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Mol Microbiol. Author manuscript; available in PMC 2012 January 1.

Published in final edited form as:
Mol Microbiol. 2011 January ; 79(2): 331–341. doi:10.1111/j.1365-2958.2010.07467.x.

Deletion of the RluD pseudouridine synthase promotes SsrA peptide tagging of ribosomal protein S7

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Summary

RluD catalyzes formation of three pseudouridine residues within helix 69 of the 50S ribosome subunit. Helix 69 makes important contacts with the decoding center on the 30S subunit and deletion of *rluD* was recently shown to interfere with translation termination in *Escherichia coli*. Here, we show that deletion of *rluD* increases tmRNA activity on ribosomes undergoing release factor 2 (RF2)-mediated termination at UGA stop codons. Strikingly, tmRNA-mediated SsrA peptide tagging of two proteins, ribosomal protein S7 and LacI, was dramatically increased in Δ*rluD* cells. S7 tagging was due to a unique C-terminal peptide extension found in *E. coli* K-12 strains. Introduction of the *rpsG* gene (encoding S7) from an *E. coli* B strain abrogated S7 tagging in the Δ*rluD* background, and partially complemented the mutant's slow-growth phenotype. Additionally, exchange of the K-12 *prfB* gene (encoding RF2) with the B strain allele greatly reduced tagging in Δ*rluD* cells. In contrast to *E. coli* K-12 cells, deletion of *rluD* in an *E. coli* B strain resulted in no growth phenotype. These findings indicate that the originally observed *rluD* phenotypes result from synthetic interactions with *rpsG* and *prfB* alleles found within *E. coli* K-12 strains.

Introduction

The tmRNA•SmpB molecular quality control system recognizes stalled translation complexes and facilitates ribosome recycling in a process termed 'ribosome rescue' (Keiler *et al.*, 1996, Moore & Sauer, 2007, Hayes & Keiler, 2010). tmRNA is a specialized RNA molecule that functions as both transfer RNA (tRNA) and messenger RNA (mRNA) during ribosome rescue. The charged tRNA-like domain of tmRNA allows binding to the A-site of paused ribosomes. The ribosome then dissociates from the message and translation resumes using a small open reading frame within tmRNA. This process results in co-translational addition of the tmRNA-encoded SsrA peptide tag to the C-terminus of the nascent polypeptide. The appended SsrA peptide targets tagged proteins for degradation by ClpXP and other proteases (Keiler et al., 1996, Gottesman *et al.*, 1998, Herman *et al.*, 1998, Choy *et al.*, 2007). SmpB is a tmRNA-binding protein that is required for delivery of tmRNA to the ribosome and for proper translation of the SsrA peptide (Karzai *et al.*, 1999, Sundermeier *et al.*, 2005).

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The tmRNA•SmpB system was originally shown to act on ribosomes stalled at the 3′ ends of truncated transcripts that lack in-frame stop codons (Keiler et al., 1996). These so-called 'non-stop' messages are produced by premature transcription termination and nuclease activity. Subsequently, it was shown that tmRNA tags full-length *E. coli* proteins at their C-termini (Roche & Sauer, 2001, Collier *et al.*, 2002), suggesting that tmRNA•SmpB is often recruited to ribosomes undergoing translation termination. In eubacteria, decoding of the three termination codons is shared by two protein release factors (RF); RF1 decodes UAG and UAA, and RF2 acts at UGA and UAA codons (Scolnick *et al.*, 1968). Work from several groups has shown that RF2 is significantly less active than RF1 in *E. coli* (Tate *et al.*, 1999, Pavlov *et al.*, 1998). Low RF2 activity results from a polymorphism at residue 246 that is unique to *E. coli* K-12 strains (Uno *et al.*, 1996, Dincbas-Renqvist *et al.*, 2000). Consistent with these observations, tmRNA•SmpB often tags full-length proteins synthesized from messages terminated with UGA codons (Roche & Sauer, 2001, Collier et al., 2002). In addition, the C-terminal residues of the nascent chain can interfere with termination at all stop codons and induce tmRNA-mediated SsrA tagging (Hayes *et al.*, 2002a, Sunohara *et al.*, 2002). These latter findings are in accord with work from Isaksson and colleagues showing that the last two residues of the nascent chain significantly influence termination efficiency (Bjornsson *et al.*, 1996, Mottagui-Tabar *et al.*, 1994). In general, inefficient translation termination promotes tmRNA-mediated SsrA peptide tagging of full-length proteins.

The RluD pseudouridine (Ψ) synthase was recently reported to play a role in translation termination. RluD catalyzes the formation of Ψ1911, Ψ1915 and Ψ1917 within helix 69 of the 50S ribosome subunit (Raychaudhuri et al., 1998). Helix 69 forms part of the universally conserved B2a bridge between the ribosome subunits, and makes functionally important contacts with the decoding center on the 30S subunit (Yusupov *et al.*, 2001). *E. coli* mutants lacking *rluD* have a profound slow-growth phenotype and show defects in ribosome assembly (Gutgsell *et al.*, 2005, Raychaudhuri et al., 1998). Ejby *et al.* demonstrated high rates of stop codon readthrough in Δ*rluD* cells, and isolated a suppressor mutation in the *prfB* gene (encoding RF2) that restored normal growth (Ejby *et al.*, 2007). These findings suggest that RF2 mediated termination is the primary defect in the Δ*rluD* mutant, and predict that SsrA tagging should be significantly increased in these cells. Indeed, we find that cells lacking RluD exhibit increased tagging of full-length proteins synthesized from messages terminated with UGA stop codons. Although deletion of *rluD* has a general effect on RF2 mediated termination, we find that the tagging of ribosomal protein S7 and the *lac* repressor (LacI) was dramatically increased in Δ*rluD* cells. Remarkably, impaired synthesis of the essential S7 protein accounts for a portion of the Δ*rluD* slow-growth phenotype. Moreover, allelic exchange of the *E. coli* B strain *prfB* gene into the K-12 Δ*rluD* mutant restored wild-type growth, and deletion of *rluD* in an *E. coli* B strain had no effect on growth under standard laboratory conditions. These results show that the originally observed Δ*rluD* growth defect is due to synthetic interactions with the *E. coli* K-12 alleles of *rpsG* and *prfB*.

Results

Deletion of rluD increases SsrA tagging of ribosomal protein S7 and lac repressor

Ejby *et al.* recently reported that deletion of *rluD* increases stop codon read-through in *E. coli* (Ejby et al., 2007), suggesting that pseudouridylation of the 50S ribosome is required for efficient translation termination. To determine whether there is a commensurate increase in tmRNA activity in cells lacking RluD, we examined SsrA peptide tagging in a Δ*rluD* mutant that expresses tmRNA(DD). tmRNA(DD) encodes the modified SsrA(DD) peptide tag, which is resistant to proteolysis and can be tracked by immunoblot (Keiler et al., 1996, Roche & Sauer, 1999). Western blot analysis of whole-cell lysates from Δ*rluD* cells showed

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two prominent tagged proteins (at ~35 and 20 kDa) that were not present in *rluD*$^+$ cells (Fig. 1). To isolate and identify these tagged proteins, we repeated the tagging experiment in Δ*rluD* cells expressing tmRNA(His_6), which encodes the SsrA(His_6) peptide tag. SsrA(His_6)-tagged proteins were enriched by Ni^{2+}-affinity chromatography, resolved by SDS-PAGE and blotted for automated N-terminal sequence analysis. Sequencing identified the tagged 20 kDa protein as ribosomal protein S7 ($P^1RRRVIGQ^8$). Additionally, the Ni^{2+}-affinity purified sample contained a protein at ~9 kDa (data not shown), which sequencing revealed to be a proteolytic fragment of S7 ($V^{79}GGSTYQ^{85}$) (see Fig. 1, labeled S7*). N-terminal sequencing of the 35 kDa species was ambiguous, so this protein was digested with trypsin and its peptides identified by mass spectrometry. This analysis identified 15 unique peptides from LacI (the *lac* operon repressor), corresponding to 30% coverage of the protein (data not shown). Examination of SsrA(DD) tagging in Δ*rluD* D*lacI* cells confirmed LacI as the 35 kDa tagged protein (Fig. 1).

Both S7 and LacI have been previously identified as SsrA-tagged proteins in *E. coli* (Collier et al., 2002, Roche & Sauer, 2001). Additionally, the *rpsG* (encoding S7) and *lacI* genes both terminate with a UGA stop codon, suggesting that the encoded proteins are tagged at their mature C-termini during inefficient RF2-mediated termination in the Δ*rluD* background. To identify the specific tagging site(s), we performed mass spectrometry on SsrA(His_6)-tagged proteins purified from Δ*rluD* cells, and found that S7 was indeed tagged at its C-terminus (Fig. 2A). Tagged LacI was not detected by this analysis, presumably because the intact protein does not ionize well in the mass spectrometer. Therefore, to test whether LacI is also tagged at its C-terminus, we examined the tagging of LacI expressed from plasmid constructs containing different stop codons. LacI was readily tagged when expressed in Δ*rluD* cells from a transcript containing the wild-type UGA stop codon (Fig. 2B). However, no tagging was observed when LacI was expressed from a message terminated by UAG (Fig. 2B). Taken together, these data strongly suggest that inefficient translation termination in Δ*rluD* cells results in SsrA tagging of full-length S7 and LacI.

Intriguingly, the S7 protein from *E. coli* K-12 strains contains 23 additional residues at the C-terminus that are not found in most other Enterobacteriaceae, nor in *E. coli* B strains. This C-terminal extension arises from an A-to-T transversion that mutates the ancestral *rpsG* stop codon to leucine (Fig. 3A). To examine the role of the S7 C-terminal tail, we cloned *rpsG* from *E. coli* X90 (a K-12 derived strain) into an expression plasmid to more easily study S7 tagging. An N-terminal His_6 epitope tag was added to facilitate S7 purification and quantitative comparison of SsrA tagging between *rluD*$^+$ and Δ*rluD* backgrounds. In agreement with previous observations, we found that K-12 derived His_6-S7 was tagged in *rluD*$^+$ cells, and that tagging increased significantly in the Δ*rluD* mutant (Fig. 3B). Replacing the *rpsG* stop codon with UAG had a minor effect on tagging in *rluD*$^+$ cells, but dramatically reduced tagging of K-12 His_6-S7 in the Δ*rluD* background (Fig. 3B). In contrast, overproduced S7 from an *E. coli* B strain was not tagged in either *rluD*$^+$ or Δ*rluD* cells (Fig. 3B). Moreover, allelic exchange of the K-12 *rpsG* gene with the *E. coli* B strain allele virtually eliminated tagging of S7 in the Δ*rluD* background (Fig. 1). These results demonstrate that S7 tagging is influenced by the stop codon and the C-terminal peptide extension found in the K-12 strain.

Deletion of rluD increases tagging of other E. coli proteins

To determine whether the termination of other proteins is affected in Δ*rluD* cells, we examined the tagging of RbsK and PhoP, which are also SsrA tagged as a result of inefficient termination at UGA (Roche & Sauer, 2001, Collier et al., 2002). Overproduced RbsK and PhoP were both tagged at higher levels in the Δ*rluD* background, suggesting that the effect on RF2-mediated termination is general (Fig. 4). However, we noted that the Δ*rluD* mutation had a profoundly negative effect on S7 expression that was not observed

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with either RbsK or PhoP (Fig. 4, Coomassie stained gel). These observations suggest that termination of S7 translation is particularly inefficient, and may limit the rate of S7 synthesis.

The C-terminal residues of the nascent chain can also influence termination efficiency (Bjornsson et al., 1996, Mottagui-Tabar et al., 1994, Janzen *et al.*, 2002), and tagging is often driven by a combination of stop codon and nascent chain effects (Hayes et al., 2002a). To control for nascent peptide effects on translation termination, we examined the tagging of *E. coli* thioredoxin (TrxA) expressed in Δ*rluD* cells. TrxA is not normally tagged in *E. coli*, and therefore the nascent chain is not thought to influence ribosome activity (Hayes et al., 2002a). His_6-TrxA expressed from a message containing a UGA stop codon was not appreciably tagged in either *rluD*$^+$ or Δ*rluD* background (Fig. 5). However, His_6-TrxA variants containing C-terminal proline residues (which interfere with translation termination) were tagged by tmRNA(DD), and this tagging was increased in the Δ*rluD* mutant (Fig. 5). In contrast, when expressed from UAG terminated transcripts, all His_6-TrxA variants were tagged at *lower* levels in the Δ*rluD* background when compared to *rluD*$^+$ cells (Fig. 5). Taken together, these results suggest that helix 69 pseudouridylation is not strictly required for efficient RF2 activity, but is critical for termination at certain suboptimal stop signals.

Influence of RF2 on translation termination

E. coli K-12 and B strains contain different *prfB* alleles that produce distinct RF2 proteins. RF2 from *E. coli* K-12 strains contains a threonine residue at position 246, whereas all other eubacteria (including *E. coli* B) have either alanine or serine at this position. This polymorphism significantly decreases the activity of K-12 RF2 (Uno et al., 1996, Dincbas-Renqvist et al., 2000). We replaced the *E. coli* K-12 chromosomal *prfB* locus with the B strain gene to determine whether this allele, like the Glu172Lys mutation, ameliorates the termination defect in Δ*rluD* cells. The resulting cells showed significantly reduced SsrA peptide tagging of both S7 and LacI (Fig. 1). Tagging of RbsK and PhoP was also reduced in *E. coli* K-12 cells carrying the B strain allele of *prfB* (Fig. 4). Because the B strain RF2 also suppressed tagging in *rluD*$^+$ cells (Fig. 4), it appears that much of the C-terminal tagging observed in *E. coli* K-12 strains is a consequence of the Thr246 polymorphism.

Effects of S7 and RF2 on the growth of Δ*rluD* cells

RluD is the only pseudouridine synthase required for normal growth in *E. coli* (Ofengand *et al.*, 2001). Given that the *rluD* deletion increases SsrA tagging of an essential ribosomal protein, we asked whether impaired S7 synthesis contributes to slow growth. As reported by Ofengand and colleagues (Raychaudhuri et al., 1998), we found that the Δ*rluD* mutant grew much more slowly than isogenic *rluD*$^+$ cells (Fig. 6A). Introduction of the *E. coli* B *rpsG* gene into the Δ*rluD* mutant produced a strain that grew more rapidly (Fig. 6B), suggesting that inefficient termination of S7 synthesis contributes to slow growth in the K-12 background. Inefficient termination and SsrA induced proteolysis are both predicted to reduce S7 levels. Additionally, tagged S7 could inhibit cell growth by impairing ribosome function. To differentiate between these possibilities, we asked whether SsrA tagged S7 is incorporated into functional 70S ribosomes. We first isolated 70S ribosomes from sucrose gradients, and then separated the 50S and 30S subunits in low Mg^{2+} buffer. 30S subunits were isolated from an additional sucrose gradient and analyzed by SDS-PAGE. S7 was identified in stained gels by comparing the ribosomal protein profiles from cells expressing the K-12 and B alleles of *rpsG* (Fig. 7). SsrA tagged S7 was not detected in 30S subunits isolated from a K-12 strain deleted for *rluD* (Fig. 7), suggesting that tagged S7 is either degraded by ClpXP prior to ribosome assembly, or excluded during ribosome assembly. Further deletion of *clpP* resulted in the accumulation of SsrA tagged S7 protein in 30S particles (Fig. 7). Similarly, Δ*rluD* cells expressing tmRNA(DD) contained SsrA(DD)

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tagged S7 in functional 30S subunits. Because Δ*rluD* cells lacking tmRNA grew at a rate similar to Δ*rluD* cells expressing either wild-type tmRNA or tmRNA(DD) (Fig. 6A), it appears that tagged S7 within the ribosome is not deleterious. In principle, inefficient termination of S7 synthesis could limit the rate of ribosome production in Δ*rluD* cells and thereby contribute to slow growth. However, production of the B-strain S7 protein in *trans* from a plasmid-borne construct did not accelerate the growth rate of K-12 Δ*rluD* cells (data not shown). Finally, we examined the growth of Δ*rluD* cells containing the *E. coli* B allele of *prfB* and found that these cells grew at the same rate as *rluD*$^+$ cells (Fig. 6C). Similarly, deletion of *rluD* in an *E. coli* B strain [BL21(DE3)] had no effect on cell growth under standard laboratory conditions (Fig. 6D).

Discussion

RluD catalyzed pseudouridylation within helix 69 of the 50S ribosome subunit was recently shown to be important for efficient translation termination in *E. coli* (Ejby et al., 2007). Cells lacking RluD exhibit increased stop codon readthrough, particularly at UGA stop codons, suggesting that RF2 mediated termination is especially sensitive to the loss of pseudouridine residues in helix 69. In accord with these observations, we find that tmRNA•SmpB activity on pre-termination ribosomes is significantly increased is Δ*rluD* cells, resulting in SsrA tagging at the C-terminus of full-length proteins. Remarkably, ribosomal protein S7 and LacI were tagged to such an extent that they were readily isolated and identified from Δ*rluD* cell lysates. The genes encoding S7 and LacI are both terminated by TGA stop codons, suggesting that tagging is due to impaired RF2-mediated termination in Δ*rluD* cells (Ejby et al., 2007). This adverse effect on termination appears to be general, because the tagging of other proteins (RbsK and PhoP) encoded by TGA terminated genes was also increased. However, other determinants are likely to modulate termination in the Δ*rluD* mutant. Work from Tate and colleagues showed that the identity of the nucleotide immediately 3′ to the stop codon affects translation termination (Poole *et al.*, 1995, McCaughan *et al.*, 1995). Moreover, Isaksson and colleagues discovered that last two residues of the nascent chain can significantly influence termination efficiency (Bjornsson et al., 1996, Mottagui-Tabar et al., 1994). Both of these determinants have been shown to affect SsrA peptide tagging in *E. coli* (Hayes et al., 2002a, Hayes *et al.*, 2002b), and are likely to exacerbate termination defects given that TrxA variants containing C-terminal proline residues were tagged at higher levels in Δ*rluD* cells. The C-terminal tail of S7 from *E. coli* K-12 strains also appears to interfere with termination, perhaps explaining why S7 is produced at significantly lower levels than either RbsK or PhoP in Δ*rluD* cells. Thus, RF2 activity is modulated by ribosome pseudouridylation, stop codon context in the message, and the identity of the nascent peptide.

In contrast to termination at UGA, we find that tmRNA•SmpB activity at UAG stop codons was slightly decreased in Δ*rluD* cells compared to *rluD*$^+$ cells. Because tmRNA•SmpB activity at stop codons is typically inversely related to translation termination efficiency (Hayes et al., 2002a, Collier et al., 2002), these data imply that RF1 is more active in the Δ*rluD* background. This finding is unexpected because Ejby *et al.* reported small increases in UAG readthrough in Δ*rluD* cells (Ejby et al., 2007). If stop codon readthrough and tagging are competing processes, then perhaps an increase in the readthrough rate could lead to a commensurate decrease in tagging. However, we saw no biochemical evidence of increased readthrough by gel or mass spectrometry analysis. Alternatively, the *rluD* deletion may actually increase the apparent rate of termination at UAG by reducing RF1 affinity for the ribosome. RF1 has significantly higher affinity for the ribosome than does RF2, and tends to remain bound to the ribosomal A-site after peptidyl-tRNA hydrolysis (Pavlov et al., 1998, Pavlov *et al.*, 1997a, Pavlov *et al.*, 1997b). In fact, overexpression of constructs with inefficient termination signals can lead to RF1 sequestration on ribosomes, thereby depleting

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the pool of RF1 available for termination (Janssen & Hayes, 2009). According to this model, decreased RF1 activity is an artifact of gratuitous protein overproduction, and deletion of *rluD* ameliorates the effect by increasing the rate of RF1 dissociation from post-termination ribosomes. Regardless of mechanism, the loss of RluD-mediated pseudouridylation clearly has a much more profound effect on RF2 function than RF1 in *E. coli* K-12.

Ribosomal protein S7 is tagged by the tmRNA system at very high levels (~40% of total S7 chains) in *E. coli* K-12 cells lacking RluD. S7 tagging is largely due to a point mutation that changes the ancestral *rpsG* stop signal to a leucine codon in K-12 strains. As a result, the *E. coli* K-12 *rpsG* gene is extended by 23 codons and terminated by a TGA stop codon. Presumably, this mutation was a recent event because nearly all other Enterobacteriaceae, including *E. coli* B strains, encode shorter S7 proteins. It appears that the C-terminal tail contributes to S7 tagging independent of stop codon identity, because the K-12 S7 protein is still tagged (in both *rluD*$^+$ and Δ*rluD* backgrounds) when expressed from a TAG terminated construct. However, S7 expressed from the ancestral *rpsG* gene is not tagged in the Δ*rluD* mutant. Remarkably, introduction of the *E. coli* B *rpsG* allele into K-12 Δ*rluD* cells increased the growth rate, suggesting that impaired termination of this one open reading frame contributes significantly to the Δ*rluD* slow-growth phenotype. S7 is an essential protein that plays a critical role in ribosome assembly and function (Mizushima & Nomura, 1970, Nowotny & Nierhaus, 1988). Therefore, increased tagging and degradation of S7 in Δ*rluD* cells could conceivably underlie slow growth. However, Δ*rluD* cells deleted for tmRNA grew at approximately the same rate as Δ*rluD* cells expressing either wild-type tmRNA or tmRNA(DD). Moreover, Δ*rluD* mutant growth was not accelerated when the B strain *rpsG* gene was supplied in *trans*, suggesting that impaired S7 synthesis is not the sole determinant of slow growth. S7 is encoded by the *str* operon together with *rpsL* and *fusA*, which are essential genes encoding ribosomal protein S12 and elongation factor-G (EF-G), respectively. Because tmRNA activity requires truncated mRNA (Ivanova *et al.*, 2004), it is possible that inefficient S7 termination induces cleavage of the *str* operon transcript (Hayes & Sauer, 2003), which would then adversely affect the synthesis of S12 and EF-G. Impaired production of ribosomal proteins may also be related to the previously reported ribosome assembly defects in Δ*rluD* cells. The nature of this defect is still not completely understood and the 39S particles were only observed in buffers containing low Mg^{2+} concentrations (Gutgsell et al., 2005). We were unable to examine ribosome assembly defects because no abnormal ribosome particles were observed in any Δ*rluD* background, even during the isolation of 30S subunits in sucrose gradients containing 1 mM Mg^{2+} (data not shown). Given that suppressor mutations in the *prfB* gene (encoding RF2) are able to fully restore growth to Δ*rluD* mutants, it seems unlikely that pseudouridylation *per se* is required for 50S assembly.

RluD is the only ribosomal pseudouridine synthase known that have an effect on cell growth (Ofengand et al., 2001). However, it appears that this slow-growth phenotype arises from a synthetic interaction with the *E. coli* K-12 allele of *prfB*. *E. coli* K-12 strains express a unique version of RF2 that is not found in other related Enterobacteriaceae. RF2 from *E. coli* K-12 has a threonine residue at position 246, whereas all other bacteria contain either an alanine or serine residue at this position. Research from Buckingham and Ehrenberg and their colleagues shows that RF2 from K-12 strains has a lower affinity for ribosomes than RF2 from *E. coli* strain B (Dincbas-Renqvist et al., 2000, Pavlov et al., 1998). Ejby *et al.* first uncovered the basis of the *rluD* growth phenotype by selecting suppressor mutations that allow rapid growth in the K-12 background (Ejby et al., 2007). They characterized a point mutation in the *prfB* gene that almost completely restored the wild-type growth rate. This mutation results in a Glu172Lys missense change, which was proposed to facilitate RF2 binding to ribosomes lacking pseudouridine residues in helix 69 (Ejby et al., 2007). Similarly, we find that introduction of the *E. coli* B *prfB* allele into K-12 Δ*rluD* mutants

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completely restores growth rate. Moreover, deletion of *rluD* in an *E. coli* B strain has no effect on growth rate. Taken together, these results indicate that the loss of helix 69 pseudouridylation has no intrinsic effect on protein synthesis under standard laboratory conditions. Rather, it is the interaction between the unmodified ribosome and a peculiar variant of RF2 that underlies slow growth. This is somewhat surprising given that helix 69 pseudouridylation is highly conserved between prokaryotes and eukaryotes (Ofengand et al., 2001). Perhaps these modifications play an important role under other growth conditions, similar to what has been found with RF2 methylation in *E. coli* K-12. PrmC-mediated methylation of RF2 is essential in cells expressing the *E. coli* K-12 version of RF2, but can be deleted in *E. coli* B strains (Dincbas-Renqvist et al., 2000, Mora *et al.*, 2007). However, *E. coli* B cells lacking PrmC do exhibit a slow-growth phenotype in defined media, demonstrating that RF methylation is critical under certain growth conditions (Mora et al., 2007). Thus, it may be that RF2 methylation and helix 69 pseudouridylation are two highly conserved processes that increase the robustness of translation termination, but are dispensable under optimal growth conditions.

Experimental procedures

Bacterial strains and plasmids

All *E. coli* strains were derivatives of either X90 [F′ *lacI*^{q} *lac′ pro′/ara Δ(lac-pro) nal1 argE*(amb) *rif*^{r} *thi-1*] or BL21 (DE3) [F^{-} *ompT gal dcm lon hsdS*$_{B}$ ($r_B^- m_B^-$)] and are listed in Table 1. The Δ*rluD::kan* gene deletion was obtained from the Keio collection (Baba *et al.*, 2006), and transferred into the X90 and BL21 backgrounds using either bacteriophage P1-mediated transduction or phage λ Red-mediated recombination. All transferred Δ*rluD::kan* disruptions were confirmed by whole-cell PCR using oligonucleotides, **rluD-Sac** (5′ - ACG GAG CTC GTG CAT GTT GAG GAT CGC G); and **rluD-Kpn** (5′ - CCA GGT ACC TAT CAC CAT GCT GAA TGA AAG C). The *E. coli* B allele of *prfB* (linked to a kanamycin resistance cassette) was introduced into *E. coli* strain X90 by Red-mediated homologous recombination as described (Hayes et al., 2002a). All kanamycin resistance cassettes were flanked with FRT sites, and where indicated, were removed using the FLP recombinase as described (Datsenko & Wanner, 2000). Allelic exchange of *rpsG* was performed as described (Link *et al.*, 1997). The *rpsG* locus (including promoter, *rpsL, rpsG* and 5′ region of *fusA*) was amplified from *E. coli* BL21(DE3) using oligonucleotides **#133** (5′-GAA GAT CTC TCG AGG CGT CTG CTC AGT GAA GGA GAC G) and **#135** (5′-GAA GAT CTG AAG GTC AGG TTA CCA ACA AAC GG) (Devaraj *et al.*, 2009), digested with XhoI and BglII, and cloned into compatible SalI and BamHI sites of plasmid pKO3 to generate pKF280-(BL21). Cell growth experiments were conducted in LB media incubated at 37 °C with aeration. Because Δ*rluD* “dust” strains are known to rapidly acquire “tiny” suppressor mutations (Raychaudhuri et al., 1998), all Δ*rluD* mutants were plated onto LB agar plates after liquid culture to quantify tiny suppressors. Growth rate data were discarded if the number of tiny suppressor colonies exceeded 5% of the total population.

The *rpsG* genes from *E. coli* X90 and BL21(DE3) were PCR amplified using oligonucleotides (restriction endonuclease sites underlined) **rpsG-Eco/Nde**, (5′ - GGA GAA TTC ATA TGC CAC GTC GTC GCG TC) and **rpsG-Xho**, (5′ - ATC CTC GAG GCG TTC AAT TTA AGT AGC CC). The resulting products were digested with EcoRI and XhoI and ligated into plasmid pCH450, a pACYC184-derived plasmid containing the *araBAD* promoter. The K-12 and B strain *rpsG* NdeI/XhoI fragments were subcloned into plasmid pSH21 (Shoji *et al.*, 2010) to generate constructs that express N-terminally His_6-tagged S7. The stop codon of K-12 *rpsG* was mutated to UAG by PCR using oligonucleotides, **rpsG-Eco/Nde** and **rpsG(uag)-Xho** (5′ - ATC CTC GAG GCG TCT AAT TTA AGT AGC CC). The resulting PCR product was digested with NdeI and XhoI and ligated to plasmid pSH21. The *phoP* gene was amplified with oligonucleotides, **phoP-Eco/Nde** (5′ - GAG GAA TTC

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ATA TGC GCG TAC TGG TTG TTG AAG), and **phoP-Xho** (5′ - TAC TCG AGT CAT CAG CGC AAT TCG AAC AG), digested with EcoRI/XhoI and ligated to pCH450. Plasmid pTrc2 (Holberger & Hayes, 2009) is a derivative of pTrc99A (GE Healthcare) that expresses LacI from the *lacI*^{q} gene. The *lacI*^{q} stop codon was mutated to UAG using the PCR megaprimer method. Plasmid pTrc2 was used as a template for PCR with primers: **lacI-UAG**, (5′ - GGA AAG CGG GCA GGC GCA ACG C) and **Trc-WT**, (5′ - ATT CCA TGG AAT TCT AGA CCT CCG TGT GAA ATT GTT ATC CGC TC). The resulting PCR product was used as a primer for a second amplification in combination with oligonucleotide, **lacI-Spe**, (5′ - TAT CCC GCC GTT AAC TAG TAT CAA ACA GGA TTT TCG C). The final product was digested with SpeI and NcoI and ligated into pTrc2 to generate plasmid pTrc2(UAG).

The *E. coli trxA* gene was PCR amplified using oligonucleotides **trxA-Nde** (5′ - GTG GAG TTA CAT ATG AGC GAT AAA ATT ATT CAC C) and **trxA-TAG** (5′ - GAG GAT CCC CTA CGC CAG GTT AGC GTC GAG), followed by digestion with NdeI and BamHI and ligation to pSH21 to generate plasmid pHis$_6$-TrxA(UAG). Plasmid pHis$_6$-TrxA(UAG) was used as the PCR template to generate all other His$_6$-TrxA expression constructs. Oligonucleotide **pET-Sph/Pst** (5′ - CAA GGA ATG GTG CAT GCC TGC AGA TGG CGC CC) was used as the forward primer in combination with the following oligonucleotides: **trxA-TGA** (5′ - GAG GAT CCC TCA CGC CAG GTT AGC GTC GAG); **trxA-P-TAG** (5′ - GAG GAT CCC CTA CGG CAG GTT AGC GTC GAG); **trxA-P-TGA** (5′ - GAG GAT CCC TCA CGG CAG GTT AGC GTC GAG); **trxA-PD-TAG** (5′ - GAG GAT CCC CTA ATC GGG GTT AGC GTC GAG); and **trxA-PD-TGA** (5′ - GAG GAT CCC TCA ATC GGG GTT AGC GTC GAG). All products were digested with SphI and BamHI and ligated to plasmid pSH21.

Protein expression and Western blot analysis

E. coli strains were grown overnight at 37 °C in LB medium supplemented with the appropriate antibiotics (150 μg/mL ampicillin, 25 μg/mL tetracycline, or 50 μg/mL kanamycin). The following day, cells were resuspended to an optical density at 600 nm (OD_{600}) of 0.05 in fresh media and grown at 37 °C with aeration. Once cultures reached OD_{600} ~ 0.6, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (1.5 mM final concentration) or 0.4% (w/v) L-arabinose. After further incubation for 90 min, cells were collected by centrifugation and frozen at −80 °C. Protein was extracted from frozen cells in urea lysis buffer [8 M urea − 150 mM NaCl − 10 mM Tris-HCl (pH 8.0)]. His$_6$-tagged proteins were purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography as described previously (Hayes et al., 2002b). For mass spectrometry analysis, SsrA(His$_6$)-tagged proteins were further purified by reverse-phase high pressure liquid chromatography as described (Garza-Sánchez *et al.*, 2006). Western blot analysis was performed using the LI-COR® Odyssey infrared imaging system according to the manufacturer's instructions with minor modifications. Briefly, 10 μg of total urea-soluble protein was resolved by SDS-PAGE, followed by electrotransfer to nitrocellulose membranes. Membranes were blocked with 4% (w/v) dried milk in phosphate-buffered saline [2.7 mM KCl − 1.8 mM KH_2PO_4 − 137 mM NaCl − 10.1 mM Na_2HPO_4 (pH 7.4)] followed by incubation overnight with anti-SsrA(DD) or anti-SsrA polyclonal antisera. DyLight-680 conjugated goat anti-rabbit (Rockland) secondary antibodies were used for fluorescence detection.

Isolation of ribosomal subunits

Strains were grown in 250 mL of LB media at 37 °C with aeration to OD_{600} = 0.50. Cells were harvested over ice, collected by centrifugation and washed once with ribosome isolation buffer [60 mM potassium acetate – 0.5 M NH_4Cl – 10 mM Tris-acetate (pH 7.0)]

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supplemented with 15 mM magnesium acetate (MgOAc). Washed bacterial pellets were resuspended in 10 mL of cold ribosome isolation buffer containing 15 mM MgOAc, and cells were lysed by two passages through a French press at 16,000 psi. Lysates were clarified by centrifugation twice at 30,000 *5g* for 15 min at 4 °C. Supernatants were layered onto 10 – 40% (w/v) sucrose gradients prepared in ribosome isolation buffer with 15 mM MgOAc. Samples were centrifuged 65,000 *5g* for 11 hr in a SW Ti-32 rotor at 4 °C. The 70S ribosomes were collected and dialyzed into ribosome isolation buffer supplemented with 0.1 mM MgOAc. Dialyzed ribosomes were then layered onto 10 – 40% (w/v) sucrose gradients prepared in ribosome isolation buffer supplemented with 1 mM MgOAc and centrifuged as described above. 30S ribosome subunits were isolated and dissociated in urea lysis buffer for SDS-PAGE analysis.

Acknowledgments

This work was supported by the National Institutes of Health through grant GM078634. We thank Kurt Fredrick for providing plasmids, Zach Ruhe and Brian Janssen for technical assistance and construction of critical strains used in this study. We extend special thanks to Referee #2 who suggested a key experiment for this study. Protein identification by N-terminal peptide sequencing and mass spectrometry was performed at the UC Davis Proteomics Core facility. Mass spectrometry was performed in the UC Santa Barbara Department of Chemistry and Biochemistry Mass Spectrometry Facility with the assistance of Dr. James Pavlovitch.

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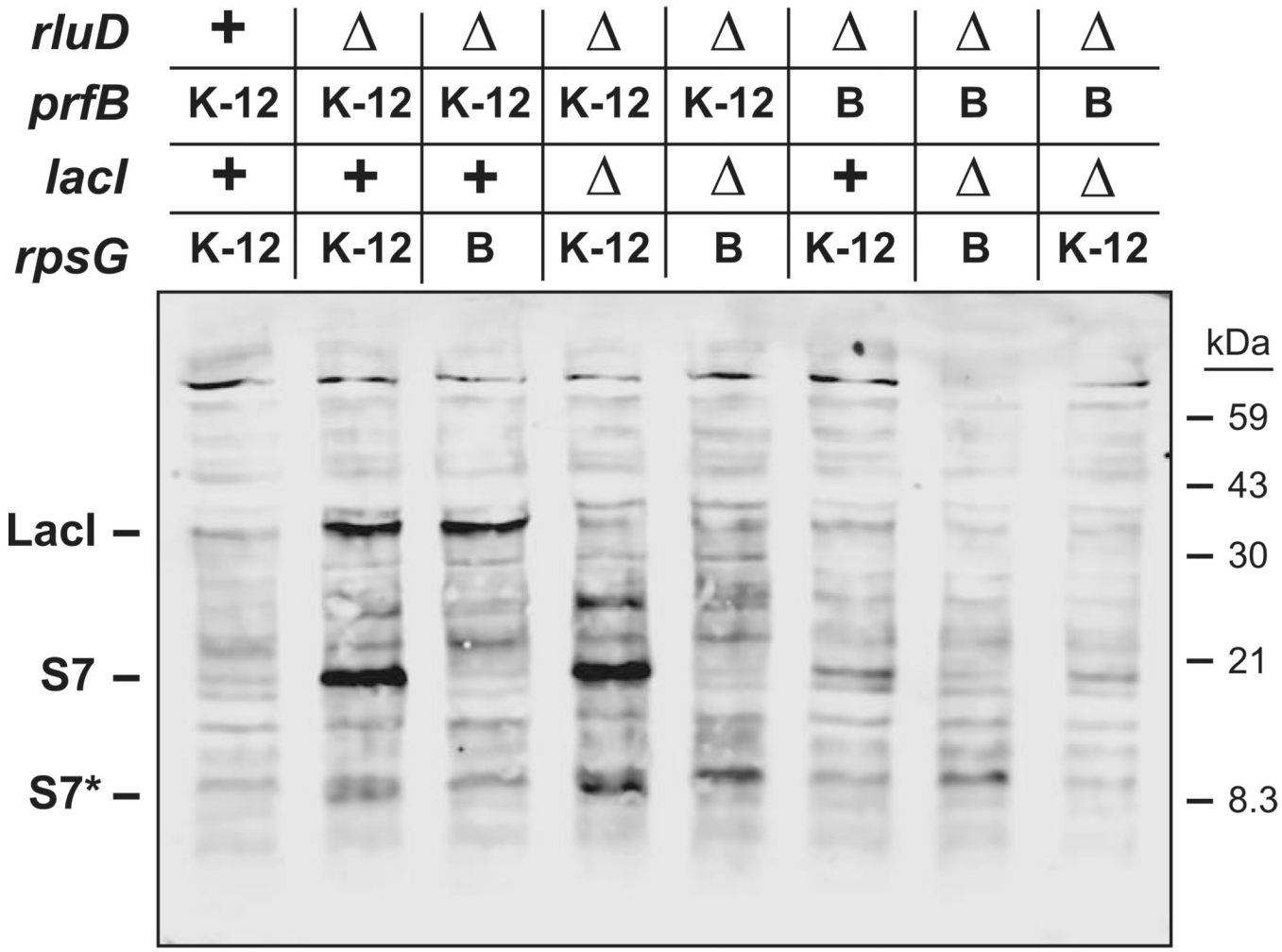

Figure 1. Deletion of *rluD* induces SsrA peptide tagging of ribosomal protein S7 and LacI

Western blot analysis of whole-cell lysates using antibodies specific for the SsrA(DD) peptide tag. All examined strains are derivatives of *E. coli* strain X90 (K-12 background) with the indicated genotypes. The migration positions of SsrA(DD)-tagged LacI, S7, and a proteolytic product of S7 (S7*) are indicated.

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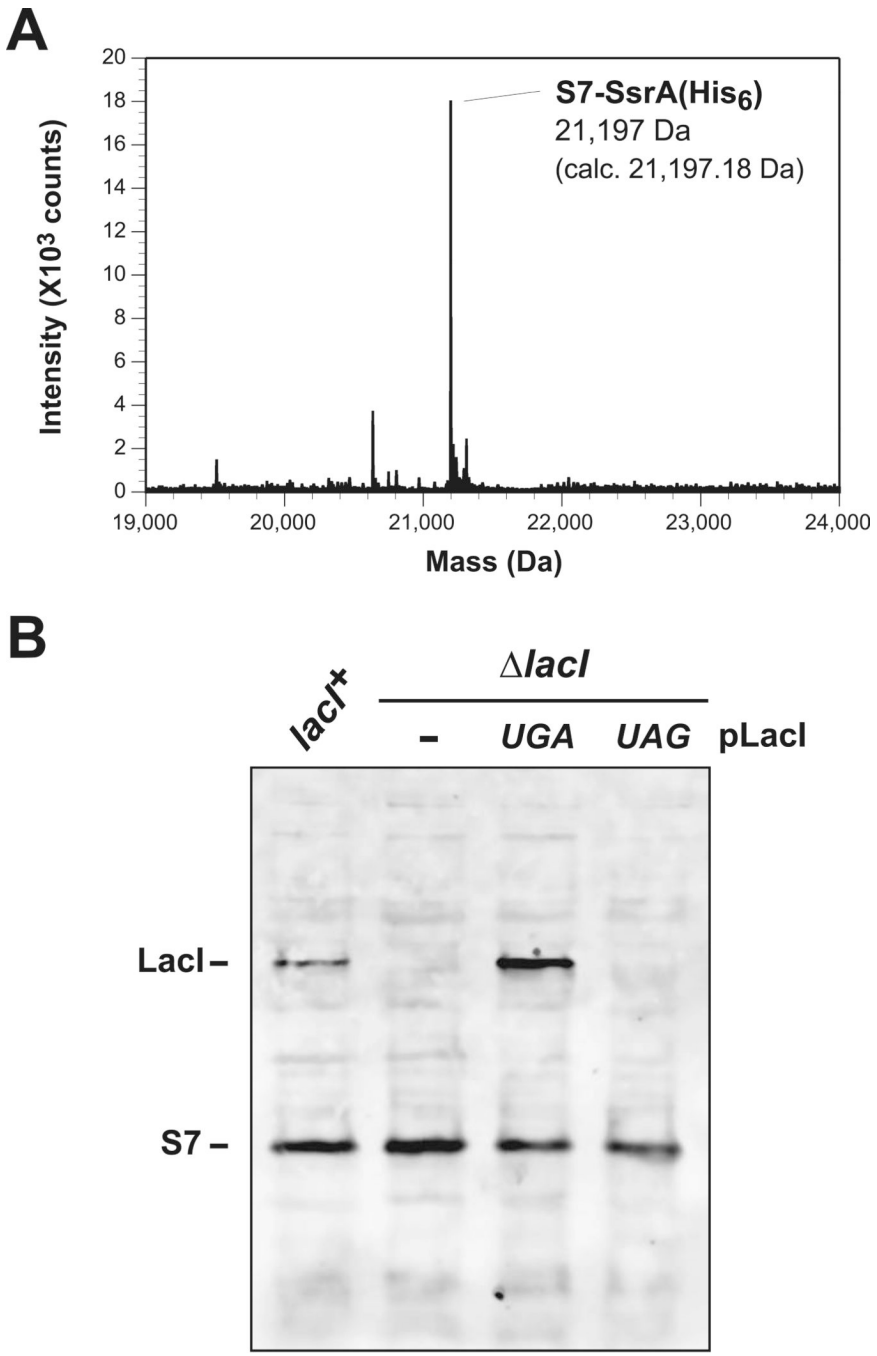

Figure 2. Ribosomal protein S7 and LacI are tagged at their C-termini in Δ*rluD* cells

A) Mass spectrometry of Ni^{2+}-affinity purified proteins from Δ*rluD* cells expressing tmRNA(His_6). The mass of the main species corresponds to SsrA(His_6) peptide addition after residue Asn179 of ribosomal protein S7. **B)** Western blot analysis of whole-cell lysates using antibodies specific for the SsrA(DD) peptide tag. The tagging of LacI was examined in D*lacI* cells expressing LacI from a plasmid (pLacI). Plasmid-encoded LacI was expressed from messages containing UGA and UAG stop codons. The migration positions of SsrA(DD)-tagged LacI and S7 are indicated.

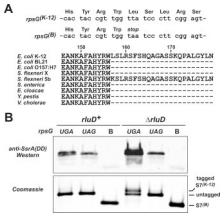

Figure 3. Ribosomal protein S7 from *E. coli* K-12 strains contains a C-terminal peptide extension

A) Alignments of *rpsG* and S7 proteins from selected Enterobacteriaceae. *E. coli* K-12 strains contains an A-to-T mutation in the ancestral *rpsG* stop codon that extends the open reading frame. Nearly all other S7 proteins from the Enterobacteriaceae lack this C-terminal tail. Amino acid residues are numbered based on S7 from *E. coli* K-12. **B)** Analysis of SsrA(DD) peptide tagging of His_6-S7 variants. His_6-tagged S7 proteins were expressed from *E. coli* K-12 and B strain *rpsG* constructs in the indicated genetic backgrounds (*rluD*$^+$ or Δ*rluD*). The K-12 version of S7 was produced from messages containing either a UGA or UAG stop codon. His_6-S7 was purified by Ni^{2+}-affinity chromatography and analyzed by SDS-PAGE and Western blot using antibodies specific for the SsrA(DD) peptide tag. The migration positions of the S7 proteins are indicated.

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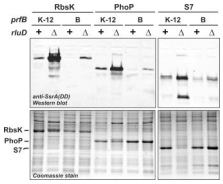

Figure 4. Tagging of other proteins is increased in Δ*rluD* cells

E. coli RbsK, PhoP and S7 proteins were overproduced from L-arabinose-inducible or IPTG-inducible plasmid systems in tmRNA(DD) cells containing the indicated alleles of *rluD* and *prfB*. Whole-cell lysates were examined by SDS-PAGE and Western blot using antibodies specific for the SsrA(DD) peptide tag. The gel migration positions of RbsK, PhoP and ribosomal protein S7 are indicated on the Coomassie stained gel.

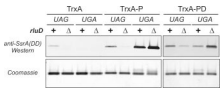

Figure 5. The nascent chain influences SsrA tagging in Δ*rluD* cells

N-terminal His_6-tagged variants of *E. coli* thioredoxin (TrxA) were overproduced in *rluD*$^+$ and Δ*rluD* cells expressing tmRNA(DD). TrxA-P contains a C-terminal Pro residue in place of Ala; and TrxA-PD contains C-terminal Pro-Asp residues in place of the wild-type Leu-Ala sequence. Each protein was expressed from messages containing UAG and UGA stop codons, and purified by Ni^{2+}-affinity chromatography for SDS-PAGE and Western blot analysis using antibodies specific for the SsrA(DD) peptide tag.

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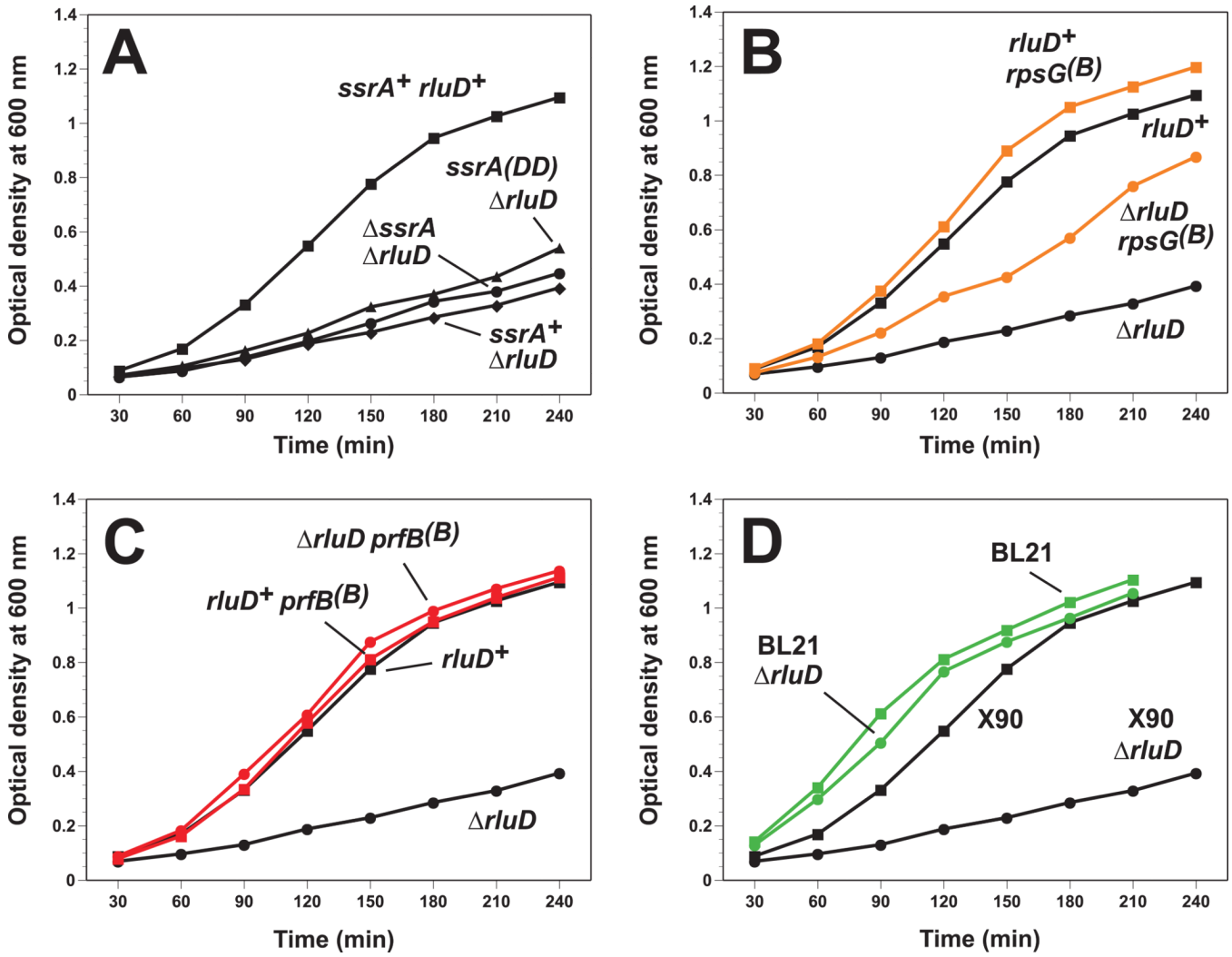

Figure 6. Effects of *rluD* deletion and *prfB* alleles on cell growth

A) Growth curves of *E. coli* strain X90 (K-12 derivative) deleted for *rluD*. The curve labeled *rluD*$^+$ was obtained from wild-type X90. The *ssrA*$^+$ allele encodes wild-type tmRNA, *ssrA(DD)* encodes tmRNA(DD), and D*ssrA* indicates cells that lack tmRNA. **B)** Growth curves of *E. coli* X90 strains (K-12 derivative) expressing *E. coli* B version of S7. Growth curves rendered in black were obtained with cells carrying the K-12 allele of *rpsG* (encoding S7), and those rendered in orange are from cells carrying the B allele of *rpsG*. **C)** Growth curves of *E. coli* X90 strains expressing the *E. coli* B version of RF2. Growth curves rendered in black are from cells carrying the K-12 allele of *prfB* (encoding RF2), and those in red are from X90 cells carrying the B allele of *prfB*. **D)** Growth curves of *E. coli* K-12 and B strains deleted for *rluD*. Curves rendered in black are from X90 cells (K-12 strain derivative) and green curves are from BL21(DE3) cells (B strain derivative).

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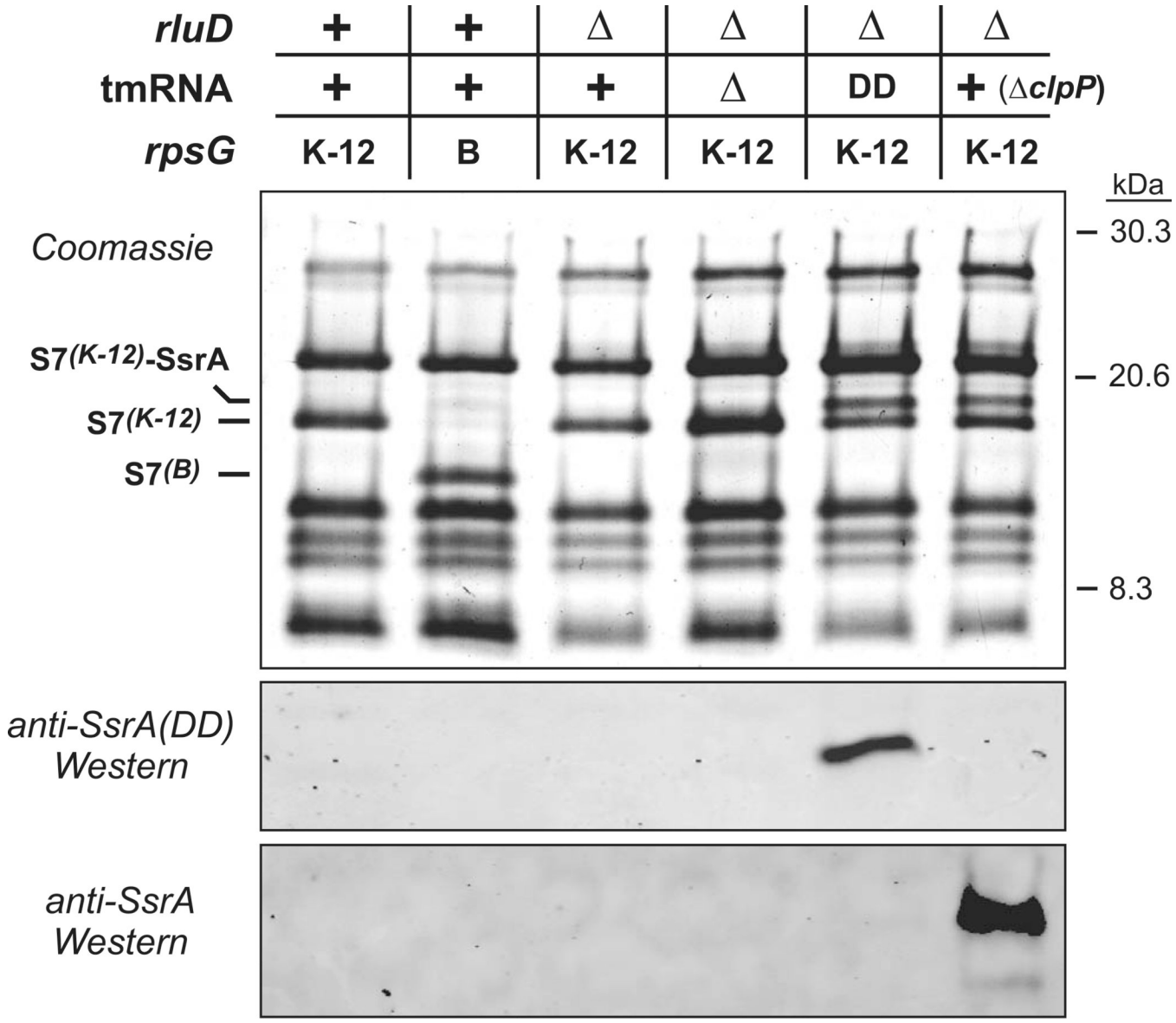

Figure 7. Incorporation of SsrA-tagged S7 into 30S ribosome subunits

30S ribosome subunits were isolated from cells of the indicated genotypes as described in the Methods. The sample in the last lane was prepared from cells deleted for the *clpP* gene. Ribosomes were analyzed by SDS-PAGE and Western blot analysis using antibodies specific for either the wild-type SsrA or SsrA(DD) peptide tags. The migration positions of ribosomal protein S7 from *E. coli* K-12 and B strains are indicated on the Coomassie stained gel. The migration positions of SsrA- and SsrA(DD)-tagged S7 from strain K-12 are also indicated.

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Table 1

Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
BL21 (DE3)	F$^-$ *omp T gal dcm lon hsdS$_B$* (r$_B^-$ m$_B^-$)	Novagen (EMD)
CH12	X90 (DE3)	(Hayes et al., 2002a)
CH113	X90 (DE3) *ssrA::cat*, Cm^r	(Hayes et al., 2002a)
CH2385	X90 (DE3) *ssrA(DD)*	(Garza-Sánchez *et al.*, 2008)
CH4374	CH2385 Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH4427	X90 *clpP::cat* Δ*rluD::kan*, Cm^r, Kan^r	This study & (Baba et al., 2006)
CH4892	CH2316 Δ*slyD* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH6960	BL21(DE3) Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH6981	CH12 *prfB(K-12)-kan*, Kan^r	This study
CH6983	CH2385 *prfB(K-12)::kan*, Kan^r	This study & (Garza-Sánchez et al., 2008)
CH6984	CH12 *rpsG(B) prfB(K-12)-kan*, Kan^r	This study
CH6987	CH12 *prfB(B)-kan*, Kan^r	This study
CH6989	CH2385 *prfB(B)-kan*, Kan^r	This study
CH6995	CH2385 *prfB(K-12)*	This study
CH7001	CH2385 *prfB(B)*	This study
CH7005	CH12 *prfB(K-12)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7006	CH113 *prfB(K-12)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7007	CH2385 *prfB(K-12)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7008	CH12 *rpsG(B) prfB(K-12)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7011	CH12 *prfB(B)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7013	CH2385 *prfB(B)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7014	CH12 *rpsG(B) prfB(B)* Δ*rluD::kan*, Kan^r	This study & (Baba et al., 2006)
CH7017	CH2385 *prfB*(*K-12*) Δ*rluD* Δ*lacI::kan*, Kan^r	This study & (Baba et al., 2006)
CH7018	CH2385 *rpsG(B) prfB(K-12)* Δ*rluD* Δ*lacI::kan*, Kan^r	This study & (Baba et al., 2006)
CH7019	CH2385 *rpsG(B) prfB(B)* Δ*rluD* Δ*lacI::kan*, Kan^r	This study & (Baba et al., 2006)
CH7020	CH2385 *prfB(B)* Δ*rluD* Δ*lacI::kan*, Kan^r	This study & (Baba et al., 2006)
Plasmids		
pHis$_6$-TrxA(UAG)	His$_6$-TrxA expression construct with TAG stop codon, Amp^r	This study
pHis$_6$-TrxA(UGA)	His$_6$-TrxA expression construct with TGA stop codon, Amp^r	This study
pHis$_6$-TrxA-P(UAG)	His$_6$-TrxA(A109P) expression construct with TAG stop codon, Amp^r	This study
pHis$_6$-TrxA-P(UGA)	His$_6$-TrxA(A109P) expression construct with TGA stop codon, Amp^r	This study
pHis$_6$-TrxA-PD(UAG)	His$_6$-TrxA(L108P, A109D) expression construct with TAG stop codon, Amp^r	This study
pHis$_6$-TrxA-PD(UGA)	His$_6$-TrxA(L108P, A109D) expression construct with TGA stop codon, Amp^r	This study
pHis$_6$-S7(K-12)	His$_6$-S7 (from *E. coli* X90) expression construct, Amp^r	This study
pHis$_6$-S7(K-12/UAG)	His$_6$-S7 expression construct with TGA stop changed to TAG, Amp^r	This study

Strain or plasmid	Description	Reference or source
pHis$_6$-S7(B)	His$_6$-S7 [from *E. coli* BL21(DE3)] expression construct, Amp^r	This study
pET21b::*rpsG*	K-12 S7 expression construct, Amp^r	This study
pCH450::*rpsG(B)*	pACYC184 derivative expressing K-12 S7 under *araBAD* promoter, Tet^r	This study
pCH450::*phoP*	pACYC184 derivative expressing PhoP under *araBAD* promoter, Tet^r	This study
pRbsK(1–6)	pTrc99a derivative expressing RbsK from construct with Arg307 and Arg309 codons synonymously recoded as CGT, Amp^r	(Hayes et al., 2002b)
pKO3	temperature sensitive allelic exchange plasmid containing *sacB repA*(ts), Cm^r	(Link et al., 1997)
pKF280-(BL21)	pKO3 containing 5′ portion of *str* operon including promoter *rpsL, rpsG* and *fusA′*, Cm^r	This study & (Devaraj et al., 2009)
pTrc2	pTrc99A derivative containing *lacI^q* with wild-type TGA stop codon, Amp^r	(Holberger & Hayes, 2009)
pTrc2(UAG)	pTrc99A derivative carrying *lacI^q* with TAG stop codon, Amp^r	This study

Abbreviations: Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant; Tet^r, tetracycline resistant

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