The i^{6}A37 tRNA modification is essential for proper decoding of UUX-Leucine codons during *rpoS* and *iraP* translation

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ABSTRACT

The translation of *rpoS* (σ^S), the general stress/stationary phase sigma factor, is tightly regulated at the post-transcriptional level by several factors via mechanisms that are not clearly defined. One of these factors is MiaA, the enzyme necessary for the first step in the N^6-isopentyl-2-thiomethyladenosinemethyladenosine 37 (ms^{2}i^{6}A37) tRNA modification. We tested the hypothesis that an elevated UUX-Leucine/total leucine codon ratio can be used to identify transcripts whose translation would be sensitive to loss of the MiaA-dependent modification. We identified *iraP* as another candidate MiaA-sensitive gene, based on the UUX-Leucine/total leucine codon ratio. An *iraP-lacZ* fusion was significantly decreased in the absence of MiaA, consistent with our predictive model. To determine the role of MiaA in UUX-Leucine decoding in *rpoS* and *iraP*, we measured β-galactosidase-specific activity of *miaA*$^-$ *rpoS* and *iraP* translational fusions upon overexpression of leucine tRNAs. We observed suppression of the MiaA effect on *rpoS*, and not *iraP*, via overexpression of tRNA^{LeuX} but not tRNA^{LeuZ}. We also tested the hypothesis that the MiaA requirement for *rpoS* and *iraP* translation is due to decoding of UUX-Leucine codons within the *rpoS* and *iraP* transcripts, respectively. We observed a partial suppression of the MiaA requirement for *rpoS* and *iraP* translational fusions containing one or both UUX-Leucine codons removed. Taken together, this suggests that MiaA is necessary for *rpoS* and *iraP* translation through proper decoding of UUX-Leucine codons and that *rpoS* and *iraP* mRNAs are both modification tunable transcripts (MoTTs) via the presence of the modification.

Keywords: tRNA modification; translation; MiaA; leu codon; RpoS

INTRODUCTION

Transfer RNA (tRNA) modifications play a critical role in the promotion of translation fidelity (Urbonavicius et al. 2001). The absence of tRNA modifications is known to increase the frequency of translational frameshifting (Björk et al. 1999; Urbonavicius et al. 2001, 2003). There are a host of tRNA modifications that are most crucial in translational frameshift suppression and the majority of them reside within the anticodon stem–loop (ASL), flanking the anticodon at positions 34 and 37, including 5-methyluridine (m^{5}U34), 5-methylcytidine (m^{5}C34), and N^6-isopentenyladenosine (i^{6}A37) (Endres et al. 2015). Certain tRNA modifications act in a regulatory manner on physiological circuits within the cell, such as DNA damage and oxidative stress in eukaryotic cells (Begley et al. 2007; Chan et al. 2010, 2012; Patil et al. 2012; Lamichhane et al. 2013a). There is limited information on the regulatory role of tRNA modifications in bacterial cells. We previously identified an important role for the *Escherichia coli* ms^{2}i^{6}A37 synthesis enzyme, MiaA, in the translation of *rpoS* mRNA (Thompson and Gottesman 2014).

RpoS (or σ^S) is the stationary phase/general stress response alternative sigma factor necessary for the adaptation of bacterial cells to the stationary phase environment (Hengge-Aronis 1993, 1996). RpoS contributes to stationary phase homeostasis by initiating the transcription of a large subset of genes that respond to limiting nutrients and increased exposure to reactive oxygen intermediates (Hengge-Aronis 1993; Tanaka et al. 1993). Since RpoS levels are important for the stationary phase stress response, RpoS levels are tightly regulated at the transcriptional level and at post-transcriptional levels (Lange et al. 1995; Brown and Elliot 1996; Hirsch and Elliott 2002). RpoS translation is stimulated by the three Hfq-dependent small regulatory RNAs: DsrA, RprA, and ArcZ in response to different environmental signals (Sledjeski et al. 1996; Majdalani et al. 1998, 2002; Mandin and Gottesman 2010). RpoS is also regulated at the level of protein stability by the ATP-dependent protease ClpXP,

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with the assistance of RssB, which serves as an adaptor protein (Gottesman 1996; Zhou and Gottesman 1998). There are three anti-adaptor proteins, IraP, IraD, and IraM, which all prevent RssB interaction with RpoS under different conditions and increase RpoS stability as a consequence (Bougdour et al. 2006, 2008).

In addition to stimulation of translational initiation by several small regulatory RNAs and regulation of RpoS stability, translation of the *rpoS* open reading frame (ORF) is also regulated by *cis* and *trans* acting elements. For example, SsrA is necessary for *rpoS* translation through a mechanism that is not completely understood (Ranquet and Gottesman 2007). The presence of rare codons within the *rpoS* ORF also positively contributes to mRNA stability by decreasing degradation by RNaseE (Kolmsee and Hengge 2011). Additionally, AceE, a subunit of pyruvate dehydrogenase, also influences *rpoS* ORF translation via an undefined mechanism (Battesti et al. 2015). Finally, the MiaA tRNA modification enzyme is required for efficient translation of the *rpoS* ORF translation (Thompson and Gottesman 2014).

MiaA is required for the first step in the formation of ms^2i^6A37 modification on tRNAs that read UUX codons (Bartz et al. 1970). Specifically, MiaA catalyzes the addition of the isopentyl group (i^6) to A37 of these tRNAs (Bartz et al. 1970). MiaB then catalyzes the addition of a methylthio group (ms^2) to complete the ms^2i^6A37 tRNA modification (Vold et al. 1979; Esberg et al. 1999). While MiaB is necessary for completing the ms^2i^6A37 tRNA modification, our initial analysis of the effect of tRNA modifications on *rpoS* suggested that MiaB was not necessary for full *rpoS* expression (Thompson and Gottesman 2014). Therefore, this work is focused on characterizing the MiaA effect on *rpoS*. MiaA is necessary for expression of genes involved in the biosynthesis of amino acids in *Escherichia coli* and *Salmonella enterica* subspecies *enterica* servar Typhimurium, including tryptophan and phenylalanine in *E. coli* as well as leucine in *S. typhimurium* (Gowrishankar and Pittard 1982; Blum 1988). The MiaA requirement for expression of the tryptophan and phenylalanine operons is due to its role in modulating transcriptional attenuation (Landick et al. 1990; Pages and Buckingham 1990). We hypothesized that leucine codon usage may play a role in the requirement for MiaA in the efficient translation of *rpoS*, due to use of UUX-Leucine rather than CXX-Leucine codons within the *rpoS* ORF (Thompson and Gottesman 2014).

Here, we test this hypothesis, both extending our previous tests with *rpoS* to another UUX-rich gene, *iraP*. We demonstrate the ability to identify putative ms^2i^6A37 modification tunable transcripts (MoTTs), via MiaA sensitivity, for a given gene based on UUX-Leucine codon usage for that gene (Endres et al. 2015). We hypothesize that genes for ORFs with high UUX Leucine codon usage, or HULC proteins, defined as having a UUX-Leucine codon usage ratio >0.22, likely have i^6A37 MoTTs. We show that *iraP* has a similar enrichment ratio for UUX-Leucine codons as *rpoS* and that its translation requires the i^6A37 tRNA modification catalyzed by MiaA. Furthermore, we demonstrate that UUX-leu to CUX-leu codon substitutions within *rpoS* and *iraP* can suppress the effects of Δ*miaA* mutants on the translation of these ORFs, suggesting that MiaA is required for *rpoS* and *iraP* translation, at least in part, for efficient decoding of UUX-Leucine codons in these two genes.

RESULTS

Replacing MiaA-sensitive leucine codons (UUGs and UUAs) with MiaA-insensitive leucine codons (CUUs and CUCs) partially suppresses the MiaA requirement for *rpoS* synthesis

We previously used an arabinose-inducible *rpoS990-lacZ* translational fusion to demonstrate the role of the MiaA-catalyzed i^6A modification on *rpoS* synthesis (Thompson and Gottesman 2014). The P_{BAD}-*rpoS990-lacZ* translation fusion contains the entire *rpoS* ORF, except for the termination codon; the small-RNA responsive 5′ UTR of *rpoS* and the native promoter are replaced by the *araBAD* promoter (P_{BAD}) (Fig. 1B; Supplemental Table S1; Thompson and Gottesman 2014). All P_{BAD}-*rpoS990-lacZ* translation fusion experiments were executed in an *rssB*$^-$ background to rule out effects on *rpoS* degradation.

We previously hypothesized that the greater than expected ratio of MiaA-sensitive leucine codon to total leucine codon usage within the *rpoS* ORF suggested that MiaA may function to ensure proper decoding of these UUX-Leucine codons within *rpoS* (Thompson and Gottesman 2014). To test this hypothesis, we constructed a P_{BAD}-*rpoS990-lacZ* translational fusion in which the first and wobble position of all MiaA-sensitive leucine codons (UUAs and UUGs) in *rpoS* were changed to create MiaA-insensitive leucine codons (CUUs and CUCs), creating a series of silent leucine mutations throughout the *rpoS990* region of the translational fusion (Table 1, *leu*3*; Fig. 1B). The rationale behind the selection of CUU and CUC codons is that they are present at frequencies similar to UUA and UUG codons (http://www.sci.sdsu.edu/~smaloy/MicrobialGenetics/topics/in-vitro-genetics/codon-usage.html). There are six codons that code for leucine, UUR, and CUX. The average frequency of decoding for leucine by CUU and CUC codons are 0.10, which is similar to the 0.11 frequency seen with UUA and UUG codons. In contrast, CUA and CUG Leu codons have average frequencies of 0.03 and 0.55, respectively.

We then measured the β-galactosidase-specific activity (as defined in Materials and Methods) as the slope of OD_{420}/OD600 of the wild-type and *leu*3* fusions in *rssB*$^-$ *miaA*$^+$ and *rssB*$^-$ *miaA*$^-$ genetic backgrounds (Fig. 1A). There was an exponential increase in the β-galactosidase-specific activity of both the wild-type P_{BAD}-*rpoS990-lacZ* and P_{BAD}-*rpoS990*(*leu*3*)-*lacZ* translational fusions in the presence of MiaA (Fig. 1A). As previously reported, the absence of

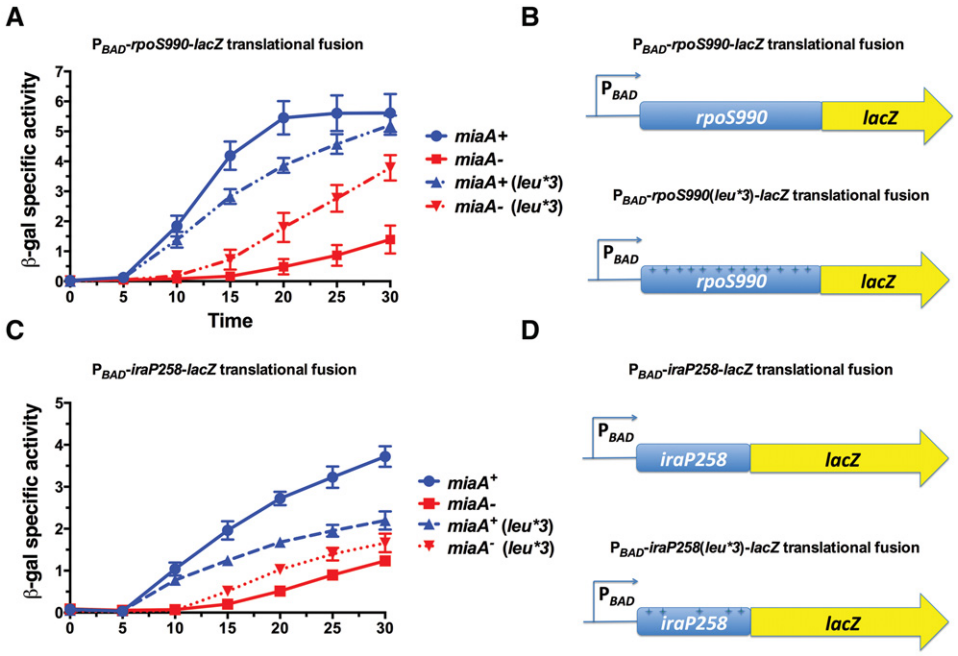

FIGURE 1. Translation of *leu*3* alleles of *rpoS* and *iraP* in the absence of ms^{2}i^{6}A37 tRNA modification. (*A*) The *rssB*$^-$ *miaA*$^+$ (KMT33001) and *rssB*$^-$ *miaA*$^-$ (KMT33002) P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion strains, as well as *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P$_{BAD}$-*rpoS990-lacZ* translational fusion strains, were assayed for β-galactosidase-specific activity following arabinose induction. β-galactosidase-specific activity is defined as the slope of OD_{420} of the collected sample cell lysate divided by the OD_{600} of the collected sample of the culture, as described in Materials and Methods. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM). (*B*) Schematic comparison of the P$_{BAD}$-*rpoS990-lacZ* and P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusions used in this experiment. The stars within the P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion represent silent leucine mutations where rare leucine codons (UUA and UUG) were changed to abundant leucine codons (CUU or CUC). (*C*) The *miaA*$^+$ (KMT45000) and *miaA*$^-$ (KMT45002) P$_{BAD}$-*iraP258*(*leu*3*)-*lacZ* translational fusion strains, as well as *miaA*$^+$ (KMT42000) and *miaA*$^-$ (KMT42002) P$_{BAD}$-*iraP258-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM). (*D*) Schematic comparison of the P$_{BAD}$-*iraP258-lacZ* and P$_{BAD}$-*iraP258*(*leu*3*)-*lacZ* translational fusions used in this experiment. The green stars within the P$_{BAD}$-*iraP258*(*leu*3*)-*lacZ* translational fusion represent silent leucine mutations where rare leucine codons (UUA and UUG) were changed to abundant leucine codons (CUU or CUC). Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM).

MiaA resulted in severely defective induction of the P$_{BAD}$-*rpoS990-lacZ* in comparison to the *miaA*$^+$ strain (Fig. 1A). Specifically, 25 min after arabinose induction (t_{25}), there was a fivefold decrease in P$_{BAD}$-*rpoS990-lacZ* expression in the absence of MiaA (Fig. 1A). The induction of the P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion was not as defective in the absence of MiaA in comparison to the wild type (1.5-fold decrease in P$_{BAD}$-*rpoS990*[*leu*3*]-*lacZ*) expression in the absence of MiaA at t_{25} (compared to fivefold in the wild-type fusion). This suggests that the presence of UUX-Leucine codons, which are sensitive to MiaA-modified leucine tRNAs, contribute to the requirement of MiaA for full *rpoS* synthesis. However, the fact that the removal of the UUX-Leucine codons did not result in full suppression of the MiaA requirement for *rpoS* synthesis suggests that MiaA does more for *rpoS* translation than just ensuring that UUX-Leucine tRNAs properly recognize their cognate codons. MiaA-sensitive codons also consist of UUX phenylalanine, UCX serine, UAX tyrosine, UGX cysteine, and UGG tryptophan. It is possible that these codons may not be sufficiently decoded in the absence of MiaA as well. Also, the expression from the *miaA*$^+$ P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion was slightly decreased in comparison to the *miaA*$^+$ P$_{BAD}$-*rpoS990-lacZ*, suggesting that the UUX-Leucine codons are required for optimal *rpoS* translation in the presence of MiaA.

TABLE 1. Silent leucine mutations in the *rpoS leu** alleles

Amino acid	Original codon	Leu* recoded codon
Leucine 11	UUA	CUU
Leucine 30	UUA	CUU
Leucine 40	UUG	CUC
Leucine 46	UUA	CUU
Leucine 55	UUG	CUC
Leucine 71	UUA	CUC
Leucine 99	UUG	CUC
Leucine 116	UUG	CUC
Leucine 167	UUG	CUC
Leucine 185	UUG	CUC
Leucine 234	UUG	CUC
Leucine 280	UUG	CUC
Leucine 313	UUG	CUC

The translation of *iraP* requires the ms^{2}i^{6}A37 tRNA modification for decoding of MiaA-sensitive leucine codons

To determine if the ms^{2}i^{6}A37 modification was required for optimal translation of other HULC proteins, we examined the leucine codon usage of several arbitrarily selected genes related to *rpoS* regulation. In addition, we acquired a table documenting the leucine codon usage of every ORF in the *E. coli* genome (Supplemental Table S7). We decided to test our hypothesis on *iraP*, which encodes an anti-adaptor that plays a role in *rpoS* stability (Bougdour et al. 2006). *iraP* is a HULC protein, with a UUX-Leucine codon usage ratio of 0.46, which is twofold greater than the expected UUX-Leucine codon ratio and higher than the UUX-Leucine codon usage ratio for *rpoS* of 0.29 (Table 3). The secondary rationales for this selection were the size of *iraP* and its involvement in the *rpoS* regulatory circuitry. The very short ORF of *iraP*, 258 nucleotides, was ideal for rapid and efficient leu codon engineering. Finally, we reasoned that linking another HULC protein with ms^{2}i^{6}A37 sensitivity would increase the possibility of identifying a physiological relationship between ms^{2}i^{6}A37 and *rpoS* expression.

We constructed an arabinose-inducible *iraP258-lacZ* translational fusion and a parallel version in which the six UUG and UUA *leu* codons were changed to CUU codons [P_{BAD}-*iraP258*(*leu*3*)-*lacZ*]. The P_{BAD}-*iraP258-lacZ* translation fusions contain the entire *iraP* ORF except for the stop codon (Fig. 1D). At 15 min, following arabinose induction, the *miaA*$^+$/*miaA*$^-$ ratio for the P_{BAD}-*iraP258-lacZ* fusions is 15:1, and the ratio decreases with time but is still two- to threefold decreased after 30 min (Fig. 1C; Supplemental Table S5). This suggests that MiaA (i^{6}A37 tRNA modification) is necessary for efficient *iraP* translation. In contrast, the P_{BAD}-*iraP258*(*leu*3*)-*lacZ* was only slightly affected by the absence of MiaA (Fig. 1C). The similar β-galactosidase-specific activity of the *miaA*$^+$ and *miaA*$^-$ P_{BAD}-*iraP258*(*leu*3*)-*lacZ* translational fusions suggests that *iraP* UUX leu to CXX leu codon mutations suppress the MiaA (i^{6}A37) requirement for *iraP* translation (Fig. 1C; Supplemental Table S5). Overall these results suggest that UUX leu codons are needed for optimal translation of *iraP* and changing UUX leu to CUX leu codon mutations decreases the efficiency of *iraP* translation in the wild-type (*miaA*$^+$) strain.

Overexpression of *leuX* ($tRNA^{LeuX}_{CAA}$) suppresses the MiaA requirement for *rpoS*, but not for *rpoS* (*leu*3*) or *iraP* translation

We hypothesized that overexpression of leucine tRNAs may suppress the effect of the absence of the i^{6}A37 tRNA modification on *rpoS* translation. If the tRNAs that read UUX leu codons are limiting, leading to the need for the modification for more efficient use, overexpression of these leucine tRNAs may suppress the MiaA requirement for *rpoS* translation. To test this hypothesis, we measured induction of P_{BAD}-*rpoS990-lacZ*, in the absence of MiaA, but with overexpression of tRNAs that recognize UUX-Leu codons (Fig. 2). Plasmids carrying either *leuX* (CAA anticodon) or *leuZ* (UAA anticodon), under control of an IPTG-inducible promoter, were transformed into *rssB*$^-$ *miaA*$^+$ and *rssB*$^-$ *miaA*$^-$ P_{BAD}-*rpoS990-lacZ* translational fusion strains. The activities of these strains were measured at 5-min intervals after addition of arabinose (Fig. 2A,C), or arabinose and IPTG (to induce the tRNAs) (Fig. 2B,D). The *leuX*-encoding plasmid had very little effect on a wild-type (*miaA*$^+$) strain, with or without IPTG induction (Fig. 2A,B). However, the *leuX*-encoding plasmid

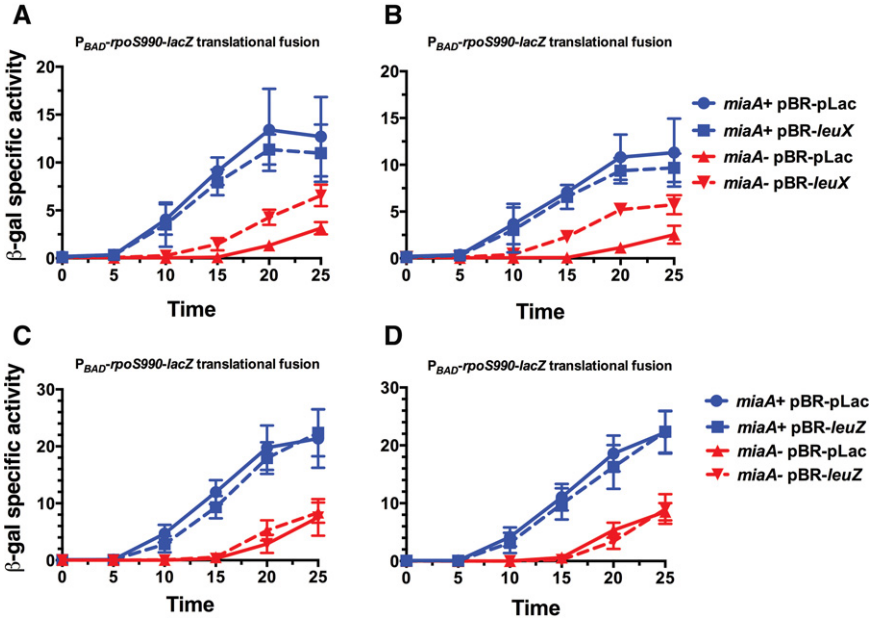

FIGURE 2. The effect of $tRNA^{leuX}$ and $tRNA^{leuZ}$ expression on wild-type and *miaA* P_{BAD}-*rpoS990-lacZ* translational fusion activity. The *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P_{BAD}-*rpoS990-lacZ* translational fusion strains containing plasmids pBR-pLac (KMT30029 and KMT30035, respectively) or pBR-pLac-*leuX* (KMT30030 and KMT30036) were assayed for β-galactosidase-specific activity following arabinose induction without (*A*) or with (*B*) IPTG. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM). The *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P_{BAD}-*rpoS990-lacZ* translational fusion strains containing plasmids pBR-pLac (KMT30029 and KMT30035, respectively) or pBR-pLac-*leuZ* (KMT30031 and KMT30037) were assayed for β-galactosidase-specific activity following arabinose induction without (*C*) or with (*D*) IPTG. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM).

significantly increased expression of the fusion in the *miaA* mutant strain (Fig. 2A,B). The increase in expression in the absence of IPTG suggests that there is leaky expression of the tRNA$^{LeuX}_{CAA}$. No increase in the β-galactosidase-specific activity of the *miaA*$^-$ P$_{BAD}$-*rpoS990-lacZ* translational fusion was observed in a parallel experiment in which *leuZ*, encoding tRNA$^{LeuZ}_{UAA}$, was overexpressed (Fig. 2C,D). Taken together, this suggests that overexpression of tRNA$^{LeuX}_{CAA}$, but not tRNA$^{LeuZ}_{UAA}$, can partially suppress the MiaA requirement for *rpoS* translation.

We hypothesized that LeuX suppression of the ms^{2}i^{6}A37 requirement for *rpoS* translation would not be observed when the MiaA-sensitive leucine codons were removed. To test this hypothesis, we transformed pBR-pLac and pBR-pLac-*leuX* into *rssB*$^-$ *miaA*$^+$ and *rssB*$^-$ *miaA*$^-$ genetic backgrounds of this P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion and measured the β-galactosidase-specific activity of the resulting strains after arabinose induction (Fig. 3A). We observed no effect of tRNA$^{LeuX}_{CAA}$ expression on the β-galactosidase-specific activity of the fusion in the presence or absence of MiaA. This suggests that the tRNA$^{LeuX}_{CAA}$ suppression of the MiaA requirement for *rpoS* translation is through the decoding of UUA's and UUG's leucine codons in the *rpoS* ORF.

We carried out similar experiments with the P$_{BAD}$-*iraP258-lacZ* translational fusions (Fig. 3B,C). Neither tRNA$^{LeuX}_{CAA}$ nor tRNA$^{LeuZ}_{UAA}$ had a significant effect on the β-galactosidase-specific activity of the P$_{BAD}$-*iraP258*-lacZ translational fusion in the absence of MiaA (Fig. 3B,C). This suggests that tRNA$^{LeuX}_{CAA}$ is not or is only a minimally effective suppressor of the MiaA requirement for *iraP* translation. Possible explanations for the difference between *rpoS* and *iraP* include the higher number of UUX leu codons in *rpoS* (13) compared to *iraP* (six) or the existence of tandem UXX-leu codons in *iraP* versus the absence of such in *rpoS* (Tables 1, 2).

Mutation of UUA-Leu to CUU-Leu or CUC-Leu codons suppress the MiaA requirement for efficient *rpoS* translation

To further characterize the role of the ms^{2}i^{6}A37 tRNA modification in the decoding of Leu codons in *rpoS* and *iraP*, we constructed translational *lacZ* fusions to alleles of *rpoS* and *iraP* where either UUA- or UUG-Leucine codons were removed and replaced with either a CUC or CUU leucine codon. We designate the UUA-Leu to CUU-Leu or CUC-Leu *rpoS* alleles as *leu*1* alleles. We designate the UUG-Leu to CUC-Leu *rpoS* alleles as *leu*2* alleles. The precise amino acid number and mutation are listed in Table 1. We then tested the β-galactosidase-specific activity of these fusions as described in the Materials and Methods section. We also repeated the assay in Figure 1A, with wild-type and *leu*3 rpoS* alleles, as a control for the *leu*1* and *leu*2 rpoS* alleles (Fig. 4A). In the presence of MiaA, the *rpoS leu*1* allele has a modest decrease in β-galactosidase-specific activity between 10 and 25 min following induction (Fig. 4B). The *miaA*$^+$ ratio of wild type to *leu*1* is increased by 1.2 to 1.5-fold at or beyond 15 min of induction (Supplemental Table S4). This suggests that the UUA-Leu to CXX-Leu silent codon mutations slightly decrease efficiency of *rpoS* translation. In the absence of MiaA, at or beyond 15 min of induction, the β-galactosidase-specific activity of the P$_{BAD}$-*rpoS990*(*leu*1*)-*lacZ* allele is at least twofold greater than the P$_{BAD}$-*rpoS990-lacZ* allele. The activities of the *miaA*$^+$ and *miaA*$^-$ P$_{BAD}$-*rpoS990*(*leu*1*)-*lacZ* translational fusion are relatively similar in comparison to the *miaA*$^+$ and *miaA*$^-$ P$_{BAD}$-*rpoS990-lacZ* translational fusion

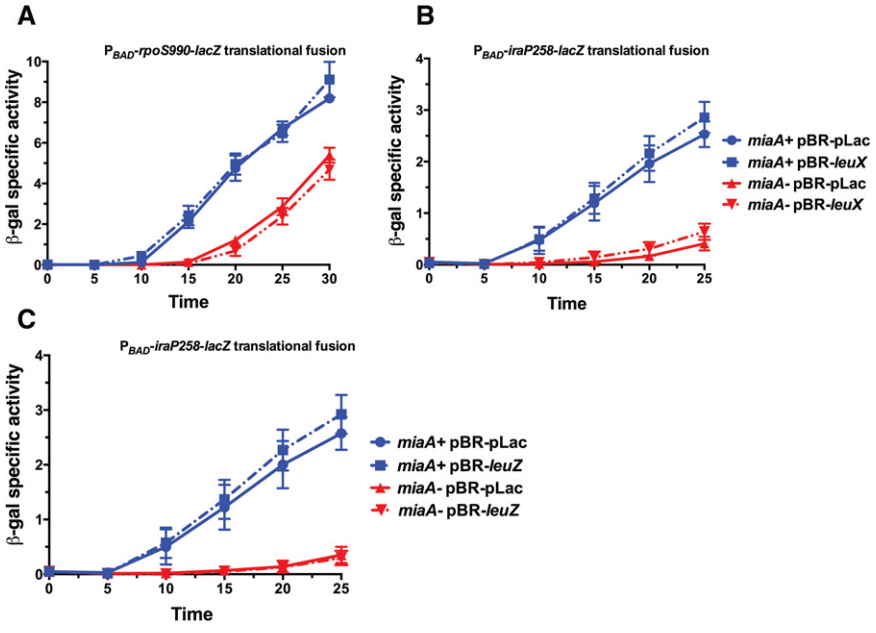

FIGURE 3. The tRNA^{leuX} suppression assay for the *miaA* effect on *rpoS* (*leu*3*) and *iraP*. (*A*) The *rssB*$^-$ *miaA*$^+$ (KMT33001) and *rssB*$^-$ *miaA*$^-$ (KMT33002) P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* translational fusion strains containing plasmids pBR-pLac (KMT30029 and KMT30035, respectively) or pBR-pLac-*leuX* (KMT30030 and KMT30036) were assayed for β-galactosidase-specific activity following arabinose and IPTG induction. (*B*) The *miaA*$^+$ (KMT45000) and *miaA*$^-$ (KMT45002) P$_{BAD}$-*iraP258-lacZ* translational fusion strains containing plasmids pBR-pLac (KMT45003 and KMT45006, respectively) or pBR-pLac-*leuX* (KMT45004 and KMT45007) were assayed for β-galactosidase-specific activity following arabinose and IPTG induction. (*C*) The *miaA*$^+$ (KMT45000) and *miaA*$^-$ (KMT45002) P$_{BAD}$-*iraP258-lacZ* translational fusion strains containing plasmids pBR-pLac (KMT45003 and KMT45006, respectively) or pBR-pLac-*leuZ* (KMT45005 and KMT45008) were assayed for β-galactosidase-specific activity following arabinose and IPTG induction. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM).

TABLE 2. Silent leucine mutations in the *iraP leu** alleles

Amino acid	Original codon	Leu* recoded codon
Leucine 8	UUG	CUU
Leucine 9	UUA	CUU
Leucine 29	UUG	CUU
Leucine 48	UUG	CUU
Leucine 80	UUA	CUU
Leucine 81	UUG	CUU

(Fig. 4B). The *miaA*$^+$/*miaA*$^-$ ratio of the P_{BAD}-*rpoS990* (*leu***1*)-*lacZ* and P_{BAD}-*rpoS990-lacZ* translational fusions, 15 min following induction, are 1.8 and 23, respectively. This ratio is much closer to 1.0-fold increase in the *leu***1* fusion as opposed to the wild-type fusion (Supplemental Table S3). This suggests that MiaA and the ms^2i^6A37 tRNA modification are necessary for efficient translation of UUA-Leucine codons within the *rpoS* ORF.

UUG-Leu to CXX-Leu codon mutations decrease *rpoS* translational efficiency

In the presence of MiaA, the *leu***2* alleles are severely defective for translational β-galactosidase-specific activity, decreased by approximately fourfold starting at 10 min after induction (Fig. 4C; Supplemental Table S4). This suggests that, independent of MiaA and the ms^2i^6A37 tRNA modification, the presence of UUG-Leucine codons is critical for efficient translation of the *rpoS* ORF. Furthermore, the β-galactosidase-specific activity of the *miaA*$^+$ P_{BAD}-*rpoS990* (*leu***2*)-*lacZ* translational fusion is nearly identical to the *miaA*$^-$ P_{BAD}-*rpoS990*(*leu***2*)-*lacZ* translational fusion (Fig. 4; Supplemental Table S3). However, the severely compromised *rpoS* translation seen in the absence of UUG possibly makes this explanation too simple. Interestingly, there is no statistically significant difference, at the 30-min time point following induction, between *miaA*$^+$ and *miaA*$^-$ activities of the *leu***1* and *leu***2* alleles of the P_{BAD}-*rpoS990-lacZ* translational fusion (Fig. 4D). In contrast, there is a statistically significant difference, at the 30-min time point following induction, between *miaA*$^+$ and *miaA*$^-$ activities of the wild-type and *leu***3* alleles of the P_{BAD}-*rpoS990-lacZ* translational fusion (Fig. 4D). The removal of UUA-Leu only or UUG-Leu codons only, as opposed to the removal of both, results in stronger suppression of the MiaA requirement during *rpoS* expression. The reason for this is unclear.

UUA-Leu to CUU-Leu or UUG-Leu to CUU-Leu codon mutations modulate *iraP* translational efficiency and partially suppress the MiaA requirement during *iraP* translation

While it is clear from data in Figure 1C that removal of both UUA-Leucine and UUG leu codons within *iraP* suppresses the MiaA effect during *iraP* translation, we sought to further characterize and define the relative contributions of UUA leu and UUG leu to the MiaA requirement for *iraP* translation just as we did for *rpoS* translation. We created two additional alleles of the P_{BAD}-*iraP258-lacZ* translational fusion, P_{BAD}-*iraP258*(*leu***1*)-*lacZ* and P_{BAD}-*iraP258*(*leu***2*)-*lacZ*. P_{BAD}-*iraP258*(*leu***1*)-*lacZ* consists of UUA-Leucine to CUU-Leucine codon changes alone (Table 2). P_{BAD}-*iraP258* (*leu***2*)-*lacZ* consists of UUG-Leucine to CUU-Leucine codon changes alone (Table 2). We measured the β-galactosidase-specific activity of these fusions as described in the Experimental Design section of Materials and Methods. Upon comparison of the *miaA*$^+$ P_{BAD}-*iraP258*(*leu***1*)-*lacZ* and *miaA*$^+$ P_{BAD}-*iraP258-lacZ* translational fusion β-galactosidase-specific activity after induction, it is clear that the *leu***1* mutation increases *iraP* translational efficiency (Fig. 5B), most evident at 15–30 min following induction (Fig. 5B). The *miaA*$^+$ P_{BAD}-*iraP258-lacZ*/P_{BAD}-*iraP258*(*leu***1*)-*lacZ* ratio is between −1.6 to −1.7 during this time period, demonstrating an increase in *iraP* translational efficiency in the absence of UUA-Leucine codons (Supplemental Table S6). The β-galactosidase-specific activity of the *miaA*$^-$ P_{BAD}-*iraP258*(*leu***1*)-*lacZ* fusion is 2.1-fold less than the *miaA*$^+$ P_{BAD}-*iraP258*(*leu***1*)-*lacZ*, after 30 min of induction (Fig. 5B; Supplemental Table S5). This is slightly smaller than the 2.8-fold decrease seen after 30 min (Supplemental Table S5). The *miaA*$^+$/*miaA*$^-$ β-galactosidase-specific activity ratio, 15 min after induction, was 1.8 in the *leu***1* allele versus 15 in the wild-type (non *leu**) allele of the P_{BAD}-*iraP258-lacZ* translational fusion (Supplemental Table S5). This suggests that the UUA-Leucine to CUU-Leucine codon changes lead to partial suppression of the MiaA requirement for *iraP* translation.

We measured the β-galactosidase-specific activity of the P_{BAD}-*iraP258*(*leu***2*)-*lacZ* translational fusion after induction, in the presence and absence of MiaA to determine the relative contribution of UUG-Leucine codons to *iraP* translation (Fig. 5; Supplemental Table S6). UUG-Leucine to CUU-

TABLE 3. Leucine codon usage in *rpoS* and *iraP*

		Actual ratio	
Leucine codon	Expected ratio	*rpoS*	*iraP*
UUA	0.11	0.09[a]	0.15[b]
UUG	0.11	0.20[a]	0.31[b]
CUU	0.10	0.11	0.15
CUC	0.10	0.02	0.08
CUA	0.03	0.00	0.00
CUG	0.55	0.57	0.31
Total	1	1	1

[a]The actual ratio of UUX Leu codons in the *rpoS* ORF is 0.29 versus an expected UUX codon usage of 0.22.
[b]The actual ratio of UUX Leu codons in the *iraP* ORF is 0.46 versus an expected UUX codon usage of 0.22.

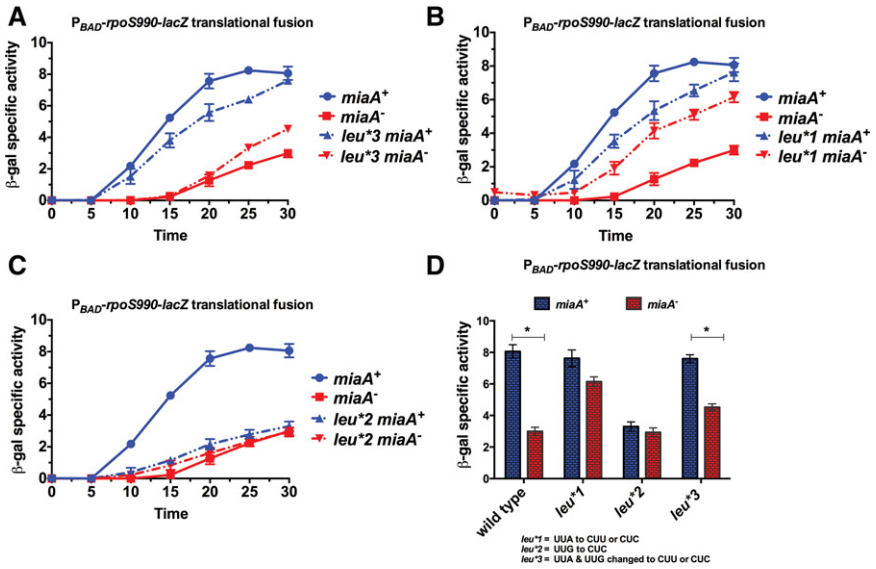

FIGURE 4. Translation of *rpoS* with UUG- or UUA-Leucine codons replaced with CXX-Leucine codons. (*A*) The *rssB*$^-$ *miaA*$^+$ (KMT33001) and *rssB*$^-$ *miaA*$^-$ (KMT33002) P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* as well as *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P$_{BAD}$-*rpoS990-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*B*) The *rssB*$^-$ *miaA*$^+$ (KMT) and *rssB*$^-$ *miaA*$^-$ (KMT) P$_{BAD}$-*rpoS990*(*leu*2*)-*lacZ* as well as *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P$_{BAD}$-*rpoS990-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*C*) The *rssB*$^-$ *miaA*$^+$ (KMT37002) and *rssB*$^-$ *miaA*$^-$ (KMT37003) P$_{BAD}$-*rpoS990*(*leu*2*)-*lacZ* as well as *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P$_{BAD}$-*rpoS990-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*D*) The *rssB*$^-$ *miaA*$^+$ (KMT36002) and *rssB*$^-$ *miaA*$^-$ (KMT36003) P$_{BAD}$-*rpoS990* (*leu*1*)-*lacZ*, *rssB*$^-$ *miaA*$^+$ (KMT37002) and *rssB*$^-$ *miaA*$^-$ (KMT37003) P$_{BAD}$-*rpoS990*(*leu*2*)-*lacZ*, *rssB*$^-$ *miaA*$^+$ (KMT33001) and *rssB*$^-$ *miaA*$^-$ (KMT33002) P$_{BAD}$-*rpoS990*(*leu*3*)-*lacZ* as well as *rssB*$^-$ *miaA*$^+$ (KMT30003) and *rssB*$^-$ *miaA*$^-$ (KMT30011A) P$_{BAD}$-*rpoS990-lacZ* wild-type translational fusion strains were assayed for β-galactosidase-specific activity following 30 min of arabinose induction. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM). The means between *miaA*$^+$ and *miaA*$^-$ strains in section *D* were analyzed for statistical significance using *t*-test and the Holm–Sidak method with *P*-values <0.05.

Leucine codons had no effect on the translation of *iraP* in a *miaA*$^+$ host (Fig. 5C; Supplemental Table S6). However, the β-galactosidase-specific activity of the *miaA*$^-$ P$_{BAD}$-*iraP258*(*leu*2*)-*lacZ* translational fusion is higher than the *miaA*$^-$ P$_{BAD}$-*iraP258*(*leu*2*)-*lacZ* (Fig. 5C). Furthermore, the *miaA*$^+$/*miaA*$^-$ ratios in the *leu*2* allele of the *iraP* fusion are less than the *miaA*$^+$/*miaA*$^-$ ratios in the wild-type allele of the *iraP* fusion (Fig. 5C,D; Supplemental Table S5). Taken together this suggests that the *leu*2* allele allows for suppression of the MiaA requirement for *iraP* translation. More precisely, MiaA is necessary for *iraP* translation in part due to decoding of UUG-Leucine codons.

MiaB is dispensible for *rpoS* and *iraP* translation

As previously discussed, the MiaA enzyme catalyzes the first step in the synthesis of the ms^{2}i^{6}A37, the addition of the i^6 to the A37 nucleotide (Bartz et al. 1970). The addition of the ms^2 group, catalyzed by the MiaB enzyme, requires the presence of the i^6 as a prerequisite (Vold et al. 1979). Although we had previously demonstrated no effect of *miaB* mutants on *rpoS* levels (Thompson and Gottesman 2014), we revisited this question using the *rpoS* and *iraP* translational fusions. We measured the β-galactosidase-specific activity of the P$_{BAD}$-*rpoS990-lacZ* and P$_{BAD}$-*iraP258-lacZ* translational, following arabinose induction (Supplemental Fig. S9). For the *rpoS* translational fusion, there was a negligible decrease in the β-galactosidase-specific activity of the fusion in the *miaB* mutant, at 15–30 min following arabinose induction. For the *iraP* translational fusion, there was a negligible decrease in the β-galactosidase-specific activity of the fusion in the *miaB* mutant, at 20–30 min following arabinose induction. Taken together, this confirms our previous finding, and shows that the ms^2 portion of the ms^{2}i^{6}A37 tRNA modification is dispensable for full *rpoS* and *iraP* translation. The i^{6}A37 portion of the ms^{2}i^{6}A37 tRNA modification is responsible for its role in translation of *rpoS* and *iraP*.

DISCUSSION

Prediction of i^{6}A37 modification tunable transcripts (MoTTs) through leucine codon usage

Endres et al. (2015) define transcripts with codon usage different from that of average transcripts, as modification tunable transcripts (MoTTs). In their model, under normal growth conditions, MoTTs are moderately expressed in the presence of tRNA modifications (Endres et al. 2015). Levels of tRNA modification, and therefore the translation of MoTTs, change during stress or damage conditions (Endres et al. 2015). Our findings for *rpoS* and *iraP* establish them as MoTTs, adding MiaA modification and UUX codon usage as new examples of this phenomenon.

We hypothesized that UUX-Leucine codon enrichment within an ORF may correlate with MiaA (i^{6}A37) sensitivity in genes other than *rpoS* (Thompson and Gottesman 2014). The data in this study strongly support the utility of using leucine codon usage ratios to predict the necessity of the i^{6}A37 tRNA modification during translation of a given protein and provides a possible method for the identification of this subclass of MoTTs as HULC genes. We observed that the ratio of UUX-Leu codons in *iraP* is 0.46, which is even greater than the 0.29 ratio seen in *rpoS* (Table 3). While both ratios are higher than the expected 0.22, the ratio is

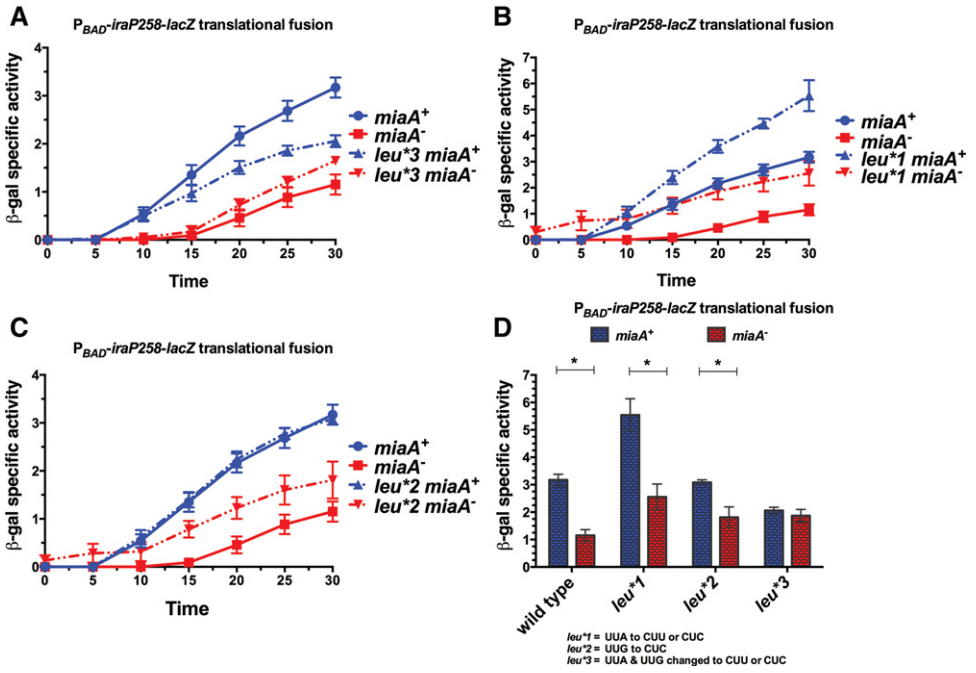

FIGURE 5. Translation of *iraP* with UUG- or UUA-Leucine codons replaced with CXX-Leucine codons. (*A*) The *miaA*$^+$ (KMT45000) and *miaA*$^-$ (KMT45002) P$_{BAD}$-*iraP258*(*leu*3*)-*lacZ* as well as *miaA*$^+$ (KMT42000) and *miaA*$^-$ (KMT42002) P$_{BAD}$-*258-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*B*) The *miaA*$^+$ (KMT43000) and *miaA*$^-$ (KMT43002) P$_{BAD}$-*iraP258*(*leu*1*)-*lacZ* as well as *miaA*$^+$ (KMT42000) and *miaA*$^-$ (KMT42002) P$_{BAD}$-*iraP258-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*C*) The *miaA*$^+$ (KMT44000) and *miaA*$^-$ (KMT44002) P$_{BAD}$-*iraP258*(*leu*2*)-*lacZ* as well as *miaA*$^+$ (KMT42000) and *miaA*$^-$ (KMT42002) P$_{BAD}$-*iraP258-lacZ* translational fusion strains were assayed for β-galactosidase-specific activity following arabinose induction. (*D*) The *miaA*$^+$ (KMT43000) and *miaA*$^-$ (KMT43002) P$_{BAD}$-*iraP258*(*leu*1*)-*lacZ*, the *miaA*$^+$ (KMT44000) and *miaA*$^-$ (KMT44002) P$_{BAD}$-*iraP258*(*leu*2*)-*lacZ*, the *rssB*$^-$ *miaA*$^+$ (KMT45000) and *rssB*$^-$ *miaA*$^-$ (KMT45002) P$_{BAD}$-*iraP258*(*leu*3*)-*lacZ* as well as *miaA*$^+$ (KMT42000) and *rssB*$^-$ *miaA*$^-$ (KMT42002) P$_{BAD}$-*iraP258-lacZ* wild-type translational fusion strains were assayed for β-galactosidase-specific activity following 30 min of arabinose induction. Each value represents the mean of at least three replicate experiments; the error bars represent the standard error of the mean (SEM). The means between *miaA*$^+$ and *miaA*$^-$ strains in section *D* were analyzed for statistical significance using *t*-test and the Holm–Sidak method with *P*-values <0.05.

much higher in *iraP*. Further bioinformatics searches, along with biochemical and genetic analysis, will be needed to further evaluate the predictive power associated with the correlation of UUX-Leu codon usage and sensitivity to the lack of MiaA for putative HULC proteins. These investigations are likely to yield more insight into the regulatory nature of the i^{6}A37 tRNA modification in *E. coli* and other biological systems.

UUG-Leucine and UUA-Leucine codon usage in *rpoS* and *iraP*

There are differences in how UUA and UUG codons are used in *rpoS* and *iraP*, in relation to the presence of the i^{6}A37 modification. Our *leu** mutations were created with the idea that UUX-Leucine to CUX-Leucine mutations would suppress the MiaA requirement for full *rpoS* translation. The *leu*3* mutation suppressed the MiaA effect in both *rpoS* and *iraP* (Figs. 4, 5). However, the suppression was more pronounced in *iraP*, as *miaA*$^+$ and *miaA*$^-$ levels were identical (Fig. 5). The reason for this is unclear, particularly since *iraP* has seven fewer UUX-Leu codons than *rpoS*. Both, *leu*1* and *leu*2* mutations, representing the UUA-CUX and UUG-CUX substitution mutations, respectively, demonstrated stronger MiaA suppression than the *leu*3* mutation for reasons that are not clear. However, this suggests that UUA-Leu and UUG-Leu substitutions, individually, are more effective at suppressing the MiaA effect than UUA and UUG substitutions collectively. It should be noted that the *rpoS* transcript is approximately three times longer than the *iraP* transcript. UUG-Leucine codons are more frequent within the *rpoS* and *iraP* transcripts than UUA-Leucine codons. The number of UUX codons in *iraP* and *rpoS* transcripts is seven and 12, respectively. There are two instances within the *iraP* transcript where UUX codons occur in tandem, once at leucine 8 and leucine 9 and again at leucine 80 and leucine 81. MiaA affects the translation of the 14 amino acid *trp* leader region, *trpL*, by influencing the translation rate of two tandem Trp codons (Trp 10 and Trp11) by ms^{2}i^{6}A37-modified tRNA^{Trp} (Landick et al. 1990). This tandem duplication of UUX-Leucine codons is not seen in the *rpoS* transcript. These two independent tandem duplications of UUX-Leucine codons in the *iraP* ORF likely contribute significantly to MiaA sensitivity during *iraP* translation. Undermodified tRNA^{Leu} may

contribute to ribosome pausing or stalling at each of these two tandem duplications contributing to decreased translational speed and accuracy.

The steady-state levels of *rpoS* have an additional level of regulatory fine-tuning

rpoS is subject to regulation at multiple levels, including the translation of the *rpoS* ORF, through SsrA, MiaA, AceE, and the presence of rare codons (Ranquet and Gottesman 2007; Kolmsee and Hengge 2011; Thompson and Gottesman 2014; Battesti et al. 2015). While there have been extensive studies on the regulation of the synthesis and activity of tRNA modification enzymes, the precise cellular or environmental signals that influence the post-transcriptional levels and post-synthesis activities of many tRNA modifications are elusive (Winkler 1998). This is also true of the i^{6}A37 modification and the activity of MiaA in *E. coli*. The leucine codon usage relationship suggests that leucine availability may provide a physiological condition whereby the i^{6}A37 modification is most critical for expression of *rpoS* synthesis. Lrp, the leucine-responsive regulatory protein, is a global regulatory protein whose regulatory action is partially dependent upon leucine (Ernsting et al. 1992; Lin et al. 1992; Platko and Calvo 1993). Mutations in *lrp*, as well as *rpoS*, confer a growth advantage during stationary phase, or GASP, phenotype (Zambrano et al. 1993; Zinser and Kolter 2000). In addition, the transcription factor LeuO regulates DsrA, one of the small RNAs that stimulate *rpoS* translation (Klauck et al. 1997; Repoila and Gottesman 2001). LeuO also regulates the leucine biosynthesis operon, *leuABCD* (Chen et al. 2001; Stratmann et al. 2012). These observations provide clues into a stationary phase network that connects leucine metabolism and *rpoS* synthesis.

There are several environmental signals that feed into the *rpoS* synthesis pathway through the small regulatory RNAs that stimulate its translation and anti-adaptors that promote its protein stability. Low temperature (DsrA), cell surface stress (RprA), aerobiosis (ArcZ), phosphate starvation (*iraP*), magnesium starvation (IraM), and DNA Damage (IraD) all act on RpoS synthesis or stability (Sledjeski et al. 1996; Majdalani et al. 2002; Bougdour et al. 2006, 2008; Mandin and Gottesman 2010). It would be useful to consider the possibility, and investigate, whether ms^{2}i^{6}A37 modification levels are modulated under these conditions that are known to stimulate Rpos levels. This may lead to greater insight into the physiological and regulatory nature of the ms^{2}i^{6}A37 tRNA modification.

MiaA is encoded directly upstream of and co-transcribed with Hfq, which is necessary for *rpoS* translation mainly through acting as a chaperone for the activating sRNAs (Tsui and Winkler 1994; Tsui et al. 1994). This tandem coding of MiaA and Hfq is highly conserved throughout the prokaryotic domain. This and our previous work offer one possible explanation, the regulation of *rpoS* translation, for this conserved synteny of *miaA* and *hfq*. It is likely that further bioinformatics and experimental analysis will uncover other mRNAs whose translation is regulated by both Hfq (possibly through Hfq-dependent sRNAs) as well as the i^{6}A37 tRNA modification.

There is some residual β-galactosidase-specific activity of the *rpoS* and *iraP leu** fusions, in which all UUX leu codons have been changed to CUX codons, in the absence of *miaA* (Figs. 1, 4, 5). This suggests that MiaA may be necessary for the decoding of other (non-leu) codons within *rpoS* and *iraP*, or have some other indirect effects on *rpoS* and *iraP* translation. We previously identified *tsaE* as a gene that now falls into the HULC designation of proteins and consequently may be an i^{6}A37 MoTT (Thompson and Gottesman 2014). The TsaE protein is also encoded upstream of *miaA* and *hfq* (Tsui and Winkler 1994). The TsaE protein, along with TsaC, TsaD, and TsaB, form an enzyme that catalyzes the addition of the N^6-L-threonylcarbamoyladenine^{37} (t^{6}A37) modification on ANN decoding tRNAs (Deutsch et al. 2012; Zhang et al. 2015). It is possible that translation of both *rpoS* and *iraP* are sensitive to loss of the t^{6}A37 modification, and that i^{6}A37 deficiency affects *rpoS* and *iraP* levels in part indirectly by limiting TsaE translation. We are currently investigating the possible role of t^{6}A on *rpoS* and *iraP* levels to test this hypothesis.

Implications for comparative functional genomics of tRNA isopentenyl transferases

We previously noted other proteins within the prokaryotic domain that are sensitive to MiaA levels (Thompson and Gottesman 2014). These proteins include *Agrobacterium tumefaciens vir* and *Shigella flexneri* VirF (Gray et al. 1992; Durand et al. 1997, 2000). In addition, *Streptomyces coelicolor bld* mutant phenotype is suppressed by overexpression of $tRNA^{Leu}_{CAA}$, which also likely contains the i^{6}A37 modification (Pettersson and Kirsebom 2011). The UUX-Leucine codon usage in *Shigella flexneri* VirF is indicative of MiaA sensitivity (Thompson and Gottesman 2014). Based on our observations in *E. coli* K12, it is likely that experimental analysis of leucine codon usage within these genes will yield insights into how UUX-Leu codons influence the translation of MiaA-sensitive proteins in other bacterial species.

The MiaA gene is also highly conserved among other biological domains, with homologs in yeast and humans, MOD5 and TRIT1, respectively (Martin and Hopper 1982; Golovko et al. 2000; Lamichhane et al. 2013b; Yarham et al. 2014). TRIT1 is particularly interesting due to its role as a putative lung adenocarcinoma tumor suppressor, as its expression was significantly down regulated in lung adenocarcinomas (Spinola et al. 2005). A genome-wide association study of a single nucleotide polymorphism (SNP) mapping to TRIT1, in different ethnic populations, demonstrated statistically significant links between a TRIT1 Phe202Leu genotype and lung cancer survival rates (Spinola et al. 2007). This suggests that

an association of leucine codon usage and tRNA isopentenyl transferase activity may be highly conserved well beyond the bacterial domain.

Expansion of the *rpoS* regulatory network in *E. coli*

Prior to this work, the mechanism by which MiaA exerted its effect on *rpoS* expression was undefined. Our data strongly suggest that the MiaA requirement for *rpoS* expression is partially through promotion of translational efficiency at MiaA-sensitive UUA- and UUG-Leucine codons in the *rpoS* ORF. The *rpoS* ORF is somewhat enriched for these leucine codons in comparison to other proteins that are gene regulators or members of the transcriptional regulatory machinery (Thompson and Gottesman 2014). Our current model for the role of MiaA in *rpoS* regulation is illustrated in Figure 6. In the presence of active, or excess MiaA, i^6A37-modified $tRNA^{Leu}_{CAA}$ efficiently recognizes UUA or UUG codons within the *rpoS* and *iraP* ORFs, leading to optimal translation of *rpoS* and *iraP* ORFs (Fig. 6). *iraP* levels contribute to *rpoS* stability, further increasing steady-state levels of *rpoS* (Fig. 6). In the absence of MiaA, or the presence of inactive MiaA, unmodified or undermodified $tRNA^{Leu}_{CAA}$ leads to the suboptimal translation of *rpoS* and *iraP* ORFs (Fig. 6). Suboptimal *iraP* levels lead to decreased *rpoS* stability, further decreasing steady-state levels of *rpoS* (Fig. 6).

MATERIALS AND METHODS

Media and growth conditions

M63-Glycerol-Sucrose-XG plates were used for positive selection of in-frame and out-of-frame P_{BAD}-*rpoS990-lacZ* translational fusion (Court et al. 2003; Mandin and Gottesman 2010). Positive selection of in-frame and out-of-frame P_{BAD}-*rpoS990-lacZ* translational fusions were confirmed by screening sucrose-resistant (Suc^R) colonies for chloramphenicol-sensitivity (Cm^S) on Luria-Bertani (LB) Lennox Agar plates supplemented with chloramphenicol to a final concentration of 25 µg/mL. LB agar plates supplemented with zeomycin (LB-Zeo) or tetracycline (LB-Tet), to a final concentration of 25 µg/mL, were used to select for transduction or recombineering of *rssB*::*tet* or Δ*miaA*::*zeo* mutations, respectively. LB agar plates supplemented with ampicillin (LB-amp), to a final concentration of 100 µg/mL were used for selection of plasmids. All cultures for β-galactosidase assays were grown in LB Lennox media (KD Medical). LB Lennox media were supplemented with ampicillin to a final concentration of 100 µg/mL selection of pBR-*leuX* or pBR-*leuZ* plasmids. For arabinose induction experiments, cells were grown in LB Lennox containing 0.2% glucose (LB-Glu) to an OD_{600} of 1.0, washed with an equal volume of fresh LB Lennox to remove residual glucose, and resuspended in LB

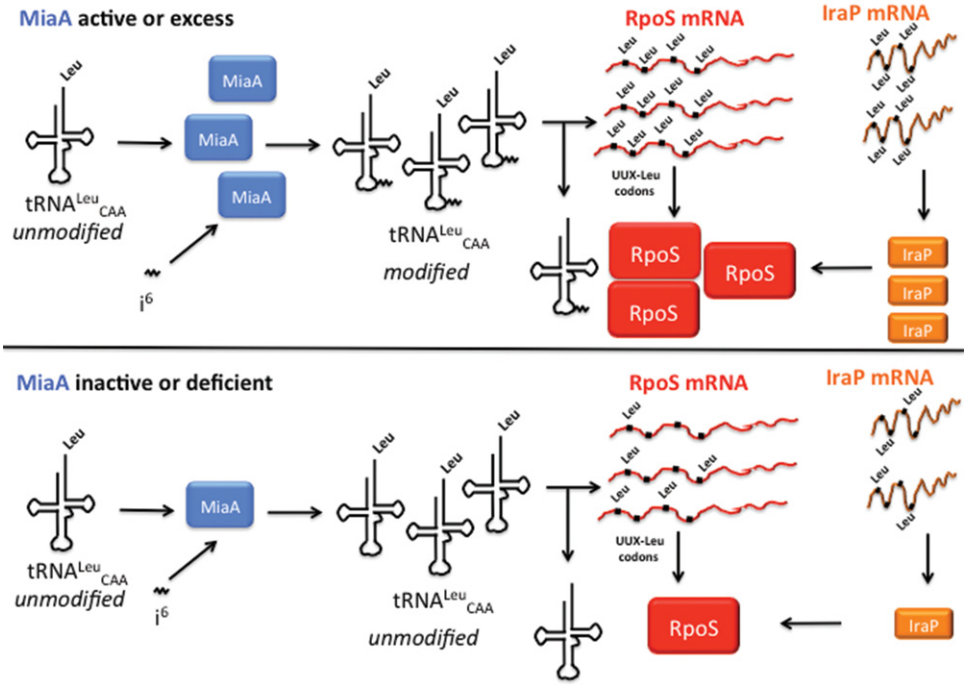

FIGURE 6. MiaA (i^6A37) regulation of *rpoS* and *iraP* model. This model illustrates our current model for the i^6A37 tuning of *rpoS* and *iraP* expression. In the presence of excess and/or active MiaA, $tRNA^{leuX}$ is i^6A37 modified, leading to optimal translation of UXX-Leucine codons with both *rpoS* and *iraP*. Since *iraP* increases *rpoS* stability, optimal amounts of *iraP* contribute to increased steady-state levels of *rpoS*. When MiaA is inactive or limiting, $tRNA^{leuX}$ is unmodified, leading to suboptimal translation of UXX-Leucine codons within both *rpoS* and *iraP*. Suboptimal translation of *iraP* will result in decreased stability of *rpoS*. These combined factors significantly decrease the steady-state levels of *rpoS*.

Lennox with 0.2% arabinose. Upon shifting cultures to LB-Ara, aliquots of the cultures were taken periodically following arabinose induction for β-galactosidase assays.

Strains and plasmids

Strains and plasmids are listed in Supplemental Table S1. All P_{BAD}-*rpoS990-lacZ* translational fusion strains used for the experiments carried a mutation in *rssB*, *rssB*::*tet*, to rule out possible effects on *rpoS* stability.

Construction of P_{BAD}-rpoS990-lacZ *translational fusions*

Arabinose-inducible translational fusions of *rpoS990* to *lacZ* were constructed by recombineering strain PM1805. Strain PM1805 contains the λ-Red proteins under control of the temperature-sensitive allele of the λ-repressor, *cI857*. PM1805 also contains the counter-selectable marker, *cat-sacB*, at the *lac* locus to allow for positive selection of recombinant fusions in the presence of sucrose. Recombineering into PM1805 requires induction of the λ-Red proteins, creation of electrocompetent cells, electroporation of allelic exchange substrates, and positive selection of fusions in the presence of sucrose. To induce the λ-Red proteins, PM1805 was grown in LB at 32°C to OD_{600} of 0.5 and shifted to 43.5°C for 15 min. The culture was then cooled in an ice-water bath. Then, washing the induced PM1805 culture in ice-cold H_2O made electrocompetent cells. Approximately 100 ng of the purified PCR product corresponding to the allelic exchange substrates were electroporated into the electrocompetent-induced PM1805 using the GenePulser (BioRad) on the Ec1 setting. Electroporated cells were recovered overnight in 10 mL of LB (Lennox) broth in a 125 mL flask at 32°C. Overnight cultures were serially diluted and spread on M63-Glycerol-Sucrose-XG plates and incubated at 30°C for 3–5 d. The purified PCR products were electroporated into electrocompetent PM1805. For construction of the wild-type P_{BAD}-*rpoS990-lacZ* translation fusion, an allelic exchange substrate was created through the amplification of a portion of the P_{BAD}-*rpoS990-lacZ* translational fusion from genomic DNA of strain KMT581 using oligonucleotide primers KT1123 and KT1124 (Table 2).

Three different derivatives of the P_{BAD}-*rpoS990-lacZ* translation fusion were also constructed: P_{BAD}-*rpoS990(leu*1)-lacZ*, P_{BAD}-*rpoS990(leu*2)-lacZ*, P_{BAD}-*rpoS990(leu*3)-lacZ* translational fusions. The P_{BAD}-*rpoS990(leu*1)-lacZ* contained UUA to CUU changes across the entire ORF. The P_{BAD}-*rpoS990(leu*2)-lacZ* contained UUG to CUC changes across the entire ORF. The P_{BAD}-*rpoS990(leu*3)-lacZ* contained both UUA to CUU and UUG to CUC changes across the entire ORF, effectively removing all MiaA-sensitive leucine codons. For the P_{BAD}-*rpoS990 (leu*1)-lacZ* P_{BAD}-*rpoS990(leu*2)-lacZ* translational fusions, synthetic (gBlock) DNA fragments (IDT Technologies) "P_{BAD}-*rpoS990(leu*1)-lacZ*_AES" and "P_{BAD}-*rpoS990(leu*2)-lacZ*_AES" (Supplemental Table S2), respectively, were PCR amplified using oligonucleotide primers KT1123 and KT1124 and used as an AES for recombineering into electrocompetent PM1800. For the P_{BAD}-*rpoS990(leu*3)-lacZ* translational fusions, two synthetic (gBlock) DNA fragments (IDT Technologies) with overlapping homology, "P_{BAD}-*rpoS990*(MiaA_Leu-) gBlockup" and "P_{BAD}-*rpoS990*(MiaA_Leu-) gBlockdown" (Supplemental Table S2), were recombined together using the Gibson Assembly Mastermix (New England Biolabs). The resulting DNA fragment was PCR amplified using oligonucleotide primers KT1123 and KT1124 (Table 2) and used as an AES for recombineering into electrocompetent PM1805.

Construction of ΔmiaA::zeo *allele by recombineering*

The *miaA* gene was deleted and replaced with a zeomycin (zeo) resistance cassette by recombineering in strain KMT194. An allelic exchange substrate for *miaA* mutagenesis was created by PCR amplification of the zeo cassette from genomic DNA from strain KMT465, using oligonucleotide primers KT1035 and KT1036 (Table 2). The PCR product was purified and ~100 ng was used for electroporation into electrocompetent KMT194 after induction of the λ-Red proteins, using a protocol identical to the one used to induce the λ-Red proteins in, and prepare electrocompetent cells of, PM1805. After electroporation, putative recombinants carrying Δ*miaA*::*zeo* mutations were selected on LB-Zeo plates. Recombinants were purified once on LB-Zeo plates and twice on LB plates. The Δ*miaA*::*zeo* mutation was confirmed by PCR and phenotypic analysis.

Construction of P_{BAD}-iraP258-lacZ *translational fusions*

We constructed arabinose-inducible translational fusions of the entire *iraP* ORF, except for the termination codon *iraP258*, to *lacZ* by recombineering strain PM1805. The methods used were identical to those listed in the construction of the P_{BAD}-*iraP258-lacZ* translational fusion strains. We also created three different derivatives of the P_{BAD}-*iraP258-lacZ* translation fusion in a manner identical to the *rpoS* fusions: P_{BAD}-*iraP258(leu*1)-lacZ*, P_{BAD}-*iraP258(leu*2)-lacZ*, P_{BAD}-*iraP258(leu*3)-lacZ* translational fusions. The P_{BAD}-*iraP258(leu*1)-lacZ* contained UUA to CUU changes across the entire ORF. The P_{BAD}-*iraP258(leu*2)-lacZ* contained UUG to CUU changes across the entire ORF. The P_{BAD}-*iraP258(leu*3)-lacZ* translational fusion contained both UUA to CUU and UUG to CUU changes across the entire *iraP* ORF, effectively removing all MiaA-sensitive leucine codons from the *iraP* portion of the fusion. For the P_{BAD}-*iraP258-lacZ*, P_{BAD}-*iraP258(leu*1)-lacZ*, P_{BAD}-*rpoS990 (leu*2)-lacZ*, and P_{BAD}-*iraP258(leu*3)-lacZ* translational fusions, synthetic (gBlock) DNA fragments (IDT Technologies) "P_{BAD}-*iraP258-lacZ*_AES," "P_{BAD}-*iraP258(leu*1)-lacZ*_AES," "P_{BAD}-*rpoS990(leu*2)-lacZ*_AES," and "P_{BAD}-*iraP258(leu*3)-lacZ* AES" (Supplemental Table S2), respectively, were PCR amplified using oligonucleotide primers KT1162 and KT1163 and used as an AES for recombineering into electrocompetent PM1800.

P1 transduction to moved marked mutations

All Δ*rssB*::*tet* and Δ*miaA*::*zeo* mutations were transferred into the wild-type and mutant P_{BAD}-*rpoS990-lacZ* or P_{BAD}-*iraP258-lacZ* translational fusion strains by bacteriophage P1 transduction and selecting for tetracycline (Tet^R) or zeomycin (Zeo^R) resistance, respectively.

DNA manipulations and cloning reactions

To determine whether overexpression of rare leucine tRNAs could suppress the MiaA requirement for *rpoS* expression, we cloned rare leucine tRNA genes, *leuX* and *leuZ*, downstream from the

IPTG-inducible P_{LlacO} promoter in plasmid pBR-pLac (Guillier and Gottesman 2006).

*Construction of pBR-*leuX

The *leuX* gene was amplified from *E. coli* K12 MG1655 chromosomal DNA using oligonucleotide primers KT1104 and KT1105 (Table 2). The PCR product was then purified using the PCR Purification Kit (Lamda Biotech), digested with restriction enzymes AatII (New England Biolabs) and EcoRI (New England Biolabs), and ligated to an AatII/EcoRI digest of plasmid pBR-pLac (Supplemental Table S1) using Quick Ligase (New England Biolabs). An aliquot of the ligation reaction was transformed into NEB5 chemically competent cells (New England Biolabs) using heat shock transformation at 42°C for 30 sec, recovered in 500 µL SOC Media, and 100 µL aliquot was spread on LB-Amp plates. Putative pBR-*leuX* transformants were screened by colony PCR using the BAC-Direct Kit and oligonucleotide primers KT1115 and KT1105. Plasmids from PCR positive transformants were sequenced to confirm the presence of the *leuX* insert. Plasmid pBR-*leuX* was transformed into wild-type and Δ*miaA*::*zeo* fusion strain using TSS transformation (Chung et al. 1989). The clones were confirmed by DNA sequencing.

*Construction of pBR-*leuZ

The steps used for construction of pBR-*leuZ* are identical to those used for the construction of pBR-*leuX* with the exception of the oligonucleotide primers used for PCR amplification (oligonucleotide primers KT1102 and KT1103) and colony PCR screening (oligonucleotide primers KT1115 and KT1103). The clones were confirmed by DNA sequencing.

β-galactosidase assays

High-throughput kinetic β-galactosidase assays were carried out in 96-well plates as previously described (Zhou and Gottesman 1998). The Filtermax F5 (Molecular Devices) multimode microplate reader was used to read microtiter plates. β-galactosidase-specific activity units are defined as the slope of OD_{420} reading divided by OD_{600} and are approximately 25-fold lower than Miller Units.

Experimental design for assays executed following arabinose induction

Briefly, samples to be assayed were grown in 5 mL of LB-Glu overnight at 37°C in a roller drum. Overnight cultures were diluted 1:1000 in 30 mL of fresh LB-Glu in a 125–mL Erlenmeyer flask and grown at 37°C in a shaking water bath at 200 rpm. When cultures reached an OD_{600} of 1.0, cells were harvested by centrifugation and resuspended in 30 mL of fresh LB supplemented 0.2% arabinose, and 100 µL aliquots of each culture were taken every 5 min for β-galactosidase assays. Samples were collected in triplicate for each individual experiment and averages were taken as a representative sample for each experiment. The data represent the mean and standard error of the mean of at least three independent replicates.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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