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Alterations in Ribosomal Protein L19 that Decrease the Fidelity of Translation

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Abstract

Ribosomal protein L19 is an essential ribosomal protein and is a component of bridge B8, one of the protein-RNA bridges linking the large and small ribosomal subunits. Bridge B8 also contributes to the accuracy of translation by affecting GTPase activation by ribosome-bound aminoacyl tRNA-EF-Tu•GTP ternary complexes. Previous work has identified a limited number of accuracy-altering alterations in protein L19 of *Salmonella enterica* and *Thermus thermophilus*. Here, we have targeted the *Escherichia coli* *rplS* gene encoding L19 for mutagenesis and have screened for mutants with altered levels of miscoding. We have recovered 14 distinct L19 mutants, all of which promote increased stop codon readthrough, but do not have major effects on subunit association or cell growth. Examination of the *E. coli* 70S ribosome structure indicates that the amino acid substitutions cluster in three distinct regions of L19 and thereby potentially affect its interactions with L14 and 16S rRNA.

Keywords

tRNA selection; Ribosomal Protein L19; Miscoding errors

1. Introduction

The ribosome exploits a variety of mechanisms to ensure fast and accurate decoding [1–3]. Despite this, a variety of chemical agents, as well as mutations in the translation machinery can affect the accuracy of protein synthesis. The identification of alterations in ribosomal proteins and rRNAs that increase or decrease the fidelity of translation has provided critical insights into the ribosome's tRNA discrimination mechanisms and with the advent of high resolution structures of the ribosome, the effects of many of these mutations can now be interpreted in structural terms [4]. Thus, mutations affecting the decoding center, or the regions of the 30S ribosomal subunit that exhibit conformational changes during the decoding process can alter the stringency of tRNA selection.

More puzzling to explain are some of the mutations in components of the large ribosomal subunit that also promote translation errors [5, 6]. Some of these likely affect the interaction of elongation factor EF-Tu with the ribosome. All elongator tRNAs are delivered to the ribosome as a ternary complex with EF-Tu•GTP and a critical step in the tRNA selection pathway is the activation of GTP hydrolysis by the GTPase Associated Center (GAC) on the 50S subunit [7, 8]. Consistent with this, mutations in components of the GAC, including the sarcin/ricin loop of the 23S rRNA and protein L7/L12 produce a range of accuracy phenotypes [9–13]. Cognate ternary complexes are far more effective GTPase activators than near-cognate complexes and sensing of correct codon-anticodon complexes in the decoding center of the 30S subunit is coupled to GTPase activation by the GAC. The GAC and decoding center are some 80Å distant and the mechanism of signal transmission between the two functional centers remains unclear.

Both the tRNA itself, as well as one or more of the intersubunit bridges connecting the large and small subunits have been proposed to serve as conduits for this signal transmission. Among the intersubunit bridges, bridge B8 has emerged as a likely candidate involved in signaling between the decoding center and the GAC. This bridge is formed by contacts between helices h8 and h14 of 16S rRNA in the 30S subunit and proteins L14 and L19 in the 50S subunit. Several *E. coli* mutations in h8 and h14 have an error-prone, or ribosome ambiguity (*ram*) phenotype and when assayed, stimulate GTPase activation by near-cognate ternary complexes [14]. L19 also contacts 16S rRNA h44; indeed, the first *ram* mutation found in *E. coli* 16S rRNA occurred at U1469 in h44 [15], close to the site of contact with L19. Intriguingly, X-ray crystallography of 16S h14 mutant ribosomes revealed that the mutant ribosomes adopt a conformation resembling that usually seen only upon binding of aminoacyl tRNA-EF-Tu•GTP to 70S ribosomes, as if the mutation predisposes the ribosome to trigger GTP hydrolysis, irrespective of whether the tRNA bound is cognate or near-cognate [16]. Alterations affecting ribosomal protein L19, one of the 50S components of bridge B8, have been isolated in selections for compensatory mutations that relieved the slow growth associated with streptomycin-resistant S12 mutants in *Salmonella enterica* and *Thermus thermophilus* [17, 18]. The *Salmonella* L19 mutants also have an error-prone phenotype, just like the h8/h14 mutants in 16S rRNA; the *T. thermophilus* mutants have not yet been assayed directly for their miscoding properties.

Given the emerging appreciation of the role of bridge B8 in modulating GTP hydrolysis by ribosome-bound ternary complexes, we have targeted the *E. coli rplS* gene encoding L19 for mutagenesis. In screens for mutants with altered decoding accuracy, we have isolated 14 error-prone mutants, affecting 11 different residues in L19.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strain MC323 (F⁻, λ⁻, *lacZ*521 (Tn10 linked), *rph*-I) was used in electroporation experiments and to screen for accuracy-altering mutations in *rplS* [19]. This strain carries a leaky UGA mutation in *lacZ* and forms pale blue colonies on X-gal containing medium. Mutations that increase or decrease UGA readthrough are readily detected on X-gal-containing media as darker and paler blue/white colonies, respectively. All mutants were

reconstructed in strain MC361(F^- , ara^- ($gpt-lac$)5 thi^- $prfB$ [*E. coli* B]), a K-12 strain that carries the fully active A246 RF2 protein from *E. coli* B [20]. Plasmids pKD4, pKD46 and pCP20 were used in recombineering procedures [21]. The ampicillin resistant *lacZ* reporter plasmids p12-6 and p34-11 were used to monitor readthrough levels in L19 mutants. These plasmids carry UAG and UGA stop codons in the 5' end of the coding region, respectively.

2.2. Random mutagenesis of *rplS*

To facilitate subsequent manipulation of *rplS* mutations, a kanamycin resistance element was inserted 60 nt downstream of the L19 stop codon, immediately after the transcription terminator, using standard recombineering techniques. A 2.1 kb fragment carrying both *rplS* and the downstream kanamycin resistance cassette was amplified using the error-prone Mutazyme polymerase (Agilent Technologies, Santa Clara, CA). Following the manufacturers' guidelines, the amplification conditions were adjusted to permit 1–2 misincorporation errors per molecule. The mutagenized PCR products were then electroporated into electrocompetent MC323 cells, expressing the λ red recombinase from plasmid pKD46 [21]. The electroporated cells were grown without selection for 90 min and then plated on solid, rich medium containing X-gal (50 mg/l) and kanamycin (25 mg/l). Colonies that differed in color from the pale blue of the wild type strain were identified and the *rplS* gene was amplified and sequenced from these isolates. P1 transduction was used to transfer the desired *rplS* mutations into MC361 and the kanamycin resistance cassette was removed by transient expression of the FLP recombinase from pCP20. The *rplS* orf and flanking regions from the reconstructed strains were then sequenced to confirm the presence of the desired mutations only.

2.3. Growth rate determinations, β -galactosidase assays and ribosome analyses

Doubling times were determined from liquid cultures grown at 37 °C in LB medium. Growth on solid medium was monitored by spotting ten-fold dilutions of overnight cultures onto LB plates and incubating for 24–72 hrs.

Cells to be assayed for β -galactosidase activity were grown in minimal (E) medium containing glucose (0.2%), thiamine (0.05 mM) and casamino acids (0.2%) [22]. Assays were carried out on permeabilized cells, using ortho-Nitrophenyl- β -galactoside as a substrate.

For analysis of ribosomes, detergent lysates were prepared from logarithmically growing cells and loaded onto 10–40% sucrose gradients in 10m M $MgCl_2$, as described previously [23]. Separation of ribosomal subunits from 70S ribosomes and polysomes was achieved by centrifugation in a Beckman SW28 rotor at 17,000 rpm for 18 hrs. The gradients were analyzed using an ISCO gradient fractionator connected to an ISCO UV6 detector.

3. Results and discussion

3.1. Targeted mutagenesis of *rplS*

Using reverse genetics, L19 has been shown to be an essential ribosomal protein and decreased expression of L19 leads to the accumulation of 50S subunit assembly

intermediates and cold-sensitive growth [24]. That said, *E. coli* strains apparently lacking L19 completely have been isolated as drug-independent revertants of erythromycin-dependent strains [25, 26]. These strains carry one or more unidentified, compensatory mutations that allow ribosome assembly and function in the absence of L19. Nonetheless, given the normally-essential nature of L19, we have used a recombineering approach for the generation of *rplS* mutations. Electroporation of MC323 with randomly mutagenized *rplS*-Kan^R fragments yielded approximately 1,000 transformants. Of these, 20 were darker blue on X-gal plates than the wild type MC323 strain. Sequencing of the *rplS* gene from these 20 colonies showed that 2 had unaltered, wild type sequences, while 14 unique base changes affecting L19 were found among the remaining 18 isolates (Table 1). 3 mutants carried the same Q40L alteration while 2 isolates had the same E33D change and a further 2 carried the same chain-terminating mutation (E111*) that shortens L19 by four residues. The Q40H mutant also contained a silent, third position change at the Ile 47codon. Two different amino acid substitutions at residues V31, R38 or Q40 were recovered. Notably, no error-restrictive mutations were isolated. Previous work in *S. enterica* has described L19 mutants with Q40H, Q40L, Q40R or G104A changes [17, 27], while recent work in *T. thermophilus* has described an L19 mutant with an E33V alteration [18]. Thus our approach has produced some of the previously-described L19 mutants as well as multiple novel mutants.

The 14 unique mutants isolated here were reconstructed for further analyses in strain MC361. Determinations of doubling times showed that at least in rich medium at 37 °C, a majority of the mutants grew at rates close to that of the wild type strain (Table 1). Only the Q40H and Q40L mutants had modestly increased (~1.2 fold) doubling times. On solid, rich medium, all mutants grew at equivalent rates at 37 °C and 42 °C (not shown) However, when grown at 20 °C on solid medium, growth of 8 mutants was clearly impaired (Fig. 1) and these mutants also grew substantially slower than the wild type in liquid LB broth at 25 °C (Fig. 1). Cold sensitivity is a characteristic of many assembly-defective ribosomal mutants [28]. However, sucrose gradient analyses of lysates from each of the 14 mutants grown at 37 °C did not reveal any detectable defects in subunit assembly or association (not shown). All mutants had levels of 30S and 50S subunits and 70S ribosomes that were indistinguishable from wild type. In addition, sucrose gradients of lysates prepared from the 8 cold sensitive mutants grown at 25 °C were indistinguishable from wild type (not shown). Thus, at 37 °C, none of the mutations has major effects on growth, 50S subunit assembly or 30S–50S association and the cold sensitivity of particular mutants is not due to ribosome assembly or association defects. Potentially, the cold sensitivity of some L19 mutants derives from indirect effects of the ribosomal mutations on gene expression, that are only manifested at low temperatures.

3.2. Miscoding in L19 mutants

The effects of L19 alterations on the accuracy of decoding were addressed using two different *lacZ* plasmids, p12-6 and p34-11 that report on UAG and UGA readthrough, respectively. All L19 mutants promoted increased readthrough of both stop codons. The highest levels of UAG and UGA readthrough (5.5–5.8-fold above wild type levels) were seen in strains carrying the Q40L alteration in L19, while the R71C mutant supported the lowest levels of readthrough (1.3–1.5-fold increases). In previous studies, substitutions at

Q40 in *S. enterica* and E33 in *T. thermophilus* L19 were also shown to suppress growth deficiencies and streptomycin resistances conferred by alterations in protein S12. Moreover, multiple rRNA mutations in the 16S rRNA components of bridge B8 have been isolated as missense suppressors [14], or have been shown to reverse the streptomycin dependence of defined S12 alterations [23]. This suggests that bridge B8 mutant ribosomes (including the L19 alterations described here) promote a variety of miscoding events and that their decoding defects are not limited to effects on stop codon readthrough.

3.3. Bridge B8 and the binding of aminoacyl tRNA-EF-Tu•GTP ternary complexes

Ribosome-dependent activation of GTP hydrolysis is a key step in the tRNA selection pathway and this process, in addition to inspection of codon-anticodon pairings in the decoding center, actively discriminates against near-cognate tRNAs. Bridge B8 components helices h8 and h14 in 16S rRNA have been implicated in EF-Tu-dependent GTPase activation and tRNA selection processes and L19 mutations likely affect fidelity by altering this same step. Although they do not contact the ternary complex directly, h8 and h14 shift positions upon ternary complex binding [29]. Moreover, movement of h14 appears to be coupled to that of the adjacent helix h5, which does contact EF-Tu, in the switch 2 region [16, 29]. Biochemical investigations have shown that truncations or base substitutions mutations in h8/h14 affect tRNA selection through accelerating GTP hydrolysis by near-cognate ternary complexes [14]. Structural analyses of one of these h14 mutant ribosomes (G347U) revealed that the mutation causes the (vacant) 70S ribosomes to adopt a conformation normally seen only upon binding of the aminoacyl tRNA-EF-Tu•GTP ternary complex. Together, these findings show that conformational rearrangements of bridge B8 are critical for GTP hydrolysis by EF-Tu and the fidelity of translation.

3.4. Structural basis for mutant phenotypes

While the 16S rRNA components of bridge B8 have been studied biochemically and structurally, less information is available on the L14 and L19 components. Nonetheless, examination of the wild-type *E. coli* 70S ribosome structure (pdb entries 2i2p and 2i2; [30]) allows a structural interpretation of the amino acid substitutions identified in this study (Fig 3A). A number of potential protein-protein or protein-rRNA interactions are visible in the vicinity of sites of amino acid substitutions (Table S1). Mutated L19 residues are located at a number of positions throughout L19 (Fig 3B), but are clustered in three regions.

One region includes the C-terminal tail of L19 (Fig 3C). This tail, extending from R102 to N114 is rich in basic residues and has the potential to make extensive contact with 16S rRNA helix 44, which would be lost with the deletion extending from E111. Insertion of rigidifying prolines as in the A107P and R108P substitutions are expected to distort this tail significantly and also affect its interaction with 16S rRNA. The R108P mutation also removes a contact with the phosphate oxygens of U1464 of 16S rRNA. U1464 is near U1469, the site of the original 16S rRNA *ram* mutation [15]. The interaction of the tail with the rest of L19 is stabilized by interactions between backbone oxygen of K110 and R87, and between R112 and both R87 and D23. V91 makes a hydrophobic interaction with L96 to further stabilize the interaction of the tail with the rest of the protein. Any amino acid

substitutions disrupting this interaction could potentially affect the L19-h44 interaction and the organization of the entire bridge.

A second cluster of substitutions occurs at an interface between ribosomal proteins L19 and L14 (Fig 3D). L19 residue R71 is part of a β -strand that forms a β -sheet with ribosomal protein L14 residues V75 to F78. Amino acid substitutions in this region could potentially destabilize this protein-protein interaction. However, the side chain of R71 is buried within the protein and makes no contacts with any other residues. The adjacent E70 form a long-range stabilizing interaction with L19-R100. The R71C substitution could potentially disrupt this interaction, thereby indirectly affecting the L14–L19 interaction.

A third cluster of substitutions arose in a loop, formed by residues 30–40, including at W30, V31, E33, R38 and Q40, that interacts with 16S rRNA h14 (Fig 3E). A backbone hydrogen bond between V31 and E33 stabilizes the loop conformation. The side chains of E33 and R38 interact with one another, while R38 contacts the phosphate oxygen of G346. How substitutions at W30, Q40 and F42 influence decoding is unclear.

In the genetic screens we and others have used, all of the alterations affecting 16S rRNA helices h8 and h14 and protein L19 promote increased miscoding, or suppress the effects of error-restrictive, streptomycin resistant/dependent S12 mutants, a phenotype usually, (but not invariably) associated with increased misreading. No bridge B8 mutants with decreased error rates have yet been recovered. This is likely a reflection of the disruptive effects of most h8/h14 and L19 mutations on B8 structure. If bridge B8 normally negatively regulates GTPase activation and GTP hydrolysis, the readily-obtained mutations that disrupt B8's structure might well be expected to promote promiscuous activation and hydrolysis. Conversely, mutations that could stabilize B8 structure, rendering it less prone to trigger GTP hydrolysis by all ternary complexes including near-cognates, are expected to be far rarer. In directed evolution experiments aimed at improving the fitness of error-prone *S. enterica* L19 mutants, mutations affecting S12 and L14 were recovered [27]. Since both of the S12 alterations isolated (L6Q and K42N) are error restrictive [17, 19], potentially the L14 mutants isolated in that study also decrease translational errors. However, the experiments to characterize their miscoding properties have not yet been attempted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. L19 substitutions confer cold-sensitive growth. 2.5 µl aliquots of overnight cultures of the indicated L19 mutants were serially-diluted and spotted onto LB plates and incubated at 37 °C (left panel) or 20 °C (right panel) and incubated for 24 or 72 hrs. respectively. WT refers to the parental MC361 strain carrying a wild type *rplS* gene. Doubling times (in min.) of the strains grown in LB broth at 25 °C are indicated on the right.

Fig. 2.

Substitutions in L19 affect the accuracy of decoding. Strains expressing wild type (WT) L19 protein or the indicated substitutions were transformed with the *lacZ* reporter plasmids p12-6 or p34-11 and assayed for β -galactosidase activity. The levels of UAG (upper panel) or UGA readthrough (lower panel) supported by the L19 mutants are represented as Miller units of β -galactosidase activity. Each bar represents the mean of at least three independent determinations $\pm$ SD.

Fig 3.

Structural basis for mutant phenotypes. (A) Crystal structure of the *E. coli* 70S ribosome (pdb entries 2i2p and 2i2t; [30]). Ribosomal proteins S12, L14 and L19 are colored yellow, light pink, and skyblue, respectively. All other ribosomal proteins are colored dark grey. 16S rRNA residues 153–169 and 332–353 (part of bridge B8) are colored pale green. Remaining rRNA is colored light grey. (B) A close-up view of intersubunit bridge B8. Elements are colored as in (A). Sites of mutations are indicated by red spheres at positions of Ca atoms. (C) Close up view of the interaction between ribosomal proteins L19 (skyblue) and 16S rRNA helix 44 (green). The C-terminal tail of L19 is shown as sticks. Sites of amino acid

substitutions are labeled. (D) L14–L19 interactions and sites of mutations (sticks). (E) Interaction of L19 and 16S rRNA helix 14, sites of mutations shown as sticks.

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Table 1

Nucleotide and amino acid alterations and doubling times of L19 mutants.

Amino acid change(s)	Nucleotide(s) altered	Doubling time (min) at 37°C
Wild type	wild type	26 ± 3
W30C	G93C	30 ± 1
V31D	T95A	26 ± 1
V31G	T95G	28 ± 2
E33D	A102T	30 ± 5
R38C	C115T	28 ± 3
R38H	G116A	30 ± 1
Q40H	G123T; C144T [‡]	33 ± 3
Q40L	A122T	32 ± 2
F42L	C129G	28 ± 2
R71C	C214T	29 ± 2
V91D	T275A	28 ± 2
A107P	G322C	31 ± 1
R108P	G326C	25 ± 2
E111 *	G334T	26 ± 1

In the E111* mutant, the G334T nucleotide change generates a stop codon that results in an L19 protein lacking the last four amino acids.

[‡]The Q40H mutant carries a silent mutation at the triplet encoding I47.