by transient expression of the FLP recombinase, from plasmid pCP20 [14]. The *rplL* coding sequence and flanking regions in all reconstructed

strains were sequenced to ensure that no unanticipated mutations were present. A subset of mutations were reconstructed in strains MC323, CSH142 [12] or MG1655 *prfA1* [13], using either electroporation of a *rplL*Kan^R fragment, or by P1 transduction, selecting transformants or transductants on LB plates containing kanamycin (25 mg/l).

2.3. Growth rate determinations and β-galactosidase assays

Doubling times were calculated from growth of cultures in LB medium at 37 °C. Growth on solid medium was monitored by streaking or spotting ten-fold dilutions of overnight cultures onto LB or minimal (E) medium plates [12] and incubating overnight at the desired temperature.

β-galactosidase assays were carried out on cultures grown in minimal (E) medium, with appropriate supplements and antibiotics. Assays on permeabilized cells were performed as described [12].

3. Results

3.1. Random mutagenesis of the rplL gene encoding bL12

Previous genetic approaches succeeded in isolating a limited number of mutations affecting the hinge and C-terminal regions of bL12. These were G74D, E82K and two complex insertion/deletion mutations affecting the A40 hinge region of the protein [9, 10]. In one selection, the starting, parental strain carried an alteration in ribosomal protein uS12, rendering it dependent on streptomycin. Selection for streptomycin independence led to the recovery of suppressor mutations affecting bL12. Other bL12 mutations were isolated fortuitously, in a strain carrying a deleterious tRNA missense suppressor [9, 10]. The genetically isolated bL12 mutants had an error-prone phenotype and promoted miscoding *in vitro* and readthrough of stop codons *in vivo* [19]. Here, we have taken advantage of this miscoding phenotype and used our previously developed approach to the isolation of accuracy-altering ribosomal mutations and applied it to *E. coli* bL12.

Electroporations of MC323 cells with mutated *rplL*-Kan^R DNA fragments (containing the *rplL* gene encoding bL12 and a linked kanamycin-resistance gene) yielded 300 kanamycin-resistant transformants. Of these, 6 had an error-prone phenotype and formed darker blue colonies than the parental MC323 strain on X-gal containing medium. Sequencing the *rplL* gene showed that these isolates encoded altered bL12 proteins with single V66F, V87I, L110Q, G114R, G114C or E118G substitutions. One mutant with an E112K alteration had an error-restrictive phenotype and formed colonies lighter in color than the starting MC323 strain.

During the course of these experiments, Ge *et al.* [7] reported on the effects of bL12 mutations on IF2 function, using an *in vitro* system. In an effort to see if the bL12 alterations that influenced IF2 functions also had effects on accuracy, 4 mutations described by Ge *et al.,* A83C, L80A, V72A and V66D were regenerated in our strains and analyzed along with the seven genetically-isolated mutants. Two of the chosen mutations, V66D and L80A had been shown by Ge *et al.* to have profoundly detrimental effects on IF2 function, the A83C

variant retained substantial IF2 interaction while the V72A mutant ribosomes had intermediate activity in IF2-dependent assays [7].

All mutations were reconstructed in MC361, our standard laboratory K-12 strain that carries a fully active RF2 protein [20]. The V66D and L110Q alterations had severely reduced growth on solid minimal and rich media at all temperatures tested and increased doubling times in liquid, rich media at 37 °C (Fig 2). The L110Q mutant also failed to grow on solid media at 42 °C, while the V66D mutant had greatly reduced growth at 30 °C and 20 °C. The V72A, L80A and A83C mutants also had increased doubling times at 37 °C.

3.2 Effects of altered bL12 on codon recognition at initiation, elongation and termination of translation.

bL12 interacts with different translation factors during initiation, elongation and termination and thus has the potential to influence each of these stages of protein synthesis. In previous studies [17], we have described the construction of various *lacZ* plasmids that report on errors and non-standard decoding events at each of these steps of translation and have we used a selection of these plasmids here to monitor the effects of bL12 alterations.

Initiation on bacterial mRNAs overwhelmingly occurs at AUG codons, preceded by a leader region of varying length. Initiation on leaderless mRNAs and from non-AUG codons are discriminated against and both initiation factors IF2 and IF3 participate in this selection [21, 22]. In previous studies, we have used initiation from non-AUG codons and from leaderless mRNAs to monitor initiation codon selection. Those *in vivo* studies showed that alterations in 16S rRNA and initiation factor IF3 increased initiation from both non-AUG codons and leaderless mRNAs [15, 17]. Other *in vitro* experiments have shown that initiation from AUU at least, is strongly dependent on IF2 function [23]. In plasmids pSG413 and pSG415, the normal AUG initiation codon for *lacZ* has been replaced by CUG and AUC, respectively [17], while plasmid pDB encodes a leaderless λ c*I*-*lacZ* fusion [16, 17]. The results of β-galactosidase assays in the bL12 mutants in Fig. 3 show that several of the mutants (V66D, L80A, A83C, V87I and G114R) affect initiation from CUG. A subset of these also affect initiation from AUC (Fig. S1), while only V66D, L110Q and G114R mutants affect leaderless mRNA expression (Fig. 3).

Expression of a wild-type *lacZ* gene initiating with an AUG codon from plasmid pLG25 did not differ substantially between wild type and mutant bL12 strains (not shown). It is notable that while non-AUG initiation is decreased in the bL12 mutants, initiation on leaderless mRNAs is increased. This in contrast to the effects of other ribosomal and initiation factor mutations, that increase both of these initiation events. The effects of altered bL12 proteins on both non-AUG and leaderless initiation events are likely mediated by initiation factor IF2. This GTPase initiation factor interacts with both subunits and stimulates the binding of fMet-$tRNA_f^{Met}$ to the 30S ribosomal P-site, as well as subsequently promoting the joining of the 50S subunit to generate a 70S initiation complex. Initiation on leaderless mRNAs has been proposed to occur either directly on pre-formed 70S ribosomes [24] or by recruitment of a 30S–fMet-$tRNA_f^{Met}$–IF2 ternary complex [25]. Importantly, the studies by Balakin *et al.* and others [24, 26] showed that initiation by pre-formed 70S ribosomes on leaderless λ c*I* mRNA was independent of all three initiation factors, including IF2. Accordingly, in

bL12 mutants where IF2 functions are likely compromised, the 70S initiation pathway may have a selective advantage over the 30S pathway in initiation on leaderless mRNAs. The differential effects of altered bL12 on leaderless *vs.* non-AUG initiations is consistent with these two non-standard initiation occurring through different pathways, each with differing requirements for IF2 functions.

Binding of a near-cognate aminoacyl-tRNA•EF-Tu•GTP ternary complex to an elongating ribosome can result in a missense error while recruitment of a near-cognate ternary complex at a stop codon can result in a readthrough event. We have used *lacZ* plasmids that report on both kinds of events to monitor elongation and termination errors. In the *lacZ* coding sequence, codon 461 (GAG) normally encodes an essential glutamine. In plasmid pAC102, this has been substituted with a GGG glycine codon and second position misreading of GGG by $tRNA^{Gln}$ is necessary to produce an active β-galactosidase [27]. Only the V66D and L110Q mutants promoted significant (ca. two-fold increased) missense decoding (Fig.4). However, the low expression levels of this *lacZ* construct may have precluded detection of miscoding by the other bL12 mutant ribosomes.

Stop codon readthrough was monitored using three different plasmids; p34–11 and pSG3/4 UGA carry in frame UGA stop codons while p12–6 carries a UAG codon. The V66D, L80A, V87I and L110Q mutants all promoted significant UAG and UGA readthrough, while V66F only stimulated UGA readthrough (Figs. 4 and S2). Little or no effects on readthrough were seen in the V72A, A83C, E112K, G114C, G114R and E118G transformants, even though the latter four mutants were initially isolated on the basis of their effects on UGA readthrough in strain MC323. To determine if these four mutants might be false positives emerging from the original screen, or if differences in RF2 proteins, or the sequences surrounding the stop codons accounted for the lack of readthrough, two sets of strain reconstruction experiments were carried out. First, the four bL12 mutations were reconstructed in strain CSH141 which is isogenic with MC361, but carries the K-12-specific RF2 allele. The CSH141 strains were then transformed with UGA (pSG3/4 UGA) and UAG (p12–6) reporter plasmids and assayed for β-galactosidase activity. No increases in readthrough were detected in the mutants, relative to the corresponding wild type bL12 strain (not shown), indicating that the lack of readthrough is not due to differences in the RF2 protein. In the second experiment, the four mutations, along with L80A were reconstructed in strain MC323 and streaked on X-gal plates to monitor UGA readthrough in the chromosomal *lacZ* gene. The L80A G114C and G114R mutants all formed colonies that were darker blue than wild type MC323, while colonies from the E112K strain were distinctly paler blue than wild type on X-gal plates. Colonies from E118G strains were indistinguishable from wild type on X-gal plates (Fig. S3). Thus, at least in the context of the *lacZ*521 UGA mutation in strain MC323, the G114C and G114R alterations confer a stop codon readthrough phenotype while the E112K mutant decreases readthrough and the E118G appears to be a false positive with no accuracy phenotype. Together these assays and strain reconstructions show that the V66D, V66F, L80A, V87I and L110Q bL12 alterations all promote substantial stop codon readthrough. The G114C, G114R and E112K substitutions also affect readthrough, but the effects are modest and limited to specific sequence contexts. The V72A, A83C and E118G substitutions do not affect stop codon readthrough or missense decoding. The effects of bL12 mutations on frameshifting were

addressed using the −1 frameshift reporter plasmid, pSG12DP. These assays (Fig. S4) showed that with the exception of slight decreases in the L80A, E112K and G114R mutants, frameshifting levels did not differ substantially from that seen in the wild type strain.

3.3 Genetic interactions of bL12 with EF-Tu and RF3

Both the V66D and L110Q substitutions have a missense decoding phenotype, suggesting that they have an altered interaction with EF-Tu. We have also addressed EF-Tu-bL12 interactions genetically by examining the phenotypes of EF-Tu/bL12 double mutants. The well-studied A375T substitution in EF-Tu renders the factor resistant to the antibiotic kirromycin, decreases its affinity for tRNA [28] and promotes miscoding [29]. In strain MC505, *tufB* has been deleted while *tufA* encodes the A375T allele of EF-Tu and thus all of the EF-Tu in the cell is of the mutant form. Each of the bL12 mutant alleles was introduced into MC505 by electroporation and growth of the resulting transformants on solid media at 37 °C was monitored. Only the V66D, V87I and L110Q bL12 mutations showed differential growth in the two strains, indicative of a genetic interaction with the altered EF-Tu. The V87I bL12/ A375T EF-Tu double mutant grew slightly, but detectably slower than either single mutant strain and the L110Q mutation is all but inviable when combined with the A375T EF-Tu allele (Fig. S5). The V66D mutants expressing wild type EF-Tu is already very slow growing (Fig. 2) and we were unable to make a stable V66D bL12/ A375T EF-Tu double mutant. The slow growing transformants that arose after electroporation rapidly accumulated suppressor mutations that relieved the slow growth. These genetic results suggest that residues V66, V87 and L110 of bL12 are all important for EF-Tu function on the ribosome.

Several of the bL12 mutants studied here have elevated rates of stop codon readthrough. The release factor RF3, which recycles RF1 and RF2 off post-termination ribosomes, has a well-described interaction with the ribosomal stalk [5]. Inactivation of RF3 increases readthrough errors [11], likely by limiting the availability of free RF1/RF2 at termination. To examine RF3's contribution to bL12-promoted readthrough events, we chose four mutants that show substantial readthrough (V66D, V66F, V87I and L110Q), constructed derivatives in which the *prfC* gene (encoding RF3) had been deleted and then measured UAG (using plasmid p12–6) and UGA readthrough (using plasmids pSG3/4 UGA and p34–11) in these strains. A comparison of UGA readthrough in strains containing pSG3/4 UGA and either expressing RF3 or lacking it, is shown in Fig. 5. The effects of RF3 on UGA readthrough using p34–11, and on UAG readthrough using p12–6 are shown in Fig. S6.

In strains containing the pSG3/4 UGA reporter, loss of RF3 in a wild-type bL12 strain increases UGA readthrough (2.5-fold), as previously observed and similar increases are seen with the other UGA and UAG reporters (Fig S6). In contrast, readthrough of UAG and UGA in the V66F and V87I mutants is unaffected by RF3 loss. Both the V66D and L110Q mutant ribosomes support high levels of UGA readthrough in strains with RF3. Loss of RF3 in the V66D and L110Q mutants either increases readthrough modestly (pSG3/4 UGA) or has little or no effect, depending on the mutant and *lacZ* reporter. The lack of effect of RF3 deletion can mean either that the substitutions in bL12 eliminate all interaction with RF3 (and so its deletion has no effect), or that a step in termination preceding RF3's action has

become rate-limiting in these mutants. Moreover, in the V66D and L110Q strains expressing RF3, the level of UGA and UAG readthrough typically already exceeds that seen in a wild type bL12 strain lacking RF3 (1141 and 695 units of β-galactosidase *vs.* 590 units, respectively, for pSG3/4 UGA-dependent readthrough). This indicates that loss of RF3-ribosome interactions alone cannot explain all of the UGA readthrough in bL12 mutants and that other aspects of termination are also affected by the substitutions. A recent cryo-EM analysis of ribosome complexes carrying RF1 and RF3 indicated a direct interaction of RF1 with bL12, a conclusion also supported by biochemical analysis [30]. An interaction between bL12 and RF1 (and presumably RF2 also) is consistent with earlier genetic work showing that an E82K substitution in bL12 suppressed the temperature sensitivity of an RF1 allele (*prfA1*) carrying a R137P substitution [13]. Each of the 11 bL12 mutations studied here was introduced into a *prfA1* strain and tested for temperature sensitivity. However, none could suppress the *prfA1* temperature sensitivity (data not shown). While our data cannot dismiss altered bL12-EF-Tu interactions as the cause of increased UGA and UAG readthrough, our results are also consistent with other interpretations, including an effect of altered bL12 on RF1/RF2 interaction with the ribosome.

4. Discussion

The initiation, elongation, termination and recycling steps of translation each involves at least one GTPase that interacts with the ribosomal stalk. Given its critical function, bL12 has been the subject of multiple biochemical investigations. Traditionally, the effects of elimination, or mutation of bL12 have been analyzed by depleting ribosomes of bL12 with a salt/ethanol extraction step and if needed, incorporating altered bL12 proteins into these treated ribosomes, in a partial reconstitution procedure. The genetic approach used here allows the analysis of mutant bL12 ribosomes without the need for depletion and reconstitution steps and avoids any problems of contamination with residual, wild type bL12 [5]. Surprisingly, at least one of the bL12 mutations (V66D) that in previous biochemical studies was shown to be nearly inactive in virtually every assay used, was viable in our system, although extremely slow-growing. This suggests that the genetic approach used here might be applied to other alterations in bL12 that greatly reduce activity.

Alterations in bL12, including the ones identified here, affect multiple factor-ribosome interactions and multiple steps of translation. Thus, mutations isolated here on the basis of their UGA readthrough phenotype also affect initiation and/or elongation, while alterations in bL12 previously shown to affect IF2-ribosome interactions also affect elongation or termination. All the mutations we have studied are in the C-terminal region. The amino acid substitutions observed here fall into two classes, based on their potential structural impact. Five of the mutated residues are located on the protein surface, and are potential sites of interactions with protein factors. These include V66, L80, E112, G114 and E118. In particular, V66D and V66F substitutions introduce a negative charge and a bulky aromatic side chain, respectively; this residue also makes hydrophobic interaction with the nearby surface residue K70, which has been mutated in other studies [8, 31]. The absence of high-resolution structures of ribosome functional complexes in which bL12 can be resolved, precludes a precise assessment of the effects of these mutations on factor-ribosome interactions. The remainder of the mutated residues, including V72, A83, V87, L110 are

located in the interior of the C-terminal domain and form a hydrophobic core of the domain. V72 is in position to make hydrophobic interactions with V87, A83 and V68. V68 in turn makes hydrophobic interaction with L110, identified in the present study. L80 makes a hydrophobic interaction with I69, where mutations have been identified by others [8, 31]. Amino acid substitutions at these positions are likely to act indirectly, by either destabilizing or statically distorting domain conformation.

The interactions of bL12 with translation factors IF2, EF-Tu, EF-G and RF3 have been mapped using NMR spectroscopy [32]. These experiments located a common binding site on bL12 for all four factors and identified V66, V68 K70, L80 and E82 as critical residues. Site-directed mutagenesis of bL12 also identified an overlapping set of residues (K65, V66, I69, K70, R73, K84) critical for EF-Tu and/or EF-G functions [8, 31]. The key, positively charged residues in bL12 that are proposed to engage in complementary charge interactions with IF2 (and potentially with the other factors) are K65, K70, R73 and K84 [7]. Although none of these four residues was mutated in this study, several of the adjacent residues (V72 L80, A83) were and the V66D alteration was analyzed in both studies. While there is overlap between residues identified as critical by biochemical and structural approaches and some of the UGA suppressor mutations isolated here, the latter also implicates L110, E112 and G114 in factor-ribosome interactions.

Previous biochemical analysis showed that bL12 mutants with amino acid substitutions in the A40 region of the protein were altered in both EF-Tu and EF-G functions [19]. V66D mutant ribosomes have now been analyzed *in vitro* for their effects on IF2 [7], EF-Tu [31] and EF-G [8] and have been found to have greatly decreased subunit joining activities, decreased association rates with aminoacyl-tRNA•EF-Tu•GTP ternary complexes and decreased association with EF-G, as well as impaired phosphate release. Surprisingly, despite the impaired activities of V66D mutant ribosomes, cells expressing the V66D bL12 are viable and, of the mutants analyzed here, have the most substantial effects on fidelity at initiation, elongation and termination phases of protein synthesis. The extensive biochemical data on V66D and the IF2-specific assays for V72A, L80A and A83C mutants all indicate that these mutations decrease translation factor-ribosome interactions. However, the *in vivo* fidelity assays presented here are not readily explained by decreased factor-ribosome association alone, but additionally suggest that the bL12 mutations also affect the subsequent, ribosome-bound functions of the factors. Experiments with ribosomes lacking the C-terminal domain of bL12 indicate a role for bL12 in promoting GTP hydrolysis by EF-Tu•GTP [6]. Effects of altered bL12 on GTPase functions of IF2 and EF-Tu may underlie the altered fidelity of initiation and elongation observed here. However, as argued above, altered RF3 functions cannot completely explain the increases in stop codon readthrough and additional aspects of termination are likely influenced by bL12.

5. Conclusions

In this work, we have developed a methodology for mutagenesis of the *rplL* gene encoding ribosomal protein bL12. This allows for the expression of mutant bL12 proteins from the chromosomal gene and the generation of homogeneous mutant ribosomes, without the need for *in vitro* reconstitution procedures.

The bL12 mutants we have generated and analyzed here affect initiation, elongation and termination, consistent with the interaction of the ribosomal stalk with multiple factors during translation. Loss of the GTPase termination factor RF3, has remarkably little effect on bL12-promoted stop codon readthrough, suggesting the involvement of additional bL12-factor interactions at termination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ribosomal protein bL12 recruits multiple translation factor GTPases to the ribosome
- Alterations in bL12 affect multiple steps of translation
- Loss of RF3 has little effect on bL12-promoted stop codon readthrough

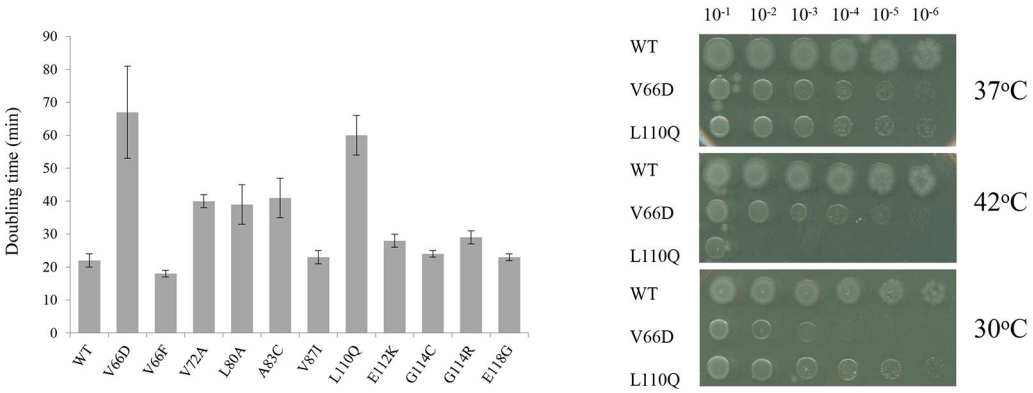

Fig. 1.

Structural interpretation of amino acid substitutions in ribosomal protein bL12. (A) The NMR structure of bL12 dimer of *E. coli*, pdb entry 1RQU [4]. One monomer is shown in blue, the second in red. (B) Closeup view of the C-terminal domain of monomer 1, showing the sites of amino acid substitutions described in this study (green) and other sites of residues mutated in other studies (grey).

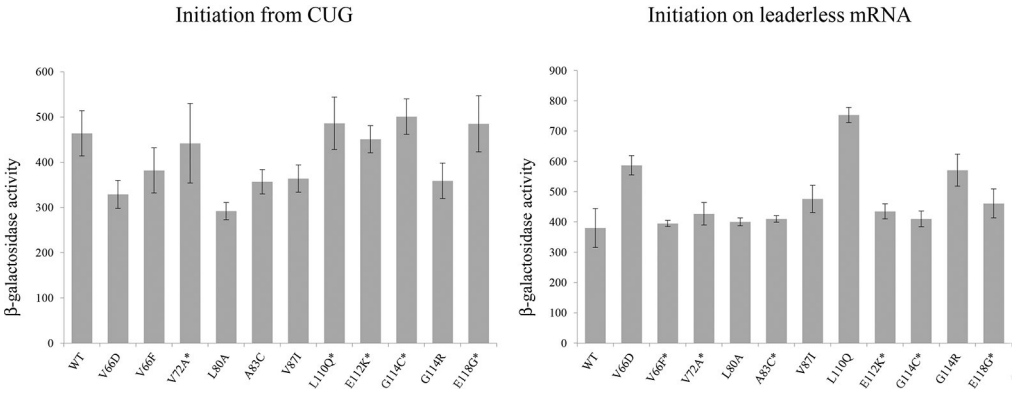

Fig. 2.

Effects of bL12 alterations on growth. The left panel shows the doubling times of wild type (WT) and bL12 mutants in LB medium at 37°C. Each bar represents the mean of at least three independent determinations ± SD. The right panel shows the growth of dilutions of overnight cultures growing solid LB medium at the indicated temperatures.

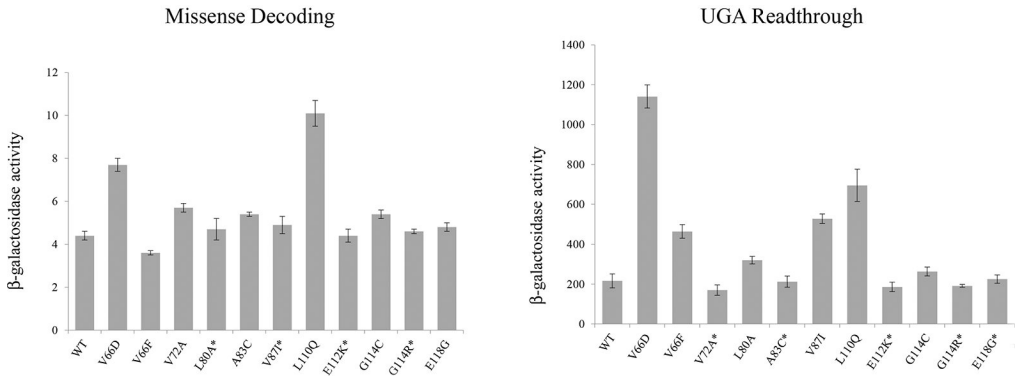

Fig. 3.

Effects of bL12 substitutions on initiation from CUG (left panel) and from a leaderless mRNA (right panel). Strains expressing wild type or altered bL12 proteins were transformed with *lacZ* plasmids pSG413 (initiation from CUG) or pDB (initiation from leaderless mRNA) and assayed for β-galactosidase activity. Bars represent the mean of at least three independent determinations of β-galactosidase activity, expressed in Miller units, ± SD. An asterisk (*) indicates that the β-galactosidase activity for the indicated mutant did not differ significantly ($P > 0.05$) from the corresponding wild type strain.

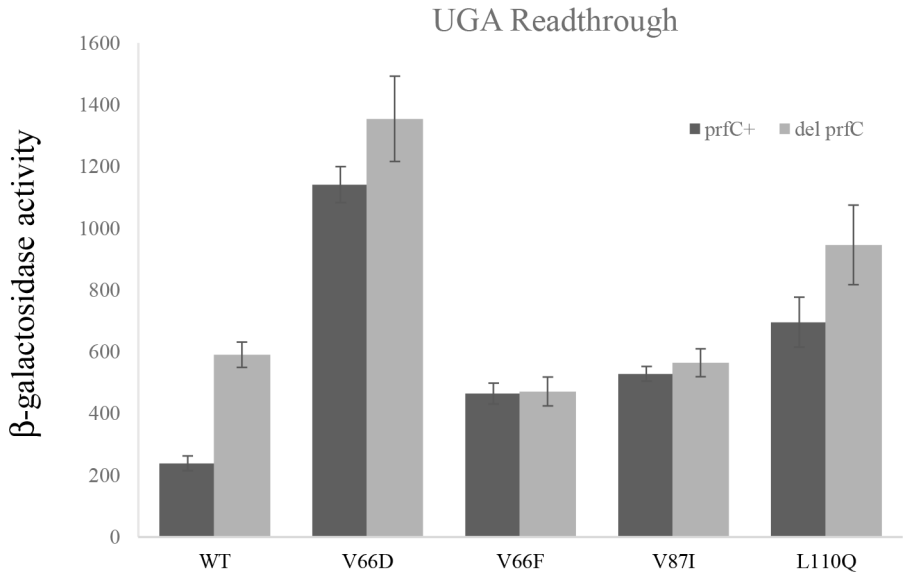

Fig. 4.

Effects of bL12 substitutions on missense decoding (left panel) and on UGA readthrough (right panel). Mutant or wild type bL12 strains were transformed with *lacZ* plasmids pAC102 (missense decoding reporter) or pSG3/4 UGA (UGA readthrough reporter) and assayed for β-galactosidase activity. Each bar represents the mean of at least three independent determinations of β-galactosidase activity, expressed in Miller units, ± SD. An asterisk (*) indicates that the β-galactosidase activity for the indicated mutant did not differ significantly ($P > 0.05$) from the corresponding wild type strain.

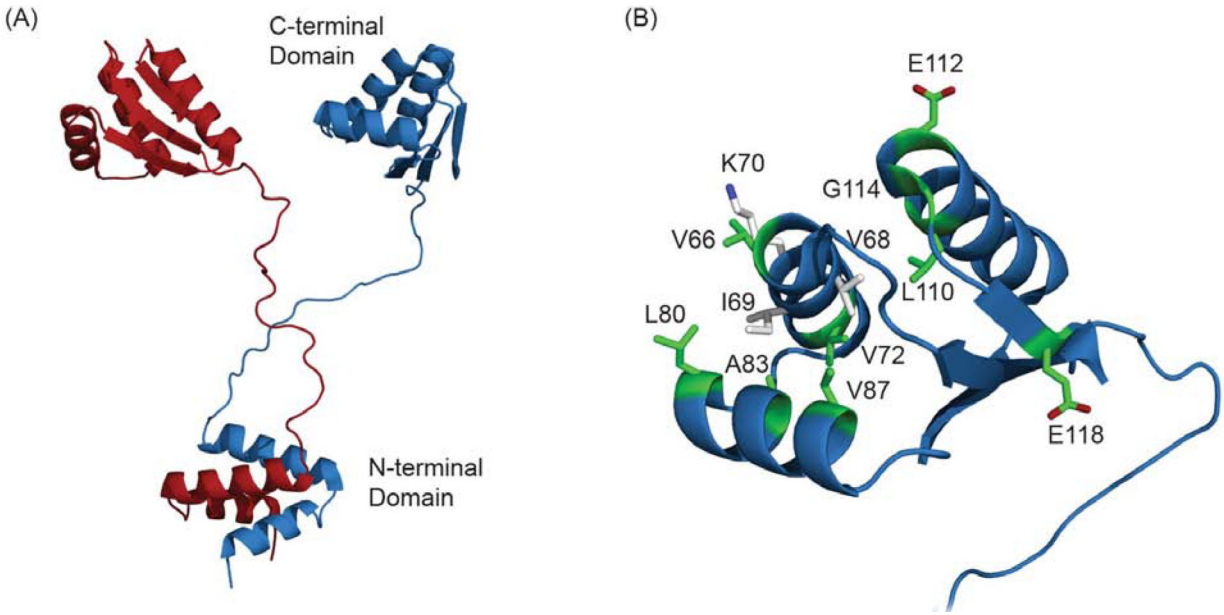

Fig. 5.

Effects of RF3 loss on bL12-dependent readthrough of UGA codons. Isogenic pairs of wild type and mutant bL12 strains either expressing RF3 (prfC$^+$) or carrying a deletion of the RF3 gene (del prfC) were transformed with the UGA readthrough reporter plasmid, pSG3/4 UGA and assayed for β-galactosidase activity. Independent activity measurements were done in triplicate and each bar represents the mean β-galactosidase activity, expressed in Miller units, ± SD.