

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Strategies Toward Improved Orthogonal Ribosomes

Permalink

<https://escholarship.org/uc/item/2vx80594>

ISBN

9798288863417

Author

Boyko, Kristina

Publication Date

2025-05-15

Peer reviewed|Thesis/dissertation

Strategies Toward Improved Orthogonal Ribosomes

By

Kristina Boyko

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Biophysics

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Jamie Cate, Chair

Professor Gloria Brar

Professor Nicholas Ingolia

Professor Alanna Schepartz

Spring 2025

Abstract

Strategies Toward Improved Orthogonal Ribosomes

by

Kristina Boyko

Doctor of Philosophy in Biophysics

University of California, Berkeley

Professor Jamie Cate, Chair

Orthogonal ribosomes are essential tools for expanding the genetic code, enabling the incorporation of non-canonical amino acids (ncAAs) into proteins, and facilitating the synthesis of novel biopolymers. However, current systems are limited by translation of endogenous mRNAs and cross-assembly with wild-type ribosomal subunits, preventing full orthogonality. This work presents multiple strategies to improve orthogonal ribosome function in *Escherichia coli* by addressing both of these limitations.

First, we investigated the role of ribosomal protein bS1 in Shine-Dalgarno (SD) independent translation, hypothesizing that its exclusion from orthogonal 30S subunits might enhance specificity for orthogonal mRNAs. We engineered 16S rRNA variants with structural extensions in helix h26 to sterically block bS1 binding. While cryo-EM and biochemical assays confirmed successful bS1 exclusion, orthogonality was not significantly improved, indicating that additional mRNA features drive SD-independent translation.

Second, we developed a novel strategy to disrupt cross-assembly by targeting inter-subunit rRNA bridges. Specifically, we mutated nucleotides in bridge B3 to prevent interaction between orthogonal 30S and wild-type 50S subunits. Cryo-EM analysis confirmed structural disruption at the interface, and we restored partial activity by rationally designing compensatory 23S rRNA mutations. To expand this approach, we screened a 23S rRNA library using a functional selection based on SecM stalling, followed by RNase H treatment to enrich for translationally competent complexes.

Finally, we applied a parallel engineering strategy to bridge B7a, introducing phylogenetically guided mutations at 16S rRNA position A702 and pairing them with complementary mutations in 23S rRNA. Although less extensively characterized than the B3 bridge, this approach further supports the feasibility of engineering orthogonal ribosome pairs through targeted subunit interface design.

Together, these findings provide a dual framework, modulating translation initiation and subunit specificity, for improving orthogonal ribosomes. This work lays the foundation for future efforts to construct fully orthogonal translation systems, enabling sophisticated genetic programs and expanded chemical capabilities in living cells.

Table of Contents

Chapter 1: Introduction

- 1.1 Overview of bacterial translation
- 1.2 Orthogonal ribosomes for genetic code expansion
- 1.3 Limitations of current orthogonal ribosome systems
- 1.4 Strategies to enhance orthogonal ribosomes

Chapter 2: Role of ribosomal protein bS1 in orthogonal mRNA start codon selection

- 2.1 Introduction
- 2.2 Results
- 2.3 Discussion and conclusion
- 2.4 Materials and methods
- 2.5 Acknowledgements

Chapter 3: Engineering approaches of the B3 bridge

- 3.1 Introduction
- 3.2 Results
- 3.3 Discussion and conclusion
- 3.4 Materials and methods

Chapter 4: Engineering approaches of the B7a bridge

- 4.1 Introduction
- 4.2 Results
- 4.3 Discussion
- 4.4 Materials and methods

Chapter 5: Conclusion and future directions

References

Acknowledgements

I am deeply grateful to all the individuals who have helped make this dream a reality. I would like to thank Jamie for his unwavering support and mentorship, your curiosity is contagious and has made even the most challenging aspects of science more rewarding. To the Cate lab, thank you for your encouragement and camaraderie; it's been a pleasure getting to know each of you and sharing so many meaningful memories. Special thanks to Chandrima for generously training me in all things structural biology, to Amos for always being open to brainstorming ideas, and to Kate for your friendship and for teaching me the importance of always digging deeper. I am also grateful to Minji and Rebecca for your enthusiasm and contributions to the projects. A heartfelt thank you to my committee members; Alanna Schepartz, Nicholas Ingolia, and Gloria Brar, for your guidance and for the honor of having your support throughout this journey. I'm especially thankful to my friend Laura; your friendship has been a constant source of strength, and I'm incredibly grateful that our paths crossed. And thank you to my instructors from Whatcom Community College and Western Washington University for all of your support.

I would also like to thank my family. Mom, thank you for always believing in me, even when I doubted myself. Dad, thank you for being my role model, I'm proud to be a scientist, but even more proud to be your daughter. To my wonderful sisters, Adena and Jessica, thank you for always being there to share a laugh. Oleg, thank you for joining me on mountain summits and ultramarathons, those have been some of my favorite memories from graduate school. And finally, to Michael; life with you is a pleasure. Looking forward to what's next!

Chapter 1: Introduction

Protein synthesis, or translation, is a tightly controlled mechanism vital for life on Earth. It involves the reading of messenger RNA (mRNA) to produce sequence defined polymers called proteins. Essential to this process is the ribosome, which is responsible for translating segments of three nucleotides, or codons, of the mRNA and quickly and accurately incorporating the correct amino acid into a polymer chain.

1.1 Overview of bacterial translation

Translation is facilitated by the ribosome; the ribosome is comprised of the large (50S) and small (30S) subunit. The 50S subunit contains 23S and 5S ribosomal RNA (rRNA) as well as 33 proteins while the 30S contains 16S and 21 proteins.¹ Translation can be broken into four phases: initiation, elongation, termination, and ribosome recycling. In bacteria, translation is initiated when the start codon of the mRNA binds to the 30S subunit, along with initiation factors 1, 2, and 3 (IF1, IF2, and IF3) forming the 30S initiation complex (30SIC).² The start codon is guided to the P-site of the ribosome by using the Shine-Dalgarno (SD) sequence, a sequence upstream of the start codon that has a complementary sequence in the 16S rRNA.¹ The start codon is decoded by initiator fMet-tRNA in the P-site. The 50S subunit then binds the 30SIC to form a full 70S initiation complex (70SIC), releasing the initiation factors and moving onto the elongation phase. Charged tRNA is brought to the ribosome in a ternary complex with elongation factor Tu (EF-Tu) and GTP to the A-site.¹ After the correct pairing of the codon and the anticodon, GTP undergoes hydrolysis and EF-Tu releases the aminoacyl end of the A site tRNA, allowing it to move to the P-site and allowing peptide bond formation to occur.³ The deacylated tRNA is then moved from the P to the E site. The peptidyl tRNA can then move from the A site to the P site. When a stop codon reaches the ribosome, it is recognized by either release factor 1 or release factor 2 (RF1 or RF2). Peptide hydrolysis is triggered by the binding of the release factors and the third release factor (RF3) binds to the ribosome and facilitates release of RF1 and RF2. The bound mRNA, deacylated tRNA, and subunits, are dissociated through ribosome recycling factor and EF-G.¹ The machinery is therefore ready for another round of translation.

1.2 Orthogonal ribosomes for genetic code expansion

The synthesis of peptides containing non-canonical amino acids (ncAAs) has vast implications and can be used in applications such as the development of novel peptide therapeutics, or implementing new chemical probes such as fluorescence or ligand binding.^{4,5} To date, ncAAs have been incorporated using post-translational protein modifications, solid-phase peptide synthesis, or selective pressure incorporation.⁴ While these methods have been successful, the speed and accuracy of the ribosome makes it a prime target for the synthesis of peptides containing ncAAs. Engineering the ribosome to incorporate ncAAs is a challenging feat and involves making mutations to the peptidyl transferase center (PTC).⁶ However, making mutations to the rRNA in the PTC can result in instability and less activity.⁷ This highlights the need for a robust orthogonal ribosome system where the endogenous translational machinery is orthogonal to the engineered machinery designed to incorporate ncAAs.⁶ An orthogonal ribosome would provide the flexibility of making mutations to the PTC without hindering the translation of endogenous proteins needed for cell survival.

1.3 Limitations of current orthogonal ribosome systems

A central aspect of engineering mRNAs encoding ncAA-containing proteins to be orthogonal to the translation machinery is to include an orthogonal ribosome binding site (RBS) on the 16S rRNA and an orthogonal SD (oSD) sequence on the mRNA, for start codon selection.^{8,9,10} To ensure that the orthogonal ribosome only translates these orthogonal mRNAs (omRNAs), 16S rRNA in the orthogonal 30S subunit (o30S) harbors an orthogonal ASD (oASD) sequence that base pairs with the omRNA. This combination of oSD in the omRNA and the oASD in the o30S subunit, in theory, should ensure that the target omRNA is only translated by the orthogonal ribosome, while the orthogonal ribosome does not translate endogenous mRNAs. However, recent studies in *E. coli* have shown that the SD sequence does not fully dictate whether an mRNA transcript is translated. Single-molecule tracking techniques in live cells revealed that translating ribosomes with oASD sequences in their 16S rRNA bind to endogenous mRNAs.¹¹ This finding is further supported by ribosome profiling experiments that reveal that different ASD sequences in 16S rRNA do not prevent orthogonal ribosomes from translating cellular mRNAs.¹² Taken together, this cross-reactivity of orthogonal ribosomes with endogenous mRNAs limits the current utility of these orthogonal systems.

In addition to cross reactivity with cellular mRNAs, current orthogonal ribosomes in bacteria introduce a covalent linkage (a “tether” or “staple”) between the 16S and 23S rRNA by transcribing the orthogonal rRNA as one transcript, thereby covalently linking the ribosomal 30S and 50S subunits.^{13,14} While these orthogonal ribosome have been used to incorporate ncAAs to some degree, they are limited by cross assembly with endogenous ribosomal subunits, preventing full orthogonality.^{15,13} Collectively, the tendency for orthogonal ribosomes to both cross react with cellular mRNAs and endogenous ribosomal subunits leaves room for engineering of a more robust orthogonal system.

1.4 Strategies to enhance orthogonal ribosomes

In *E. coli*, ribosomal protein bS1 is thought to contribute to translation initiation using mechanisms independent of the SD sequence.¹⁶ Ribosomal protein bS1 is composed of multiple RNA-binding domains and is, in some cases, thought of as an additional translation factor due to its reversible binding to the ribosome.¹⁶ Protein bS1 also has high affinity for mRNA and is thought to facilitate translation for highly structured mRNAs.¹⁷ However, the translation of leaderless mRNAs, as well as of mRNAs that have an RBS close to the 5' end, is independent of bS1.¹⁷ Recent cryo-EM structures of *E. coli* ribosomes with truncated ribosomal protein bS1 reveal that bS1 binds to mRNA, just upstream of the SD sequence.¹⁸ In addition to binding to the mRNA, bS1 simultaneously binds to ribosomal protein uS2 adjacent to the SD-ASD interaction site, as well as to 16S rRNA in this region.¹⁹ Taken together, these mechanistic and structural findings have led to the model that bS1 acts to recruit mRNA to the ribosome in an SD-independent manner. We reasoned that the ability of ribosomal protein bS1 to recruit mRNAs to the ribosome in an SD-independent manner might be responsible for the lack of orthogonality seen with present orthogonal ribosomes and omRNAs. We therefore tested whether orthogonal ribosomes could be engineered to rely more on SD and ASD binding, by engineering an orthogonal 16S rRNA that sterically prevents bS1 binding to the 30S ribosomal subunit. In this case, ribosomal protein bS1, an essential protein in *E. coli*, would continue to bind to the endogenous ribosome and translate endogenous mRNAs.¹⁶ However, the orthogonal ribosomes that have an orthogonal ASD and translate omRNAs, would not bind bS1 and eliminate this aspect of bS1's contribution to mRNA recruitment to the ribosome.

In addition, to overcome the limitation of cross assembly with endogenous subunits we proposed a new approach to augmenting ribosome orthogonality, using mutations to a central rRNA contact, or “bridge” between the ribosomal subunits. There are twelve instances in the bacterial ribosome where the small and large subunit interact, six of which are rRNA-rRNA bridges and potential targets for generating a new orthogonal ribosome.²⁰ We proposed to mutate bridge B3, which occurs at the center of the ribosomal subunit interface and is essential for subunit association.^{20,21} Bridge B3 involves two conserved A-minor interactions between 16S rRNA nucleotides A1418 and A1483 in helix h44 and 23S rRNA helix H71.²⁰ We reasoned, based on previous experiments and structural analysis that mutations A1418G and/or A1483G should sterically disrupt the subunit interface.^{21,22} We hypothesized that we could make the mutations mentioned above to prevent binding of orthogonal 16S rRNA to wild-type 23S rRNA and then engineer an orthogonal 23S to bind to the orthogonal 16S rRNA. We engineered the orthogonal 23S rRNA by introducing a sequence library in the nine nucleotides that are involved in the B3 bridge on the 23S rRNA side and selected for active mutants *in vitro*.

Additionally we attempted to engineer an orthogonal B7a bridge, one of the first bridges to form during translation and is essential for subunit joining.²⁰ Structurally, position A702 in h23 of 16S rRNA forms an A-minor interaction with H68 in the 23S rRNA.²⁰ Upon modification of the N1 position of A702, 70S formation decreases.^{23,21} Interestingly, position 702 in B7a is not phylogenetically conserved. In bacteria, the position is found to be an adenosine in over 95% of sequences. However, in archaea, it is most commonly found to be a uradine.²⁴ In eukaryotes, the most common base at 702 is a guanosine at around 72%. Similarly, there is covariation found on the 23S rRNA side of the bridge.²⁴ We hypothesized that we could introduce the mutations at position 702 to either U or G (A702U or A702G) to prevent binding to the bacterial large subunit. In conjunction, we hypothesized that we could restore 70S formation by introducing compensatory mutations to the 23S rRNA side of the bridge that correspond to A702U or A702G. Taken together, these experiments aim to improve the utility of orthogonal ribosomes for the purpose of genetic code expansion by addressing the limitations of Shine-Dalgarno independent translation as well as cross-assembly with endogenous subunits.

Chapter 2: Role of ribosomal protein bS1 in orthogonal mRNA start codon selection

In many bacteria, the location of the mRNA start codon is determined by a short ribosome binding sequence that base pairs with the 3'-end of the 16S ribosomal RNA (rRNA) in the 30S subunit. Many groups have changed these short sequences, termed the Shine-Dalgarno (SD) sequence in the mRNA and the anti-Shine-Dalgarno (ASD) sequence in 16S rRNA, to create “orthogonal” ribosomes to enable the synthesis of orthogonal polymers in the presence of the endogenous translation machinery. However, orthogonal ribosomes are prone to SD-independent translation. Ribosomal protein bS1, which binds to the 30S ribosomal subunit, is thought to promote translation initiation by shuttling mRNA to the ribosome. Thus, a better understanding of how the SD and bS1 contribute to start codon selection could help efforts to improve the orthogonality of ribosomes. Here we engineered the *Escherichia coli* ribosome to prevent binding of bS1 to the 30S subunit, to separate the activity of bS1 binding to the ribosome from the role of the mRNA SD sequence in start codon selection. We find that ribosomes lacking bS1 are slightly less active than wild-type ribosomes *in vitro*. Furthermore, orthogonal 30S subunits lacking bS1 do not have improved orthogonality. Our findings suggest that mRNA features outside the SD sequence and independent of bS1 binding to the ribosome likely contribute to start codon selection and the lack of orthogonality of present orthogonal ribosomes.

2.1 Introduction

Translation initiation in bacteria involves the formation of a 30S initiation complex, in which the start codon of the mRNA pairs with the initiator fMet-tRNA anticodon in the P site of the small (30S) ribosomal subunit. The positioning of the start codon in the 30S initiation complex is aided by the SD sequence 5' of the start codon, which base pairs to the complementary ASD sequence in 16S rRNA. Characteristics of the SD sequence, including its presence or absence, spacer length from the SD sequence to the start codon, or SD sequence composition, can determine translation efficiency. For example, many bacteria encode leaderless mRNAs, or mRNAs that do not contain an identifiable SD sequence, and only 54% of *E. coli* genes have an SD sequence.²⁵ RNA-seq analysis has also revealed that leaderless mRNAs can comprise up to 70% of transcripts in other bacteria as well as archaea.²⁶ In mRNAs possessing an SD sequence, the number of nucleotides between the start codon and the SD sequence affects the levels of protein expression.²⁷ Finally, nucleotides which compose the SD sequence can also affect translation. For example, in *E. coli* the canonical SD sequence (5'-UAAGGAGG-3') yields better translation efficiency than a 5'-AAGGA-3' sequence.²⁸ Taken together, these findings suggest that the role of the SD sequence in the mechanism of translation initiation does not follow a simple set of rules, and can vary widely across bacteria.

Mechanism of gene expression in *E. coli* have been the most widely studied in bacteria, making this model organism the most popular host for synthetic biology applications. These include efforts to expand the genetic code to enable noncanonical amino acid incorporation into proteins for the synthesis of non-proteinogenic polymers by the ribosome.⁵ To enable genetic code expansion, many groups have engineered cells with an orthogonal ribosome, a secondary ribosome to translate orthogonal mRNAs independent of the endogenous translation machinery, along with orthogonal mRNAs (omRNAs).¹⁰ The orthogonal ribosome can be engineered to incorporate non-canonical amino acids while the endogenous ribosome can continue translating cellular mRNAs, preventing cellular growth defects.^{13,14} A central aspect

of engineering mRNAs encoding non-canonical proteins to be orthogonal to the endogenous translation machinery is to include an orthogonal ribosome binding site (RBS) on the 16S rRNA and an orthogonal SD (oSD) sequence on the mRNA, for start codon selection.^{8,9,10} To ensure that the orthogonal ribosome only translates these omRNAs, 16S rRNA in the orthogonal 30S subunit (o30S) harbors an orthogonal ASD (oASD) sequence that base pairs with the omRNA RBS. This combination of oSD in the omRNA and oASD in the o30S subunit, in theory, should ensure that the target omRNA is only translated by the orthogonal ribosome, while the orthogonal ribosome does not translate endogenous mRNAs.

Multiple examples of orthogonal RBS have been reported, in which both the 16S rRNA and mRNA RBS sequences have been engineered. Approaches to identify these sequences involved positive and negative selections as well as computational methods.^{10,29} However, recent studies in *E. coli* have shown that the SD sequence does not fully dictate whether or not an mRNA transcript is translated. Single-molecule tracking techniques in live cells revealed that translating ribosomes with oASD sequences in their 16S rRNA bind to endogenous mRNAs.¹¹ This finding is further supported by ribosome profiling experiments that reveal that different ASD sequences in 16S rRNA do not prevent orthogonal ribosomes from translating cellular mRNAs.¹² Taken together, this cross-reactivity of orthogonal ribosomes with endogenous mRNAs limit the current utility of these orthogonal systems (Figure 1A).

In *E. coli*, ribosomal protein bS1 is thought to contribute to translation initiation using mechanisms independent of the SD sequence.¹⁶ Ribosomal protein bS1 is composed of multiple RNA-binding domains and is, in some cases, thought of an additional translation factor due to its reversible binding to the ribosome.¹⁶ Protein bS1 also has high affinity for mRNA and is thought to facilitate translation for highly structured mRNAs.¹⁷ However, the translation of leaderless mRNAs, as well as of mRNAs that have an RBS close to the 5' end, is independent of bS1.¹⁷ Recent cryo-EM structures of *E. coli* ribosomes with truncated ribosomal protein bS1 reveal that bS1 binds to mRNA, just upstream of the SD sequence.¹⁸ In addition to binding to the mRNA, bS1 simultaneously binds to ribosomal protein uS2 adjacent to the SD-ASD interaction site, as well as to 16S in this region.¹⁹ Taken together, these mechanistic and structural findings have led to the model that bS1 acts to recruit mRNA to the ribosome in an SD independent manner.

We reasoned that the ability of ribosomal protein bS1 to recruit mRNAs to the ribosome in an SD-independent manner might be responsible for the lack of orthogonality seen with present orthogonal ribosomes and omRNAs. We therefore tested whether orthogonal ribosomes could be engineered to rely more on SD and ASD binding, by engineering an orthogonal 16S rRNA that sterically prevents bS1 binding to the 30S ribosomal subunit. In this case, ribosomal protein bS1, an essential protein in *E. coli*, would continue to bind to the endogenous ribosome and translate endogenous mRNAs.¹⁶ However, the orthogonal ribosomes that have an orthogonal ASD and translate omRNAs, would not bind bS1 and eliminate this aspect of bS1's contribution to mRNA recruitment to the ribosome (Figure 1A). Using cryo-EM and *in vitro* and cell-based translation assays, we found that preventing bS1 binding to the 30S subunit does not affect ribosome orthogonality *in vitro* or *in vivo*, indicating that SD-independent translation is not solely dependent on bS1 binding to the ribosome.

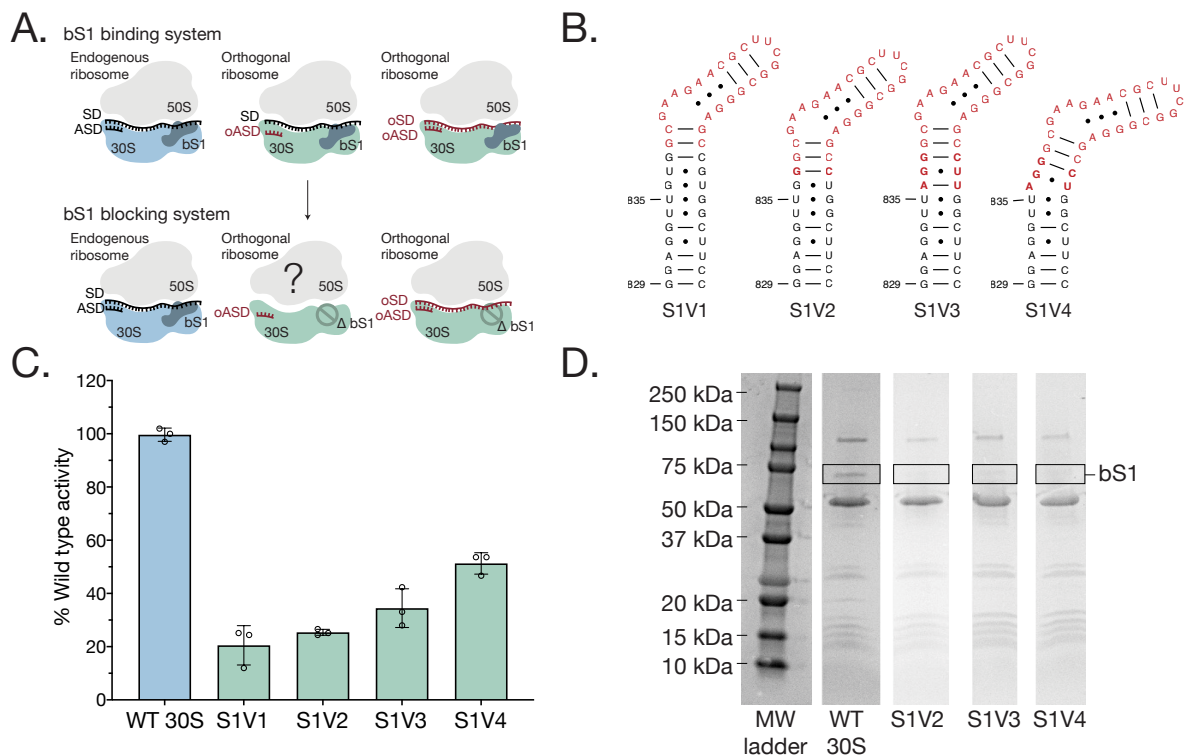


Figure 1. Model and characterization of bS1 blocking ribosomes. **A.** Proposed model of orthogonal ribosomes in a bS1 binding system and a bS1 blocking system. Current orthogonal ribosomes that undergo SD independent translation translate orthogonal mRNA (right) as well as endogenous mRNA (middle), possibly due to bS1. A bS1 blocking system would prevent binding of bS1 to orthogonal ribosomes, which could prevent translation of endogenous mRNA (middle) while encouraging translation of orthogonal mRNA (right). Endogenous mRNA is shown in black, and orthogonal mRNA in red. **B.** Secondary structure of all versions of the bS1 blocking mutants inserted into helix h26 of *E. coli* 16S rRNA. Nucleotides colored red are added bases. Nucleotides in bold highlight differences between versions S1V1-S1V4. **C.** *In vitro* activity assay of wild-type 30S and bS1 blocking mutants with wild-type 50S subunits. Activity corresponds to luminescence of synthesized nanoluciferase, normalized to wild-type ribosomes. Error bars indicate standard deviation for n=3 replicates. **D.** 10% SDS PAGE gel of wild-type 30S subunits and versions S1V2-S1V4 of the bS1 blocking mutants. The black box highlights bS1. The relative intensity of the maltose and MS2 binding fusion protein used for purification, as well as the other ribosomal proteins, is consistent across all lanes, indicating that the same amount of protein was loaded for each sample.

2.2 Results

Previous structural studies have shown that bS1 binds to the 30S subunit near helix h26 in 16S rRNA.^{18,19} Given that helix h26 contains variable regions that are surface exposed and are not phylogenetically conserved,³⁰ we introduced an rRNA extension to helix h26 between positions U835 and G851 to attempt to sterically block bS1 binding to the mutant ribosomes.³¹ However, we kept the conserved loop of the helix, which contains a stable UUCG tetraloop, constant (Figure 1B).³² To extend h26, we searched for an RNA motif with structural and folding properties that have been well characterized. We tested four different variable sequences that form kink-turn (k-turn) motifs to identify an RNA structural element that could sterically prevent bS1 from binding the 30S subunit (S1V1-S1V4).³³

We first inserted a k-turn derived from an engineered RNA that introduced 14 additional bases into helix h26 in 16S rRNA (S1V1).³⁴ However, the S1V1 30S subunit was

only 20% active compared to tagged wild-type 30S subunits, as shown by *in vitro* translation of a nanoluciferse reporter (Figure 1C). We therefore tried introducing a G-C base pair at the base of the helix h26 extension to both stabilize and extend the helix, to create mutant S1V2. To test for bS1 depletion from the S1V2 30S subunit, we expressed and purified ribosomes via an MS2 hairpin on the 16S rRNA.³⁵ After purification, we isolated and resolved the proteins from the samples on a 15% SDS-PAGE protein gel. As a control, we also tagged wild-type 16S rRNA and purified wild-type 30S subunits adjacent to the mutant ribosomes. The gel shows a band for bS1 in the wild-type 30S at the expected molecular weight of 61.2 kDa, consistent with previous studies.³⁶ However, this band is missing in the S1V2 mutant sample (Figure 1D). While it is likely that some percentage of bS1 was depleted during 30S subunit purification, 30S subunits with the wild-type 16S rRNA control that underwent the same purification scheme still contained subunit bS1, as evidenced by the solid band for bS1 in the SDS-PAGE gel. This indicates that the S1V2 rRNA extension played a role in depleting bS1. However, while S1V2 successfully prevented bS1 from binding the 30S subunit, this mutant was only 25% as active as wild type 30S subunits (Figure 1C). To search for more active 30S subunits, we hypothesized that we could further extend the helix to prevent bS1 binding and introduced an additional A-U pair at the base of the helix (S1V3, Figure 1B). The S1V3 mutation successfully disrupted bS1 binding to the 30S subunit (Figure 1D) and was about 35% as active as wild-type ribosomes (Figure 1C).

We determined a cryo-EM structure of a 70S ribosome with the S1V3 30S subunit and found that ribosomal protein uS2 was missing from the 30S subunit (Figure 2A). This suggested that the A-U base pair extension may have extended and rotated the k-turn into a position that collides with uS2. To mitigate this potential collision, we removed the uridine from the A-U base pair in mutant S1V4, such that stacking of the A in the RNA helix would angle the helix away from ribosomal protein uS2. This final variant, S1V4, also successfully blocked bS1 from binding the 30S subunit (Figure 1D) and was about 50% as active as wild-type 30S subunits. To ensure the S1V4 30S subunit maintained structural integrity, i.e. with respect to ribosomal protein uS2, we determined the cryo-EM structure of S1V4 to a global resolution of 1.8 Å and a local resolution of 4.4 Å around the h26 extension (Figure 2B). The structure reveals well resolved density for the base of helix h26. However, as the helix continues into the k-turn the density becomes weaker, indicating a flexible k-turn extension (Figure 2C). Cryo-EM density is lacking for ribosomal protein bS1 but is present for uS2 indicating that the extension successfully prevents bS1 binding while not disturbing binding of uS2 to the ribosome (Figure D).

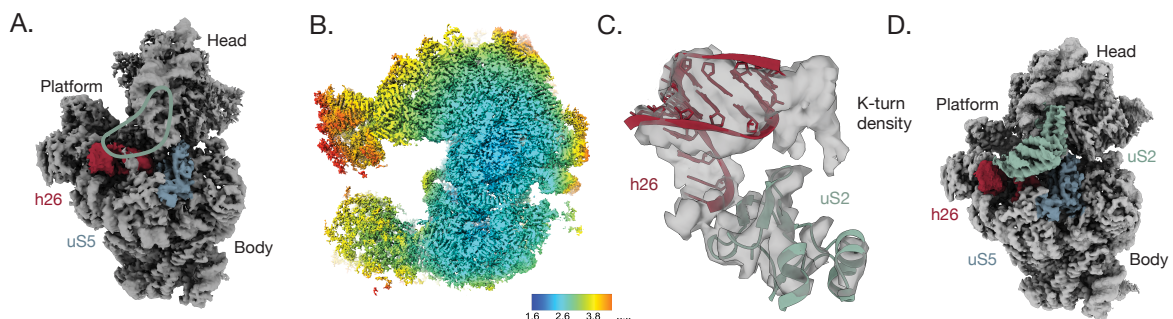


Figure 2. Structure of S1 blocking ribosomes. **A.** Cryo-EM density of 30S subunit S1V3, with density for ribosomal protein uS2 missing. Density is clearly resolved for protein uS5 and rRNA helix h26 (blue and red, EMD 47168). The green outline indicates where uS2 density would be. **B.** Local resolution of the S214 30S subunit cryo-EM map. A cross-section of the local resolution was made in ChimeraX and the color key is reported in Ångstroms. Local resolution was measured using the Local Resolution

estimation in Relion 4.0-beta-2.³⁷ **C.** Cryo-EM density of the h26 k-turn extension (unmodeled) and uS2, in 30S subunit S1V4. The cryo-EM maps was blurred with a B-factor of 61 Å². **D.** Cryo-EM density of 30S subunit S1V4. Density for uS2 is indicated in green and is well resolved. Density of uS5 is shown in blue and rRNA helix h26 is shown in red (EMD 47169).

To test our hypothesis that preventing bS1 binding to the 30S subunit would improve ribosome orthogonality, i.e. make ribosomes more dependent on the SD-ASD interaction, we used a previously-developed oASD sequence in both the wild-type 16S and S1V4 16S rRNA backgrounds.²⁹ We also cloned the corresponding oSD sequence into a nanoluciferase reporter (Figure 3). Using a PURE *in vitro* transcription and translation (IVTT) system supplemented with purified 30S and 50S subunits, we measured nanoluciferase luminescence from IVTT reactions containing wild-type or S1V4 30S subunits, and mRNAs in all four possible combinations of wild-type SD and ASD, and oSD and oASD pairings. In the case of the wild-type 30S subunit, translation of the wild-type SD reporter is robust and the translation of the orthogonal SD-ASD pair is about 50% of wild-type levels (Figure 3). Consistent with published results, there is no detectable translation for non-cognate SD-ASD pairs when using wild-type 30S subunits capable of binding bS1.³⁷ We observed similar results with S1V4, although there is slight cross-reactivity between the wild-type ASD and oSD. These results suggest that the SD-ASD interaction can be made highly orthogonal *in vitro*, and prevention of bS1 binding to the 30S subunit is inconsequential and possibly deleterious to orthogonality.

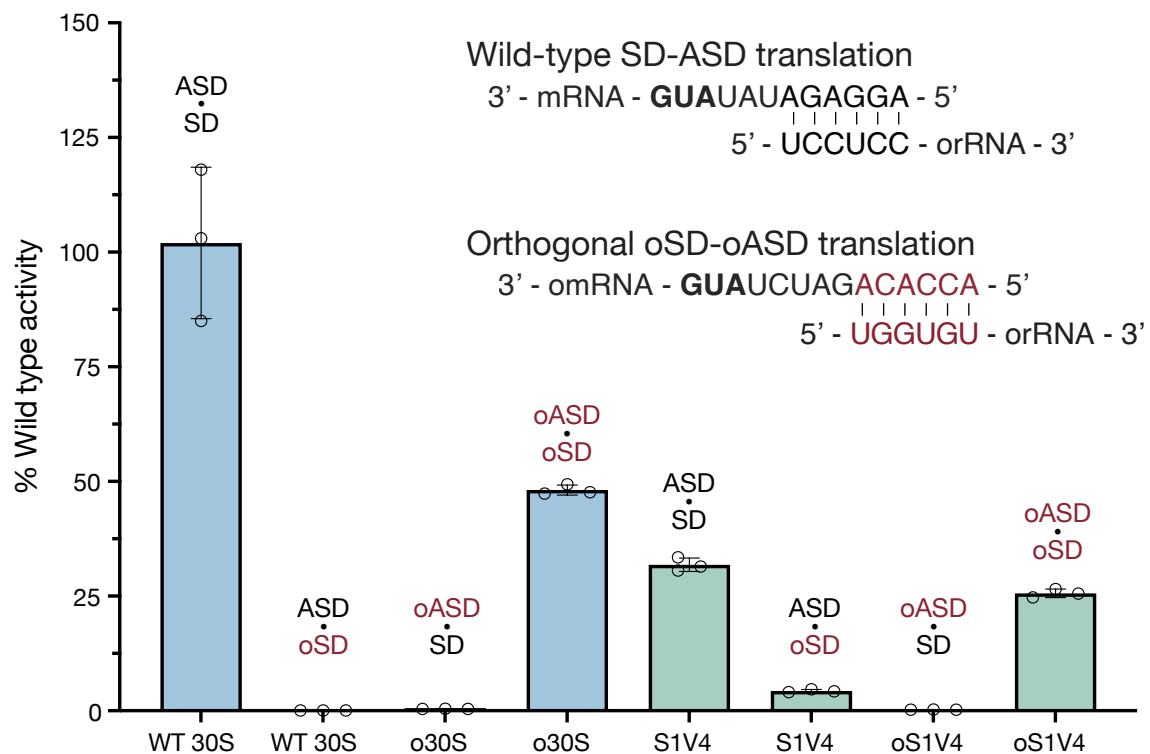


Figure 3. *In vitro* activity of 30S subunit S1V4. Activity of 30S ribosomal subunits with wild-type or orthogonal ribosome binding site elements in an *in vitro* nanoluciferase translation assay. Normalized luminescence of wild-type 30S subunits (blue) with a wild-type ASD sequence and oASD sequence with corresponding nanoluciferase reporters (black and red). The activity of 30S subunit S1V4 is shown in green with a wild-type ASD sequence and oASD sequence with corresponding nanoluciferase reporters (black and red). The activity is normalized to wild-type ribosomes. Error bars indicate the standard deviation for n=3 measurements.

Although preventing bS1 binding to the 30S subunit did not improve ribosome orthogonality *in vitro*, under these conditions there is no competition between mRNAs for ribosome binding as would occur in cells.³⁸ We therefore sought to understand how preventing ribosomal protein bS1 binding to the 30S subunit would affect the orthogonality of ribosomes *in vivo*. To accomplish this, we developed a two-plasmid system based on previously designed reporters.²⁹ In this system, GFP is encoded on a plasmid under the control of an arabinose inducible promoter, while the ribosomal RNA operon is encoded on a plasmid under control of an IPTG inducible promoter. In this system, wild-type GFP is translated from an mRNA harboring a wild-type SD, using ribosomes carrying the wild-type ASD (Figure 4A). In the orthogonal case, oGFP is translated from an mRNA harboring an orthogonal SD and should only be translated by ribosomes with an orthogonal ASD (Figure 4A). With the set of mRNA and ribosome plasmids harboring all combinations of SD, ASD, wild-type 16S, and S1V4 16S sequences, we co-transformed eight different variations of ribosome and GFP plasmids to test for ribosome orthogonality.

We measured the fluorescence of GFP four hours after inducing GFP and ribosomal RNA expression with arabinose and IPTG, respectively. We also assessed the level of exogenous 16S rRNA expression using RT-PCR (Figure 5). Cells from both wild-type 30S subunits and S1V4 30S subunits harboring the wild-type ASD and expressing the wild-type SD mRNA had comparable levels of fluorescence, which likely derives mostly from endogenous ribosomes given the level of exogenous 16S rRNA expression (Figure 4B, Figure 5). We also observe high levels of fluorescence from cells expressing GFP from the mRNA with a wild-type SD (wild-type GFP) and o30S and oS1V4 30S, again likely due to the mRNA being translated by endogenous ribosomes. Interestingly, in the case of cells expressing oGFP mRNA, the amount of translation by ribosomes formed from oS1V4 30S subunits is comparable to that of cells expressing o30S subunits (Figure 4C), consistent with the *in vitro* translation results. The negligible amount of GFP cross-translation by wild-type ASD containing ribosomes of oSD mRNA is also comparable between wild-type and S1V4 30S subunits (Figure 4C). Taken together, these results indicate that the oSD and oASD sequences function similarly, independent of whether bS1 is able to bind to the 30S subunit or not.

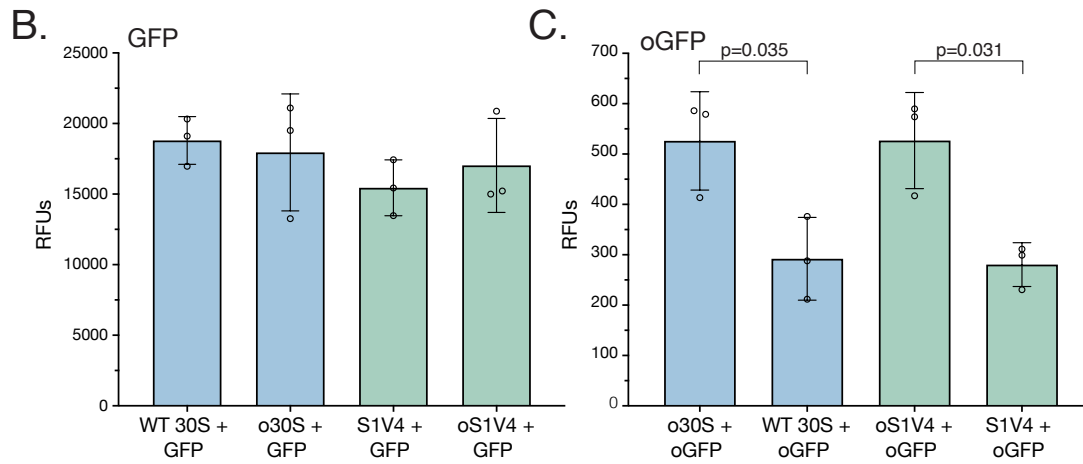
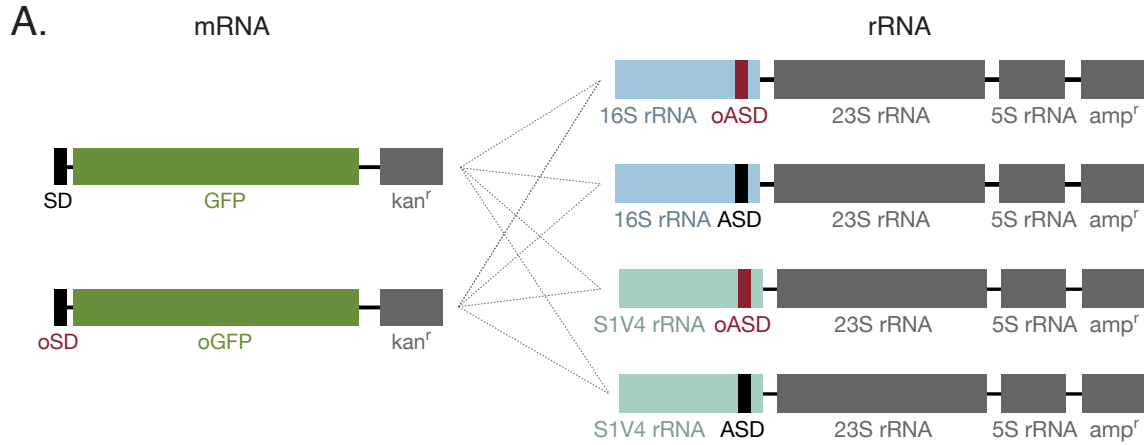


Figure 4. *In vivo* activity of S1V4 30S subunits. **A.** Two-plasmid reporter system with an arabinose inducible promoter upstream of GFP or oGFP, and an IPTG inducible promoter upstream of wild-type or S1V4 16S rRNA sequence, either with an ASD or oASD at the 3' end. All 8 combinations used in reporter assays are indicated by connecting lines. **B.** *In vivo* translation of wild-type GFP by wild-type 30S (blue), o30S (blue), S1V4 (green), and oS1V4 (green). Error bars indicate standard deviation for n=3, p-values > 0.29. **C.** *In vivo* translation of orthogonal GFP by o30S (blue), wild-type 30S (blue), oS1V4 (green), and S1V4 (green). Error bars indicate standard deviation for n=3 measurements. Measurements reported in relative fluorescence units (RFUs).

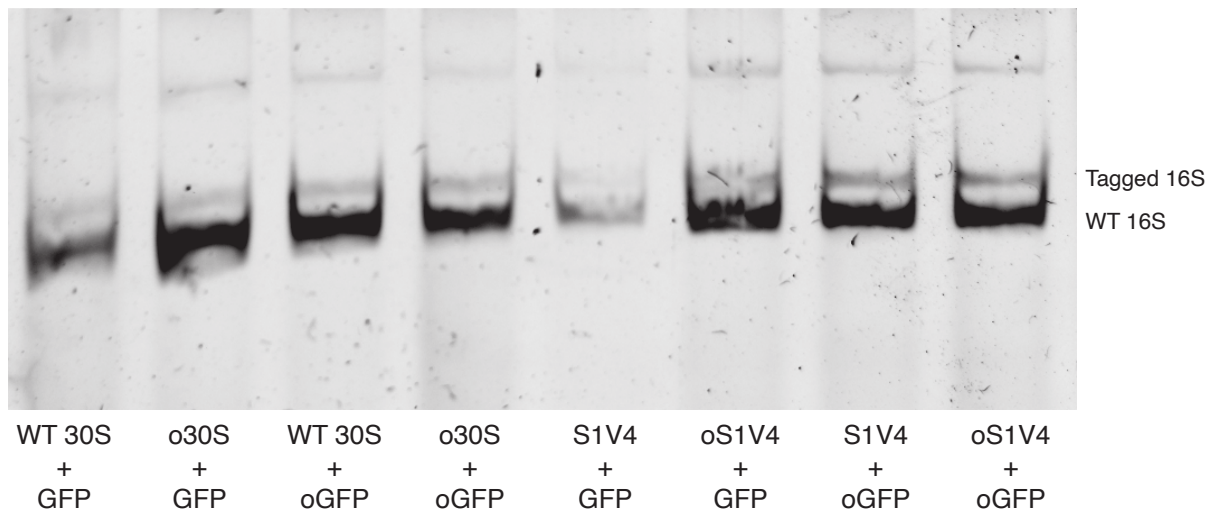


Figure 5. RT-PCR of mutant ribosomes. 5% TBE gel of RT-PCR of mutant ribosomes used in *in vivo* experiments. RNA was extracted from cells four hours after induction with IPTG and arabinose.

The lower band indicates wild-type 16S and the higher band is from the tagged 16S. The expected difference is 35 base pairs. Expression was quantified by integrating the gel bands, excluding the edges.

2.3 Discussion and conclusion

While orthogonal ribosomes play an essential role in efforts to expand the genetic code, they are limited by Shine-Dalgarno independent translation. This has been shown by single molecule tracking of orthogonal ribosomes in live cells, which revealed that 30S subunits, regardless of having an orthogonal ASD, still translate endogenous mRNAs.¹¹ This is further supported by ribosome profiling experiments, using ribosomes with altered SD sequences, where it was shown that the SD sequence does not dictate the ability of the ribosome to translate endogenous mRNA.¹² One possible reason for the cross-reactivity of endogenous mRNAs with orthogonal ribosomes is the role ribosomal protein bS1 plays in translation initiation. It has been hypothesized that bS1, which harbors nucleic acid binding domains, shuttles mRNAs to the ribosome by binding the 5' region of the mRNA.^{19,18} This could also occur with orthogonal ribosomes, preventing full orthogonality of the translation systems by allowing orthogonal ribosomes to translate endogenous proteins.

Here, we tested the hypothesis that preventing bS1 from binding to the *E. coli* 30S ribosomal subunit would enable orthogonal ribosomes to be more dependent on the mRNA SD sequence. Since bS1 is essential in *E. coli*,¹² we devised a strategy that would leave endogenous bS1 and endogenous ribosomes intact, yet allow us to test the role of bS1 binding to orthogonal ribosomes. In structures of the 30S ribosomal subunit, protein bS1 binds predominantly to ribosomal protein uS2, and extends in the direction of 16S rRNA helix h26. We took inspiration from RNA nanostructures and identified RNA k-turns as promising candidates to insert into h26 to sterically block bS1 from binding uS2.³⁴ We engineered 16S rRNA using a prototypical k-turn in four different extensions of helix h26. Using a combination of biochemistry and cryo-EM, we were able to identify one variant (S1V4) that prevented bS1 binding to the 30S subunit *in vitro* while remaining translationally active, and without substantially perturbing ribosome structure, i.e. uS2 binding.

We assessed the orthogonality of ribosomes harboring S1V4 30S subunits using both *in vitro* and *in vivo* translation reactions,²⁹ and found that ribosome orthogonality is not improved by preventing bS1 binding to the 30S subunit. In fact, the *in vitro* reactions, in which no mRNA competition is involved, suggest preventing bS1 binding to the 30S subunit might actually decrease SD-dependent orthogonality. In addition, preventing bS1 binding to the 30S subunit also decreased translational activity when compared to wild-type 30S subunits. Given the role of bS1 in shuttling mRNA to the ribosome,¹⁷ depleting bS1 may decrease ribosome activity because mRNA is not shuttled to the ribosome as readily.

It is possible that other characteristics of mRNA may have an impact on SD independent translation, independent of the role of bS1. For example, recent studies have shown that adenosines at positions -3 and -6 relative to the start codon, increase translation efficiency.³⁹ This sequence pattern is prevalent in *E. coli*, and could enable orthogonal ribosomes to translate these endogenous mRNAs, regardless of the absence or presence of bS1. Based on a large screen of over 200,000 sequences, unstructured UTRs also increase translation efficiency,⁴⁰ a result confirmed by ribosome profiling experiments.¹² Thus, mRNAs with unstructured UTRs could also enable binding to orthogonal ribosomes. Taken together, these features of mRNA outside of the SD-ASD interaction and independent of bS1 binding to the

30S subunit suggest that further improvements to ribosome orthogonality may require a deeper understanding of translation initiation mechanisms in *E. coli*.

2.4 Materials and Methods

Plasmid preparation

A pLK35 plasmid with an MS2 tagged located in 16S rRNA helix h6 was a gift from the Schepartz lab at UC Berkeley.³⁵ The helix h26 extensions were introduced using the In-Fusion Cloning Kit (Takara Bio) with the primers listed below. The oASD was also cloned into the wild type plasmid as well as S1V4 using the same method and the following primers:

	Forward Primer (5' to 3')	Reverse primer (5' to 3')
S1V1	AAGAACGCTTCGGCGGGAGC CGTGGCTTCCGGAGCTAAC	CGCCGAAGCGTTCTTCGCCA CAACCTCCAAGTCGACATCG
S1V2	AAGAACGCTTCGGCGGGAGC CTGGCTTCCGGAGCTAACGC	CGCCGAAGCGTTCTTCGCC AACCTCCAAGTCGACATCG
S1V3	AAGAACGCTTCGGCGGGAGC CTTGGCTTCCGGAGCTAACGC	CGCCGAAGCGTTCTTCGCC TAACCTCCAAGTCGACATCG
S1V4	AAGAACGCTTCGGCGGGAGC CTGGCTTCCGGAGCTAACGC	CGCCGAAGCGTTCTTCGCC TAACCTCCAAGTCGACATCG
oASD	GGATCTGTGGTCCTTAAAGA AGCGTACTTTGTAGTG	AAGGACCACAGATCCAACCG CAGGTTCCC

Reporter constructs

An oSD was cloned into a nanoluciferase reporter using the In-Fusion Cloning Kit (Takara Bio) and the primers listed below. A GFP gene block with overhangs (Twist) was cloned into plasmid pJH474_pTech_Para-supP (Addgene) using the In-Fusion Cloning Kit.⁴¹ An oSD was cloned into the GFP reporter using the primers listed below.

MBP-MS2 protein expression and purification

A pMAL-c2 plasmid (NEB) encoding an N-terminally 6xHis tagged MBP-MS2 fusion protein was transformed into *E. coli* BL21 cells.⁷ A colony from the transformation was grown overnight in 10 mL of LB broth containing 100 µg/mL ampicillin. The culture was transferred to 1 L of ZYM-5052 autoinduction media⁴² with 100 µg/mL ampicillin and grown overnight at 37 °C with shaking at 175 RPM. Cells were pelleted by centrifugation (6000 xg, 4 °C, 20 minutes), resuspended in lysis buffer (20 mM HEPES pH 7.5, 250 mM KCl, 10 mM imidazole), and then lysed using sonication. The resulting lysate was clarified by centrifugation (33746 xg, 4 °C, 45 minutes) and subsequently filtered through a 0.2 µm filter. The lysate was loaded onto a gravity column with 3 mL of Cobalt Resin (Thermo Scientific). The column was washed four times with three column volumes of lysis buffer. The protein was eluted into 1 mL fractions using an elution buffer (20 mM HEPES pH 7.5, 250 mM KCl, 2 mM BME, 500 mM imidazole). The purified protein was buffer exchanged to MS2-150 Buffer (20 mM HEPES pH 7.5, 150 mM KCl, 1 mM EDTA) and concentrated to 10 mg/mL using 30 kDa spin filters.

Mutant ribosome expression and purification

Plasmids containing the 16S rRNA variants designed to block bS1 binding were transformed into *E. coli* Mach1 competent cells (Thermo Fisher) and cultures were grown overnight in 10 mL of LB broth containing 100 µg/mL ampicillin. Cultures were transferred to

1 L LB containing 100 µg/mL ampicillin and incubated at 37 °C with shaking at 175 RPM. Cultures were induced with Isopropyl β- d-1-thiogalactopyranoside (IPTG) at OD₆₀₀ = 0.4, with a final IPTG concentration of 0.5 mM, for 4 hours. Cells were pelleted, resuspended in ribosome buffer A-1 (20 mM Tris HCl pH 7.5, 100 mM NH₄Cl, 1 mM MgCl₂, 0.5 mM EDTA), and then lysed using sonication. The resulting lysate was clarified by centrifugation (33746 xg, 4 °C, 45 minutes) and loaded into Ti-45 tubes. A sucrose cushion was prepared by adding 24 mL buffer B (20 mM Tris HCl pH 7.5, 0.5 mM EDTA, 100 mM NH₄Cl, 10 mM MgCl₂, 0.5 M sucrose) and 17 mL buffer C (20 mM Tris HCl pH 7.5, 0.5 mM EDTA, 60 mM NH₄Cl, 6 mM MgCl₂, 0.706 M sucrose) to the Ti-45 tubes and the ribosomes were pelleted by centrifugation (27000 RPM or 57,000 xg, 4 °C, 15 hours). Crude ribosomes were resuspended in ribosome buffer A-1, clarified by centrifugation (21130 xg, 4 °C, 25 minutes), and subsequently filtered using a 0.2 µm filter.

Crude ribosomes (>60 mg) were diluted to 15 mg/mL in ribosome buffer A-1, and purified at 4 °C. A 5 mL MBP-trap column (Cytiva) was buffer exchanged to MS2-150 buffer and 10 mg of MBP-MS2 was diluted to 3 mL in MS2-150 buffer before being loaded onto the column. The column was washed with 5 column volumes of ribosome buffer A-1 and crude ribosomes were then slowly loaded onto the column. The column was washed with another 5 column volumes of ribosome buffer A-1 and then 10 column volumes of ribosome buffer A-250 (20 mM Tris HCl pH 7.5, 250 mM NH₄Cl, 1 mM MgCl₂, 0.5 mM EDTA). Ribosomes were eluted into 1 mL fractions using an elution buffer containing 10 mM maltose (20 mM Tris HCl pH 7.5, 100 mM NH₄Cl, 1 mM MgCl₂, 0.5 mM EDTA, 10 mM maltose). The purified ribosomes were buffer exchanged to ribosome buffer A-1, concentrated to 3000 nM using 100 kDa spin filters (using the approximation of 1 A₂₆₀ = 72 nM), and flash frozen in liquid nitrogen.

The extent of bS1 depletion in the wild-type 16S rRNA and the S1 blocking mutants was analyzed by a 15% SDS-PAGE gel. The samples were diluted to 100 nM based on A₂₆₀ absorbance and 10 µL of each sample were heat shocked at 95 °C for five minutes before adding loading dye and loading on the gel. After staining with Coomassie blue dye, the amount of bS1 in each sample was quantified by band intensity using ImageJ. A box was made around the region of each bS1 band as well as above the band and the gel analysis tool was used to plot the intensity. The box above the band was used to subtract the background from the signal. This process was repeated for each lane and the resulting values were normalized to the wild type bS1 value.

Wild type ribosome expression and purification

Wild type 30S and 50S subunits were expressed and purified as previously described.⁴³ Briefly, *E. coli* MRE600 cells were grown for four hours before being pelleted and resuspended in ribosome buffer A-1 and subsequently sonicated. The lysate was clarified by centrifugation and crude ribosomes were isolated using a sucrose cushion at 4 °C. The crude ribosomes were then separated on a 20-40% sucrose gradient using a dissociation buffer (20 mM Tris-HCl pH 7.5, 60 mM NH₄Cl, 1 mM MgCl₂, 5 mM EDTA) and individual subunits were isolated via fractionation. The subunits were buffer exchanged to ribosome buffer A-1 for storage.

Ribosome purity assay

60 µg of purified mutant ribosomes were heated at 95 °C for 5 minutes. Lithium chloride precipitation solution (Invitrogen) was then added at 2X the volume of ribosomes on ice and the samples were incubated at -20 °C for one hour to precipitate the rRNA. The rRNA was pelleted by centrifugation (21130 xg, 4 °C, 25 minutes) and the supernatant was vacuum aspirated. The rRNA was washed with 40 µL 70% ethanol, pelleted by centrifugation (21130

ng, 4 °C, 5 minutes), and the supernatant was vacuum aspirated. The resulting rRNA was resuspended in RNase-free water. Primers flanking the MS2 tag in the 16S rRNA, shown below, were used in a reverse transcription reaction using the OneStep RT-PCR kit (Qiagen). The resulting cDNA was resolved on a 10% TBE-gel and visualized with SYBR Safe DNA Gel Stain. For RT-PCR, 1 mL of cells were spun down after four hours of induction and lysed via a 5 minute heat shock at 98 °C. The debris was pelleted and the RT-PCR was performed as described above.

Reassociation sucrose gradients

20-40% sucrose gradients (20 mM Tris-HCl pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA) were prepared in SW 41 Ti tubes. 500 nM 50S and 1000 nM 30S were incubated at 37 °C for 45 minutes before being added to the gradients (20 mM Tris-HCl pH 7.5, 60 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA). The gradients were run overnight at 4 °C in an SW 41 Ti swinging bucket rotor (Beckman Coulter) and subject to $5.45 \times 10^6 \times$ total centrifugal force (27,000 rpm, 16 hours). The gradients were analyzed on a Biocomp fractionator.

Cryo-EM data acquisition and processing workflow

Movies were collected on a 300 kV Titan Krios microscope with a BIO-energy filter and Gatan K3 camera as previously described,²² using a super-resolution pixel size of 0.41 Å and a physical pixel size of 0.82 Å. SerialEM⁴⁴ was used for automated data collection over a defocus range of -0.5 to -1.5 μm with an electron dose of 40 e-/Å² over 40 frames.

Raw movies were imported into CryoSPARC 4.⁴⁵ The movies were motion corrected using Patch Motion Correction before CTFFind4⁴⁶ was used to estimate the CTFs of micrographs. Particles were picked using Template Picker with a 70S ribosome 2D template. Particles were extracted and Fourier-cropped to 1/8 of the box size. 2D classification was run with 100 classes and 32 classes that were consistent with 70S ribosomes were selected for a second round of 2D classification. The particles were re-extracted and Fourier-cropped to 1/4 of the box size and particles were subjected to heterogeneous refinement using a 70S ribosome map from Watson et al.²² Classes consistent with 70S ribosomes were re-extracted at the full box size and then subjected to homogeneous refinement. For the S1V3 and S1V4 maps, local refinement was done with a mask over the 30S subunit. The pixel sizes for the maps were calibrated using a simulated map from PDB 7K00. For modeling, the coordinates from the 30S subunit in 7K00 were used as an initial model. Real-space refinement in PHENIX was used to refine the model.⁴⁷ Local resolution was determined using Local Resolution Estimation in Relion.⁴⁸ Figures were prepared in ChimeraX.⁴⁹

In vitro translation reactions

An *in vitro* translation reaction using a ΔRibosome PURExpress kit (NEB) was prepared in a total volume of 10 μL containing the following components: 2 μL solution A, 0.63 μL factor mix, 0.13 μL RNase inhibitor, and 25 ng/μL nanoluciferase DNA template. The reaction also included 250 nM 50S ribosomal subunits and 500 nM 30S ribosomal subunits. The remaining volume consisted of Milli-Q water. The reaction was incubated at 37 °C for 45 minutes. 2 μL of the reaction product was combined with 30 μL of a 1:50 dilution of Nano-Glo substrate (Promega) into a 364-well microplate, and luminescence was detected with the Tecan Spark Microplate Reader.

In vivo translation reactions

E. coli NEBExpress cells were co-transformed with two plasmids using heat shock. One plasmid contained an aminoglycoside phosphotransferase gene for kanamycin resistance

and expressed superfolder GFP under control of an arabinose inducible promoter and an orthogonal (p15A) origin of replication. The second plasmid harbored a beta-lactamase gene for ampicillin resistance and expressed an *rrnB* ribosomal RNA operon with MS2-tagged 16S rRNA. Cells were grown overnight in 1 mL of LB containing 100 µg/mL ampicillin and 100 µg/mL kanamycin. 100 µL of the overnight culture was transferred to 3 mL LB containing 100 µg/mL ampicillin and 100 µg/mL kanamycin and allowed to grow until they reached a cell density of $OD_{600} = 0.2$. Cultures were then induced with 0.5 mM IPTG and 0.1% arabinose final concentrations. Cultures were grown for 4 hours at 37°C with shaking at 175 RPM. Cells were then pelleted and resuspended in 250 µL 1x PBS (pH 7.2), which was transferred to a clear 96-well microplate. GFP fluorescence and the OD_{600} were measured with the Tecan Spark Microplate Reader. GFP fluorescence was normalized to the OD_{600} optical density.

2.5 Acknowledgements

The work in this chapter has been published in ACS Biochemistry, “Role of Ribosomal protein bS1 in Orthogonal mRNA Start Codon Selection”.⁵⁴

Chapter 3: Engineering approaches of the B3 bridge

3.1 Introduction

Currently, engineering orthogonal ribosomes rely on a covalent linkage between the 16S and 23S rRNA that physically link the 30S and 50S subunits together.^{14,13} This allows for the orthogonal ribosomes to function in the cell parallel to the endogenous ribosomes, but with minimal interruption of the endogenous translational machinery. However, in practice, these ribosomes have decreased overall activity and they are limited by their capacity to cross-assemble with endogenous ribosomal subunits.⁵⁰ Transcribing the 23S and 16S rRNA as a single transcript may also prevent proper ribosome biogenesis and lead to improper folding of the subunits.⁵⁰ While these ribosomes can incorporate non-canonical amino acids to some extent, they are limited by their capacity to cross-assemble with endogenous subunits (Figure 6A).¹⁵

To address the challenge of cross-assembly with endogenous ribosomal subunits as well prevent mis-assembly of the subunits, we proposed a novel strategy to enhance ribosome orthogonality by targeting a key rRNA contact, a “bridge” that connects the small and large ribosomal subunits. In bacteria, there are twelve such inter-subunit bridges, six of which are formed between rRNA elements and are potential sites for creating orthogonal ribosomes. We focused on bridge B3, located at the core of the ribosomal subunit interface and crucial for subunit association. Bridge B3 is formed through two conserved A-minor interactions between nucleotides A1418 and A1483 in helix h44 of 16S rRNA and helix H71 of the 23S rRNA. Based on prior structural and experimental evidence, we predicted that substituting A1418 and/or A1483 with guanosine (A1418G and A1483G) would sterically hinder subunit pairing. Our hypothesis was that these mutations would prevent the orthogonal 16S rRNA from associating with wild-type 23S rRNA, allowing us to engineer a matching orthogonal 23S rRNA. To do this, we solved the structure of the 70S ribosome with the A1418G 30S subunit and the wild-type 50S subunit, and rationally designed a 50S mutant that restored activity of A1418G 30S subunits. In addition, we created a sequence library targeting the nine nucleotides in the 23S rRNA involved in the B3 bridge and screened for functional variants *in vitro*.

3.2 Results

Previous studies show modification of the N1 position of A1418 and A1483 in the 16S rRNA using DMS prevent 70S formation.²¹ To mimic these results, we made A to G point mutations at position 1418, 1483, as well as combined both 1418 and 1483 in 16S rRNA. In the wild-type rRNA, A1418 and A1483 in the 16S rRNA interact with bases G1948 and G1959 on the 23S rRNA, respectively (Figure 6B). We hypothesized that A to G mutations would have a similar effect on 70S formation with wild-type 50S due to the exocyclic amine group at N2 in a guanosine at positions A1418 and/or A1483 causing a steric clash with G1948 or G1959 on the 23S rRNA side (Figure 6C).

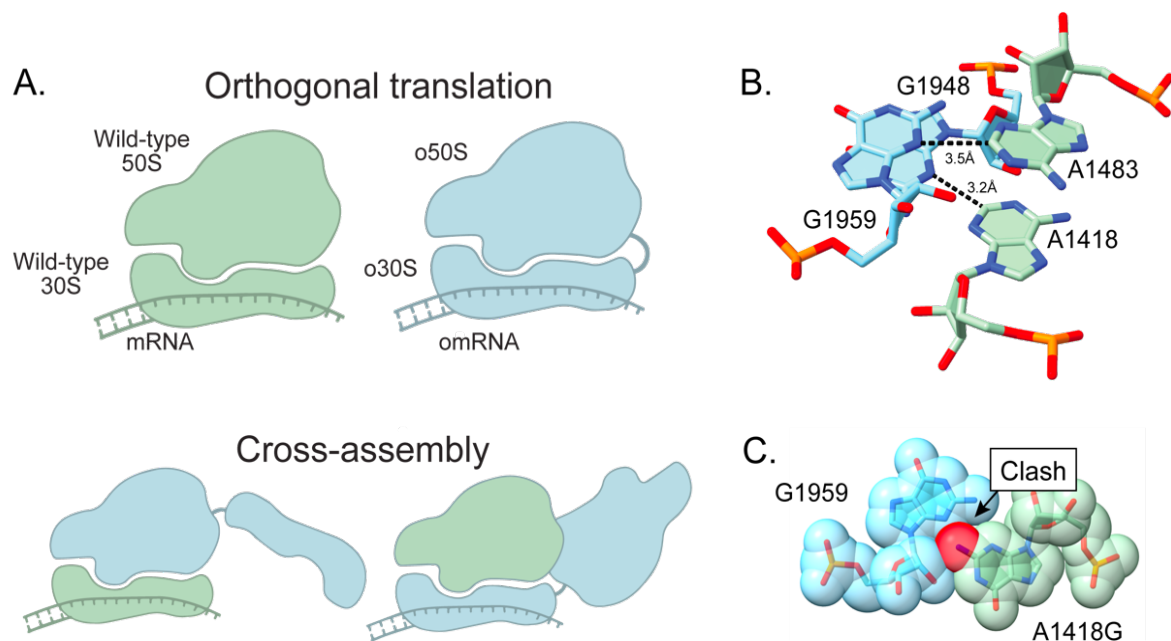


Figure 6. Cross-assembly of tethered ribosomes and proposed mutations. A. Model of orthogonal translation with tethered ribosomes and potential cross assembly with endogenous subunits. Orthogonal subunits are shown in blue and wild-type subunits are shown in green. B. Structure of the wild-type B3 bridge (PDB 7K00). 16S bases A1418 and A1483 are shown in green and 23S bases G1959 and G1948 are shown in blue. B. Model of the A1418G point mutation showing a steric clash with base G1959.

The extent of 70S formation with wild-type 50S subunits was determined by reassociation sucrose gradients using 10 mM MgCl_2 and excess 30S subunits. As hypothesized, mutant 30S subunits A1418G, A1483G, as well as the double mutant, prevented 70S formation with wild-type 50S subunits (Figure 7A). To assess whether these mutants are translationally active, we tested their activity using a PURE *in vitro* transcription and translation (IVTT) system with a nanoluciferase reporter (Figure 7B). The resulting activity assays revealed negligible activity for A1483G and double-mutant 30S subunits, and with some activity above background for the A1418G mutant, consistent with the reassociation gradients. We believe the remaining activity for A1418G 30S subunits may be a result of assisted 70S formation by tRNA, mRNA, and translation factors.

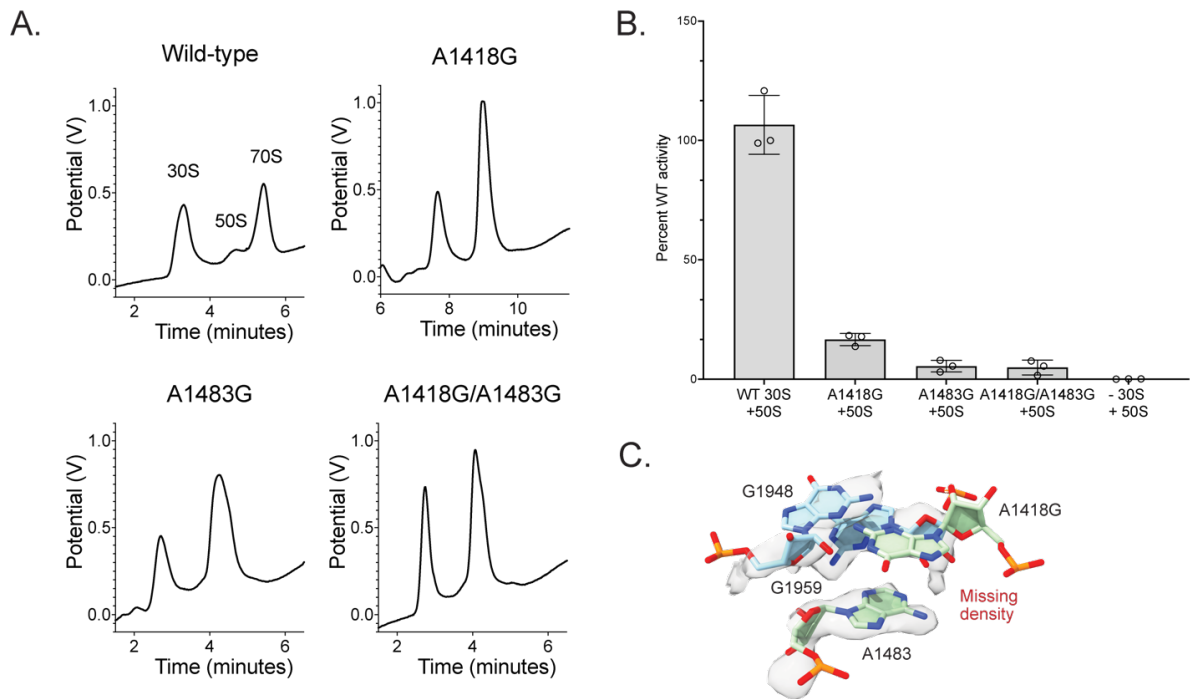


Figure 7. Characterization of the 16S rRNA B3 mutants. A. Reassociation sucrose gradients of wild-type, A1418G, A1483G, and A1418G/A1483G 30S subunits with 1000 nM 30S subunit, 500 nM 50S subunit, and 10 mM MgCl₂. B. Nanoluciferase *in vitro* translation assay of wild-type, A1418G, A1483G, and A1418G/A1483G 30S subunits with wild-type 50S subunits. The activity is normalized to wild-type ribosomes. Error bars indicate the standard deviation for n=3 measurements.

Using 20 mM MgCl₂, we forced association of A1418G 30S and wild-type 50S subunits to determine the cryo-EM structure of the resulting 70S ribosome. The structure revealed well resolved density for surrounding bases, including A1483, G1948, and G1959 (Figure 7C). However, the density for A1418G is missing, indicating the point mutation causes disorder in this region (Figure 7C). Taken together, these results indicate that 70S formation with wild-type 50S subunits can be disturbed with single point mutations at A1418 and A1483 that cause disorder in the 16S rRNA.

We hypothesized that we could rationally design compensatory mutations on the 23S rRNA side of the bridge based on the structure of A1418G-containing 70S ribosomes. The model suggests that the additional exocyclic amine group introduced by the A1418G mutation causes disorder in the surrounding region (Figure 7C), particularly near position 1948 in 23S rRNA. We reasoned that introducing mutations at position 1948 to accommodate A1418G might help restore some functional activity. Furthermore, since position 1948 is stabilized by interactions with nucleotide 1958,²⁰ we ensured that these interactions would remain intact. We designed three different mutants that introduce point mutations at positions 1948 and 1958 that we called the UG mutant (G1948U/C1958G), AU mutant (G1948A/C1958U), and the UA mutant (G1948U/C1958A) (Figure 8A).

To determine the extent of orthogonality that the rationally designed mutants have with A1418G and with wild-type 30S subunits, we tested the mutants using an *in vitro* translation reaction. The activity assay revealed that the UG mutant and the AU mutants were not active with either A1418G or the wild-type 30S subunit (Figure 8B). The UA mutant was active with the A1418G 30S subunit and restored activity to about 30% of wild-type activity (Figure 8C). However, the UA mutant is also active with wild-type 30S subunits (Figure 8C). These data

are further supported by reassociation gradients that show 70S formation of the UA mutant with both A1418G and wild-type 30S subunits (Figure 8D).

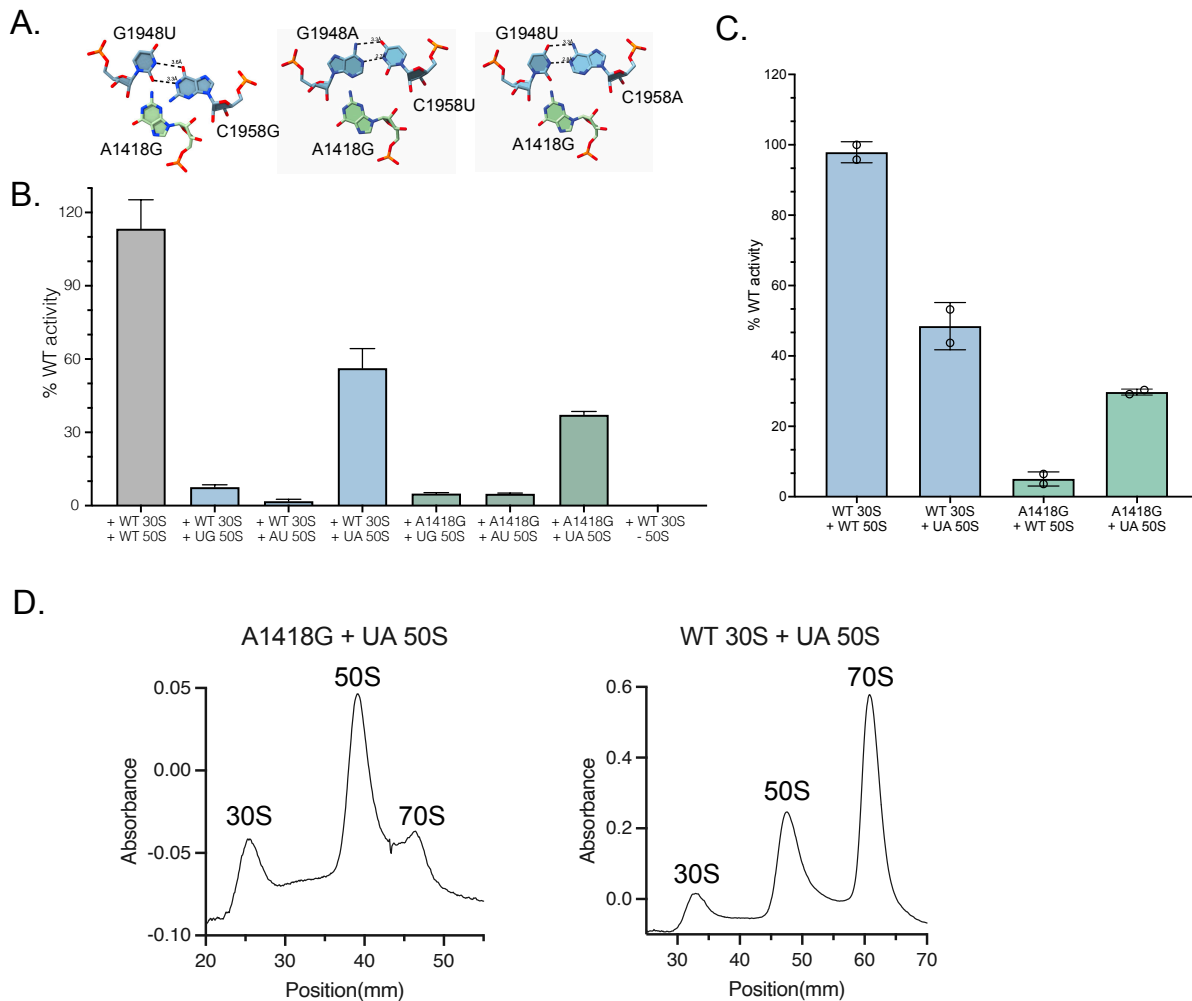


Figure 8. Characterization of the rationally designed 23S rRNA B3 mutants. A. Model of the rationally designed mutants, UG, AU, and UA. The 23S bases are shown in blue and the 16S base, A1418G, is shown in green. The models were made based on a previously determined structure (PDB 7K00). B. Nanoluciferase *in vitro* translation assay of wild-type 30S with wild-type 50S subunit (gray), and the UG, AU, or UA mutant 50S subunits, with either wild-type 30S (blue) or A1418G (green) subunits. Error bars indicate the standard deviation for n=3 measurements and the activity is normalized to wild-type translation. C. Nanoluciferase *in vitro* translation assay of wild-type 30S (blue) and A1418G (green) 30S subunits with either wild-type 50S or the UA 50S mutant subunits. Error bars indicate the standard deviation for n=2 measurements and the activity is normalized to wild-type translation. D. Reassociation sucrose gradient with A1418G with UA 50S subunits (top) and wild-type 30S with UA 50S subunits (bottom). Gradients were run with 1000 nM 30S subunit, 500 nM 50S subunit, and 10 mM MgCl₂.

Taken together, these data indicate that, based on the cryo-EM structure of the 70S ribosome composed of A1418G 30S and wild-type 50S subunits, we can rationally design compensatory mutations in the 23S rRNA to restore partial activity of A1418G 30S subunits. While the UA mutant is active with the A1418G mutation, it is also active with wild-type 30S subunits rendering only partial orthogonality. While we did not achieve full orthogonality using a rationally designed approach the results showed that activity can be restored in the A1418G mutant 30S subunits.

After we had established that we could successfully prevent 70S formation with wild-type 50S subunits using point mutations in the 16S rRNA bridge and can achieve partial orthogonality, we then sought to engineer compensatory mutations using a library approach. We introduced a randomized library in 23S rRNA by PCR amplification with a forward primer that had Ns spanning positions U1946-G1949 and C1957-C1961 (Figure 9A). The phylogenetically conserved bases between 1950 and 1956 were kept constant and are not involved in the formation of the B3 bridge.²⁰ The library was cloned into a plasmid with an MS2 tagged 23S rRNA and transformed into *E. coli*, and the resulting transformants were pooled. The resulting cells were induced to express 23S rRNA. The MS2-tagged 50S subunits were purified, and cDNA amplified from the B3 region was sent for deep sequencing to ensure library diversity. The input library, out of 105,634 total reads, had 4593 unique sequences. To select for variants in the library that are active, we used a SecM stalling sequence with an N-terminal FLAG tag.⁵¹ We hypothesized that 50S mutants that are able to translate with A1418G, A1483G, A1418/1483G, or wild-type 30S subunits would be stalled at the SecM stalling sequence and could be pulled down using the N-terminal FLAG tag while the sequences that are not active would be discarded in the flow through and washes.

An *in vitro* translation reaction using PURE Express and a SecM stalling DNA template was carried out with the purified B3 library, alongside IVTs with A1418G, A1483G, A1418/1483G, or wild-type 30S subunits. After the translation reaction, stalled ribosomes were isolated using anti-FLAG magnetic beads. The rRNA of the B3 region in the flow through, washes, and elutions, were amplified using RT-PCR and run on a 10% agarose gel (Figure 9B). The expected PCR product in the elutions were excised and sent for deep sequencing.

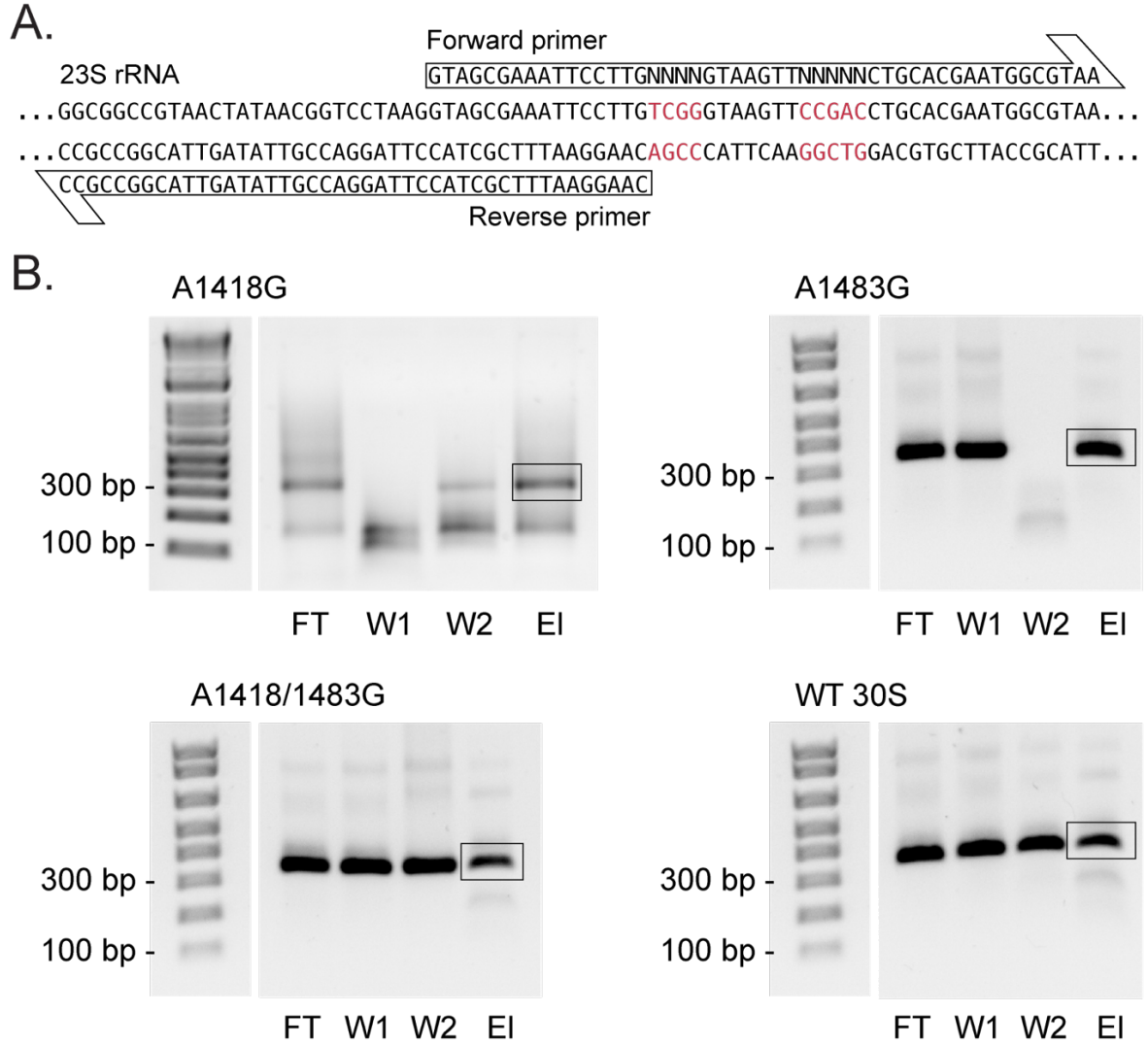


Figure 9. Primer design for library introduction and RT-PCR gels. A. The B3 library was introduced in the 23S using PCR with randomized nucleotides, while maintaining the conserved GUAAGUU region. The randomized nucleotides are shown as Ns and the corresponding nucleotides in the wild-type 23S rRNA are shown in red. B. 10% agarose gel of the SecM stalling pull down showing the flow through (FT), wash 1 and wash 2 (W1 and W2), and the elution (EI). The expected PCR product is 322 bp and the regions indicated in the black squares were extracted for deep sequencing. All lanes were loaded with normalized amounts of input RNA.

We cloned two of the top hits in the A1418G IVT (Figure 10A). Interestingly, the wild-type 23S rRNA sequence was not found in the input library, however, it was found as the second hit in the A1418G SecM stalling sequences. We hypothesize that this may be carry over of wild-type 50S subunits from purification of the A1418G mutant. We tested the top hits in *in vitro* translation reaction assays using full length nanoluciferase (Figure 10B). Unfortunately, the two hits were not translationally active with A1418G 30S subunits (Figure 10B). We hypothesized that this may be due to the ability of the “inactive” 50S subunits and 30S subunits to associate on the mRNA at the start codon, but not to be able to translate the full SecM polypeptide. This would result in the inactive complexes copurifying with active ribosomes on the same mRNA that are pulled down with the SecM FLAG peptide.

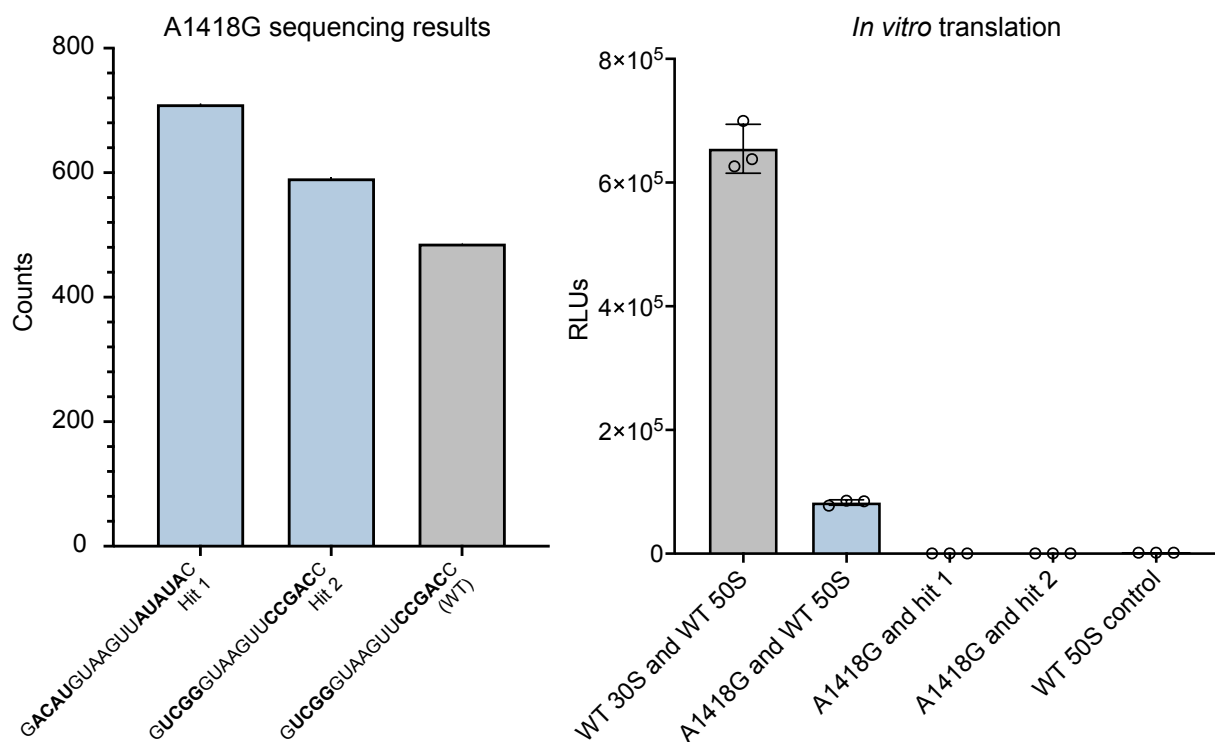


Figure 10. SecM stalling results of the B3 library with A1418G. A. Number of sequences in the A1418G SecM IVT in the two top sequences (hit 1 and hit 2) shown in blue and the wild-type sequence frequency, shown in gray. B. Nanoluciferase in vitro translation of wild-type 30S with wild-type 50S (gray), and A1418G with wild-type 50S, hit 1, and hit 2 (blue). Error bars indicate the standard deviation for $n=3$.

We hypothesized we could deplete ribosomes that associate with mRNA but that are inactive or poorly active in translation elongation, based on the fact they would reside 5' of the active ribosome stalled at the 3' end of the SecM mRNA. To do so, we introduced an RNase H treatment step after the IVT of the SecM polypeptide, but before the FLAG purification. The RNase H template targeted the mRNA using a DNA template 36 nucleotides upstream of the A site codon of stalled SecM (5'-GACGGGCGTGCTGAA-3'), which we predicted would reside 5' of the stalled ribosome. To test this strategy, we first tested the RNase H digestion by mixing equimolar amounts of hit 1 and wild-type 50S with A1418G and ran the RT-PCR as previously described, using primers that overlap the MS2 tag in the 23S rRNA (Figure 11A). The samples were run on a 10% TBE gel to distinguish MS2 tagged 23S rRNA (hit 1) or the native 23S rRNA (WT 50S). The resulting gel showed that when hit 1 was incubated with WT 50S and A1418G 30S subunits, all the 50S subunits came out in the washes (Figure 11, left). This supports our hypothesis that the poorly-active hit 1 50S subunit may be loosely associating with the mRNA in complexes with with A1418G 30S subunits, but gets clipped off by RNase H and comes out in the flow-through and wash fractions. By contrast, wild-type 50S and 30S subunits are resistant to the RNase H treatment. While some sample does come through the flow-through and wash fractions, possibly due to removal by RNase H, there is still a significant amount of ribosomes that come out in the elution (Figure 11, right).

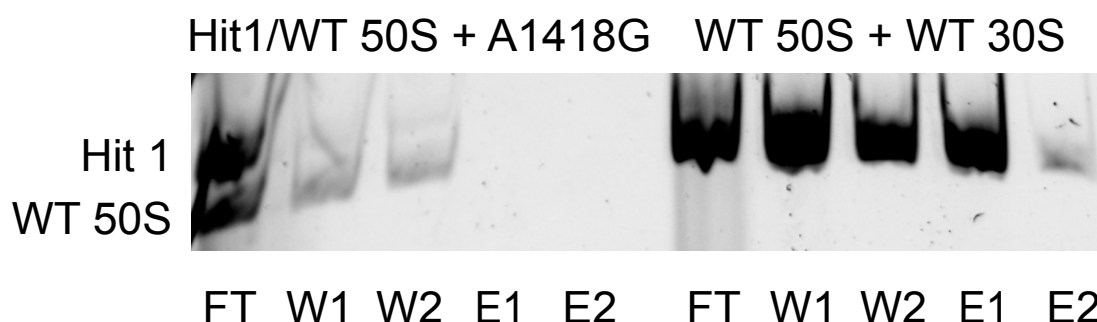


Figure 11. RT-PCR of SecM stalling after RNase H treatment. A. 10% TBE gel of the FLAG pulldown after RNase H treatment. The first five lanes of the gel show the FLAG pull down of the IVT mixture of hit 1 and wild-type 50S subunits with A1418G 30S subunits. The second five lanes are the FLAG pull down of the IVT mixture of wild-type 50S and wild-type 30S subunits as a control. The gel shows the flow through (FT), two washes (W1 and W2), and two elution fractions (E1 and E2). The positions of MS2-tagged hit 1 50S subunit (Hit 1) is labeled in the first sample, as well as the wild-type 50S subunit (WT 50S).

3.3 Discussion and conclusion

Here, we employed a novel strategy to enhance orthogonality of engineered ribosomes by targeting rRNA interactions, specifically bridge B3, rather than relying on a covalent linkage between the 16S and 23S rRNA. We first demonstrated that point mutations at conserved nucleotides A1418 and A1483 in helix h44 of 16S rRNA effectively disrupted 70S formation with wild-type 50S subunits. Cryo-EM analysis of a 70S ribosome composed of A1418G 30S and wild-type 50S subunits revealed local disorder at the B3 interface, supporting our hypothesis that the mutation sterically impairs subunit association.

Using this structural insight, we rationally designed compensatory mutations in the 23S rRNA to accommodate the A1418G mutation in 16S rRNA. Of the three tested mutations, G1948U/C1948A restored partial activity of A1418G 30S subunits, but unfortunately was also active with the wild-type 30S subunit, resulting in only partial orthogonality. While we were able to engineer some activity back into the A1418G 70S complex, we attempted to improve specificity using a library-based approach.

We generated a randomized 23S rRNA library targeting the B3 bridge and used a SecM stalling assay to pull out sequences that can translate with the A1418G, A1483G, and/or the A1418/1483G 30S subunits. Initial deep sequencing analysis of the eluted 70S complexes yielded promising results, but further characterization revealed that these sequences probably belonged to 50S subunits that can associate and initiate on mRNA with A1418G subunits but are not translationally active enough to synthesize a full SecM stalling peptide. By incorporating RNase H digestion targeting the mRNA upstream of the stalling sequence, we were able to optimize conditions that should selectively deplete inactive complexes and allow us to enrich for translationally active mutants in the future.

Overall, our findings demonstrate that bridge B3 is a viable target for orthogonal ribosome engineering. We provide evidence that single-point mutations in conserved rRNA contacts can disrupt 70S complex formation and that partial orthogonality can be achieved using a structure-based approach. We also outline a method for using a library approach to screen for mutants that may achieve full orthogonality. However, the results of this assay remain to be characterized.

Future work could focus on expanding the search space of compensatory 23S rRNA variants using larger or more targeted libraries. The approach could also be expanded by introducing a 16S rRNA B3 library that may further establish orthogonality. Additionally, *in vivo* validation will be necessary to assess orthogonality in the complex cellular environment and determine compatibility with non-canonical translation systems. Ultimately, our approach lays the groundwork for developing truly orthogonal ribosomes that could be used for orthogonal translation and the translation of non-canonical amino acids.

3.4 Materials and methods

Plasmid preparation

MS2-tagged 16S rRNA with A1418G, A1483G, and A1418/1483G mutations were prepared as previously described in chapter 2 “*Plasmid preparation*” using the primers listed below. To make the double mutant, the A1483G primers were used after the A1418G mutation had been introduced. Similarly, MS2-tagged 23S rRNA harboring the UG, AU, and UA mutations were introduced as previously described using the primers listed below.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
A1418G	ACCATGGGGGTGGGTTCG AAAAGAAGTAGGTAGC	ACCCACCCCCATGGT GTGACGGGCG
A1483G	GATTCATGGCTGGGGTGA AGTCGTAACAAGG	CCCCAGCCATGAATCA CAAAGTGGTAAGCGCC
UG	GTCTGGTAAGTTCGGAC CTGCACGAATGGCGT	CCGAACCTACCAGACAAGG AATTTTCGCTACCTTAGGA
AU	GTCAGGTAAGTTCTGAC CTGCACGAATGGCGT	CAGAACTTACCTGACAAGG AATTTTCGCTACCTTAGGA
UA	GTCTGGTAAGTTCAGAC CTGCACGAATGGCGT	CAGAACTTACCTGACAAGG AATTTTCGCTACCTTAGGA

MBP-MS2 protein expression and purification

MBP-MS2 was prepared as described in chapter 2 “*MBP-MS2 protein expression and purification*”.

B3 library plasmid preparation

The B3 library was cloned into a pLK35 plasmid with an MS2-tagged 23S rRNA helix using the primers listed in Figure 3A by using in-fusion cloning.⁵² The 2 μ L of the in-fusion product was transformed into Stellar Competent cells (Takara) and resuspended in 1 mL of SOC. The transformation was plated on two Corning 245 mm Square BioAssay dishes with LB agarose and 100 μ g/mL ampicillin. The resulting transformants were pooled and grown in LB media supplemented with 100 μ g/mL ampicillin overnight. The overnight culture was minipreped and the plasmid was transformed in NEB Express Iq cells and plated on two Corning 245 mm Square BioAssay dishes with LB agarose and 100 μ g/mL ampicillin. The transformants were pooled and a seed culture was grown with 100 μ g/mL ampicillin overnight. Cultures were transferred to 3 L LB containing 100 μ g/mL ampicillin and incubated at 37 °C with shaking at 175 RPM. Cultures were induced with Isopropyl β -d-1-thiogalactopyranoside (IPTG) at OD₆₀₀ = 0.4, with a final IPTG concentration of 0.5 mM, for 4 hours. Cells were pelleted, resuspended in ribosome buffer A-1 (20 mM Tris HCl pH 7.5, 100 mM NH₄Cl, 1 mM MgCl₂, 0.5 mM EDTA), and then lysed using sonication. The B3 library ribosomes were further purified using the method outlined in chapter 2 “*Mutant ribosome expression and purification*”.

To assess library diversity, the rRNA was extracted as described in chapter 2 “*Ribosome purity assay*”. 500 ng of rRNA was added to a One-Step RT-PCR kit (Qiagen) with the following primers that contain Illumina stubs, which are listed in bold. The resulting RT-PCR product was run on a 10% agarose gel and gel extracted (Qiagen) before being submitted for iSeq Illumina deep sequencing.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
B3 amp 150	GCTCTTCCGATCTGGTTAG CGCAAGCGAAGCT	GCTCTTCCGATCTCGCGGGT ACACTGCATCT

Mutant ribosome expression and purification.

A1418G, A1483G, A1418/1483G 30S subunits, as well as the AU, UA, and UG mutant 50S subunits, were expressed and purified as outlined in chapter 2 “*Mutant ribosome expression and purification*”. The extent of wild-type contamination was also determined using the method in chapter 2, “*Ribosome purity assay*”.

Sucrose gradients and in vitro translation reactions

Reassociation sucrose gradients and *in vitro* translation reactions were carried out as described in chapter 2 under “*Reassociation sucrose gradients*” and “*In vitro translation reactions*”.

SecM stalling and selection analysis

A 25 μ L *in vitro* translation reaction was run containing 250 nM B3 library, 250 nM 30S subunit, 10 μ L solution A, 3 μ L factor mix, and 400 ng of SecM stalling template. The mixture was incubated for 2 hours at 37 °C. To deplete 30S subunits that may associate on the 5' mRNA, the IVT reaction was treated with RNase H (NEB) and an antisense DNA oligonucleotide targeting 36 nucleotides upstream of the A site codon (5'-GACGGGCGTGCTGAA-3'). The RNase H treatment was incubated at 37 °C for 20 minutes. 100 μ L of anti-FLAG magnetic beads (Millipore) buffer-exchanged into FLAG buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 250 mM NH₄Cl, 0.5 mM EDTA) were added to the IVT mixtures, and incubated at room temperature for 20 minutes with occasional agitation. The supernatant was collected and the beads were washed with 1.5 mL of FLAG buffer twice. To elute the stalled ribosomes, 100 μ L of FLAG wash buffer with 0.6 mg/mL of 3X FLAG peptide was added to the beads and incubated at room temperature for 20 minutes with occasional agitation. The supernatant was collected from each wash and elution fraction, and 40 μ L of each fraction were heat shocked at 98 °C for 5 minutes. 40 μ L of 5 M LiCl was added to each fraction and incubated at -20 °C for 1 hour. The fractions were pelleted at 14,000 xg for 25 minutes, 4 °C before the supernatant was removed. The remaining pellet was washed with cold 70% ethanol, and the resulting pellet was centrifuged under vacuum for 5 minutes at 45 °C. The pellet was then resuspended in RNase free water and 500 ng of rRNA was added to an RT-PCR reaction (Qiagen) using the following primers to amplify the B3 region of 23S rRNA.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
B3 amp 300	GCTCTTCCGATCTCGGTGTGA CGCCTGCC	GCTCTTCCGATCTTCCTACAC ATCAAGGCTCAATGTTCAGTG

The amplified cDNA was gel extracted and purified as described above. The purified cDNA product was amplified for Illumina deep sequencing using the B3 amp 150 primers listed previously and gel purified before being sent for Illumina deep sequencing.

Deep sequencing analysis

The FASTQ files the input B3 library, as well as from the A1418G, A1483G, A1418/1483G, WT 30S SecM stalling reactions were mapped to a wild-type 42 base reference sequence using Geneious Prime.⁵³ The mapped reads were sorted by frequency and the top 100 sequences were analyzed across all five samples. Listed below are the total number of sequences and the number of unique sequences in each sample.

	Total reads	Number of unique reads
B3 input	105634	4593
A1418G	357742	135546
A1483G	451123	14826
A1418/1483G	593607	17323
WT 30S	537636	14335

Cryo-EM data acquisition and processing workflow

Cryo-EM data of A1418G with wild-type 50S was collected and processed as previously described in chapter 2 under “*Cryo-EM data acquisition and processing workflow*”.

Chapter 4: Engineering approaches for an orthogonal B7a bridge

In addition to engineering the B3 bridge in the 70S ribosome, we attempted to design an orthogonal B7a bridge to prevent cross-assembly with endogenous subunits. Rather than introducing a steric clash, we pursued a phylogenetic engineering approach. Here, we show that this approach can result in functional ribosomes.

4.1 Introduction

Bridge B7a is one of the more peripheral contacts between 23S rRNA and 16S rRNA and forms within 9.4 ms of 70S formation.⁵⁵ The bridge is comprised of A702 in h23 of 16S rRNA, which stacks with A1848 in H68 of 23S rRNA, and also forms an A-minor interaction with positions G1846-C1894 (Figure 12A).²⁰ While A1848 in 23S rRNA is highly conserved, mutation at this base does not affect subunit association.²³ However, upon modification of the N1 position of A702 with DMS, 70S ribosome formation is prevented.^{21,23}

Position 702 is also not phylogenetically conserved across all domains of life and is mostly found as a uradine in archaea and a guanosine in eukaryotes.²⁴ Given that position 702 is essential for subunit association in *E. coli*, we reasoned that making A702U and A702G mutations in the *E. coli* 16S rRNA would prevent binding to wild-type 23S rRNA. We also hypothesized that the 23S side of the B7a bridge could be engineered to bind A702U and A702G mutants by making compensatory mutations found in archaeal and eukaryotic sequences (Figure 12B). Here, we show that partial orthogonality can be achieved using these phylogenetic approaches, but further sequence improvements will need to be made to achieve full orthogonality.

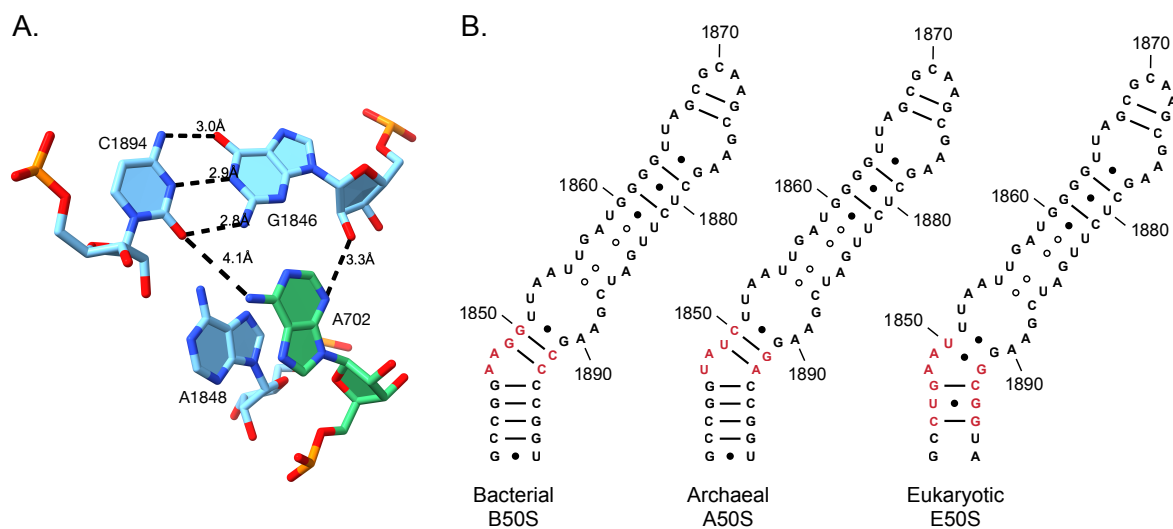


Figure 12. 23S rRNA B7a mutations. A. Interactions in the *E. coli* B7a bridge (PDB 7K00). The 23S sequences are shown in blue and base A702 in the 16S is shown in green. B. Sequences for the 23S side of the B7a bridge are shown for the bacterial (B50S), archaeal (A50S), and eukaryotic (E50S) ribosomes. Bases that differ between the samples are shown in red.

4.2 Results

We first introduced mutations at position 702 in 16S rRNA: A702U and A702G. For clarity, we refer to these as U702, G702, and the wild-type as A702. We then cloned

corresponding B7a bridge mutations into 23S rRNA, referred to as B50S (bacterial), A50S (archaeal), and E50S (eukaryotic) based on the source sequence (Figure 12B).

To test subunit compatibility, each 30S variant (A702, U702, G702) was combined with each 50S variant (B50S, A50S, E50S) and analyzed for 70S complex formation using sucrose gradients (Figure 13A). As expected, the A702/B50S pair showed robust 70S formation. G702 associated with both B50S and A50S but not with E50S, suggesting possible disorder or incompatibility in the E50S construct. U702 showed association with A50S and B50S, indicating that A50S is more permissive to variation at position 702, while E50S is largely inactive regardless of the 16S rRNA sequence.

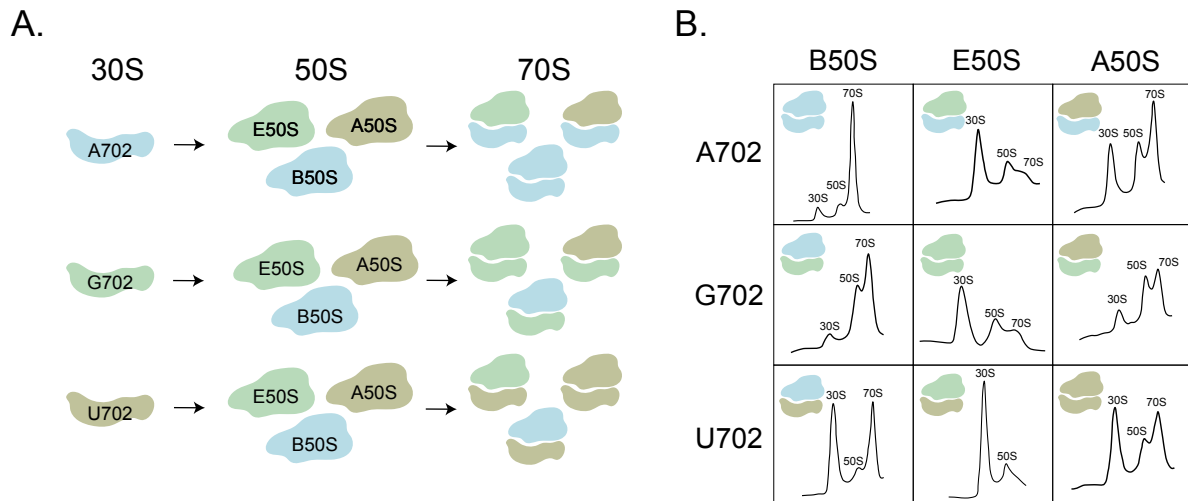


Figure 13. Investigating the orthogonality of the B7a bridge mutants. A. Matrix scheme for analyzing association and activity. Bacterial mutations are shown in blue, eukaryotic mutations are shown in green, and archaeal mutations are shown in brown. The individual small and large subunits were tested in all combinations for complex and activity analysis. B. Association sucrose gradients of the matrix. Subunit association was evaluated using 10 mM MgCl₂, 750 nM 50S subunit, and 1500 nM 30S subunit.

We next corroborated these findings using an *in vitro* nanoluciferase translation assay. To avoid variability due to transcription rate, we used pre-transcribed full-length nanoluciferase mRNA. We also titrated 30S concentration in each reaction to determine whether an optimal concentration could improve orthogonality (Figure 14). The translation results were consistent with the sucrose gradients: E50S showed little to no activity with any 16S rRNA variant, while B50S and A50S supported translation with A702, U702, and G702.

These findings confirm that the E50S construct results in an inactive ribosome, whereas A50S is active and can pair functionally with U702. However, A50S also remains reactive with wild-type bacterial 16S rRNA, indicating that full orthogonality has not yet been achieved and that additional mutations may be required to further restrict cross-reactivity.

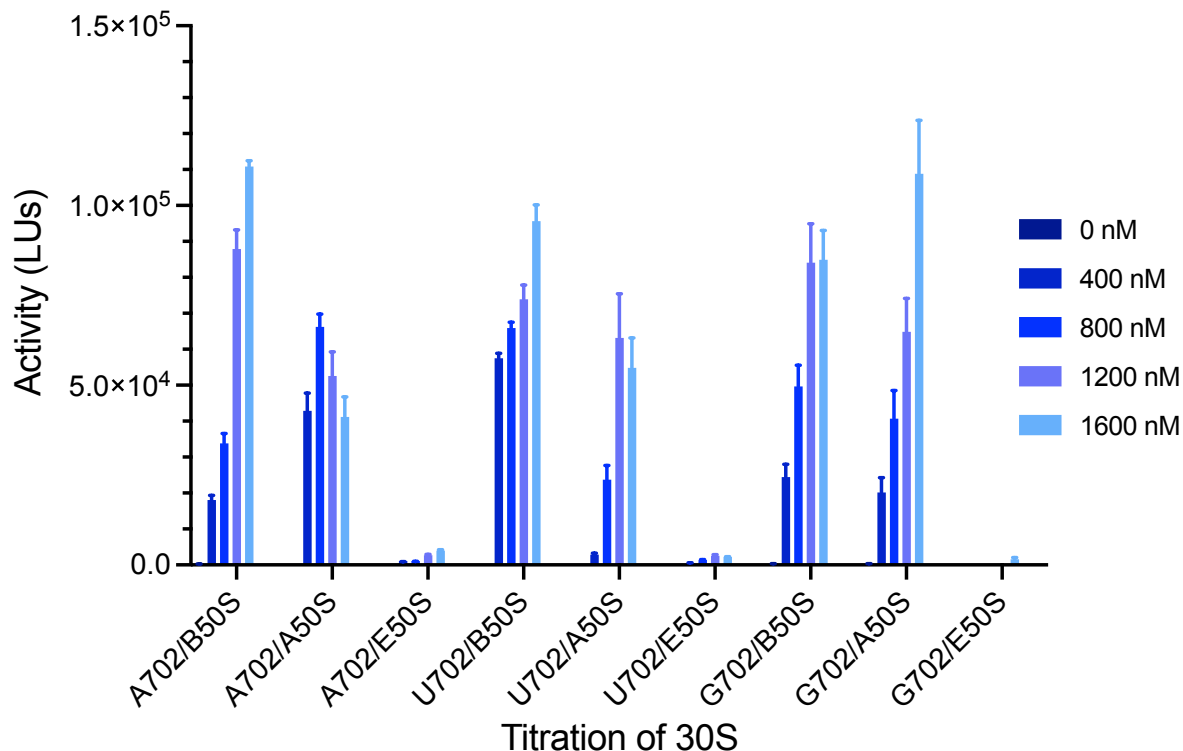


Figure 14. In vitro transcription of nanoluciferase by the B7a bridge mutants.

Luminescence was measured at increasing concentrations of 30S (0-1600 nM) for each set in the matrix. Error bars indicate technical repeats of n=3.

4.3 Discussion and Conclusion

Here we explored a phylogenetically guided approach to engineering the B7a bridge as a means of achieving orthogonality between orthogonal and endogenous ribosomal subunits. While our previous work on bridge B3 relied on introducing steric clashes to prevent subunit association and then using a rationally designed/library search for compensatory mutations, this approach leveraged evolutionary sequence diversity at position 702 of the 16S rRNA and its complementary bases in the 23S rRNA. We hypothesized that incorporating naturally occurring sequence variants from archaeal and eukaryotic ribosomes, we could enable selective association between subunits, thereby generating orthogonal ribosomes.

Our results demonstrate that the B7a bridge can tolerate mutations at A702 and that compensatory mutations in the 23S rRNA based on the archaeal sequences (A50S) can support subunit association and translational activity. Specifically, the U702 mutation in 16S rRNA showed association and activity with both the A50S and B50S. In contrast, the E50S was largely inactive, regardless of the 16S partner, indicating potential misfolding of the 23S rRNA.

Despite the promising pairing of U702 with A50S, we observed significant cross-reactivity between the archaeal and bacterial components, indicating that full orthogonality was not achieved. Both U702 and G702 maintained some capacity to pair with the wild-type bacterial B50S, and A50S was permissive to interaction with multiple 16S variants. These results suggest that the B7a bridge alone may not be sufficient to enforce strict orthogonality, likely due to its peripheral role in subunit stabilization and the compensatory flexibility of other intersubunit contacts.

Additionally, the activity assays reinforced the results of the sucrose gradients. The use of a titration series of 30S concentrations further highlighted that changes in small subunit abundance did not enhance specificity, suggesting that the observed cross-assembly is not simply a concentration-driven effect but rather an intrinsic property of the B7a interface.

Taken together, these findings demonstrate that phylogenetic engineering of the B7a bridge can yield active and partially orthogonal ribosomal pairs. Future efforts may benefit from combining mutations across multiple bridges or incorporating additional structural elements, such as rRNA expansion segments or engineered protein interactions, to increase selectivity and reduce cross reactivity. Moreover, structural studies of the engineered ribosomes may provide further insight into the molecular basis of cross-reactivity and guide the rational design of more isolated ribosomal subunits. This work expands the framework for orthogonal ribosome engineering and illustrates the utility of phylogenetically informed engineering.

4.4 Materials and Methods

Plasmid preparation

MS2-tagged 16S rRNA with the U702 and G702 mutations were prepared as described in chapter 2 under “*Plasmid preparation*” by Hector Torres Vera. MS2-tagged 23S rRNA with the A50S and E50S mutations were prepared as described in chapter 3 under “*Plasmid preparation*” using the primers listed below.

	Forward primer (5' to 3')	Reverse primer (5' to 3')
E50S part 1	GGTGCCTGAATTAATTGA TGGGGTTAGCGCAAGC	ATTAATTCAGGCACCGGGCA GGCGTCACACC
E50S part 2	TTGATCGAAGGCGGTAA ACGGCGG	TTTACCGCCTTCGATCAAGA GCTTCGCTTGC
A50S part 1	TGCCGGTATCTTAATTGATG GGGTTAGCGCAAGCG	ATTAAGATACCGGCACCGGG CAGGCGTCACACC
A50S part 2	ATCGAAGGACCGGTAAACG GCGGCCGTAAC	TACCGGTCCTTCGATCAAGAG CTTCGCTTGC

Mutant ribosome expression and purification.

A702, U702, G702, B50S, A50S, and E50S mutant subunits were expressed and purified as outlined in chapter 2 “*Mutant ribosome expression and purification*”. The extent of wild-type contamination was also determined using the method in chapter 2, “*Ribosome purity assay*”.

Sucrose gradients and in vitro translation reactions

Reassociation sucrose gradients and *in vitro* translation reactions were run as described in chapter 2 under “*Reassociation sucrose gradients*” and “*In vitro translation reactions*” with slight modifications. Reassociation sucrose gradients were run with 750 nM 50S and 1500 nM 30S subunits. The nanoluciferase DNA template was transcribed by T7 polymerase and gel purified before being added to the IVT.

5. Conclusion and Future Directions

The ability to expand the genetic code through orthogonal translation holds immense promise for biotechnology ranging from site-specific incorporation of non-canonical amino acids to the synthesis of entirely new polymers.^{4,5} However, the current bacterial orthogonal ribosomes are limited by their tendency to engage in Shine-Dalgarno independent translation and cross-assemble with endogenous subunits. Here, we sought to explore and address these limitations by using new strategies to enhance ribosome orthogonality at both ribosomal protein and rRNA levels.

We first investigated the role of ribosomal protein bS1 in Shine-Dalgarno independent translation and its potential contribution to the lack of orthogonality in existing systems.⁵⁴ Through a structure-guided approach, we generated a mutant 16S rRNA that sterically blocked bS1 binding to the 30S subunit. Although these ribosomes were translationally active and excluded bS1 binding to the 30S subunit, our *in vitro* and *in vivo* analysis revealed that orthogonality was not significantly improved. These results indicate that other features such as mRNA sequence context or secondary structure likely drive SD-independent translation and will need to be considered in future efforts to enhance orthogonal ribosome performance.

To explore the problem of subunit cross-assembly between orthogonal and endogenous subunits, we pursued a novel strategy targeting ribosomal bridge B3. We demonstrated that, based on previous studies, point mutations in the 16S rRNA could selectively prevent 70S assembly with wild-type 50S subunits. Using cryo-EM and forced association, we confirmed disorder at the interface and identified a rationally designed 23S mutant that partially restored function with the engineered 30S. While this mutant also retained activity with the wild-type 30S, resulting in only partial orthogonality, the approach validated the B3 bridge as a viable engineering target. We further extended this concept by constructing a 23S rRNA library in bridge B3 nucleotides and implementing a SecM stalling based functional selection assay, coupled with RNase H treatment to enrich for truly orthogonal ribosome pairs. This platform provides a tool for the discovery of subunit specific pairs that may ultimately enable a fully orthogonal ribosome.

Finally, we extended our bridge-targeting strategy for orthogonality to the B7a bridge using a phylogenetically guided approach. Guided by evolutionary sequence variation at position 702 of 16S rRNA, we introduced substitutions (A702U and A702G) designed to sterically disrupt B7a formation with the wild-type 23S rRNA. We then paired these 30S mutants with compensatory mutations on the 23S side of the bridge based on natural covariation observed in archaea and eukaryotes. While the B7a mutants were not as extensively characterized as those of B3, the strategy represents an orthogonal and potentially complementary path to developing ribosomes with selective subunit interactions. Together, the B3 and B7a engineering efforts demonstrate that bridge-directed ribosome design is a promising avenue for improving subunit specificity and building modular translation systems. Taken together, the work presented here established a framework for investigation ribosome orthogonality including translation initiation via bS1 exclusion and preventing cross-assembly by engineering the interface of the 23S and 16S rRNA. These complementary strategies highlight the need for holistic approaches that integrate molecular engineering, structural biology, and systems-level understanding of translation.

Moving forward, several avenues can be pursued to build upon the foundation laid by this work. First, further dissection of SD-independent translation mechanisms is warranted.

Systematic characterization of 5' untranslated region (UTR) features including nucleotide context, folding energy, and RNA-protein interactions could reveal sequence rules or structural motifs that drive promiscuous ribosome recruitment. Such insights could inform the design of omRNAs that are more selectively recognized by orthogonal ribosomes, while also enabling predictive modeling of mRNA-translation dynamics.

With the RNase H treatment method in place, the strategy for targeting bridge B3 can now be pursued by performing the SecM stalling experiment on the B3 library with A1418G, A1483G, and/or A1418/1483G mutant 30S subunits. The approach could also be expanded by introducing 16S and 23S rRNA libraries simultaneously that sample a wider sequence space and increase the likelihood of isolating highly specific subunit pairs.

Lastly, it will be critical to test the performance of improved orthogonal ribosomes in the context of ncAA incorporation and non-proteinogenic peptide synthesis. Integration with engineered tRNA/synthetase pairs and evolved translation factors may uncover synergistic effects that improve yield and fidelity. Additionally, *in vivo* evolution or continuous directed evolution strategies could accelerate the development of orthogonal systems optimized for complex translational tasks.

In summary, this work represents a step forward in the engineering of orthogonal ribosomes, offering mechanistic insights and experimental strategies that open the door to more robust platforms. The continued refinement of these systems will be central to unlocking the full potential of genetic code expansion and synthetic translation in living cells.

References

- (1) Kaczanowska, M.; Rydén-Aulin, M. Ribosome Biogenesis and the Translation Process in Escherichia Coli. *Microbiol. Mol. Biol. Rev. MMBR* **2007**, *71* (3), 477. <https://doi.org/10.1128/MMBR.00013-07>.
- (2) Gualerzi, C. O.; Pon, C. L. Initiation of mRNA Translation in Bacteria: Structural and Dynamic Aspects. *Cell. Mol. Life Sci. CMLS* **2015**, *72* (22), 4341. <https://doi.org/10.1007/s00018-015-2010-3>.
- (3) Pape, T.; Wintermeyer, W.; Rodnina, M. V. Complete Kinetic Mechanism of Elongation Factor Tu-Dependent Binding of Aminoacyl-tRNA to the A Site of the E. Coli Ribosome. *EMBO J.* **1998**, *17* (24), 7490–7497. <https://doi.org/10.1093/emboj/17.24.7490>.
- (4) Birch-Price, Z.; Hardy, F. J.; Lister, T. M.; Kohn, A. R.; Green, A. P. Noncanonical Amino Acids in Biocatalysis. *Chem. Rev.* **2024**, *124* (14), 8740–8786. <https://doi.org/10.1021/acs.chemrev.4c00120>.
- (5) Shandell, M. A.; Tan, Z.; Cornish, V. W. Genetic Code Expansion: A Brief History and Perspective. *Biochemistry* **2021**, *60* (46), 3455–3469. <https://doi.org/10.1021/acs.biochem.1c00286>.
- (6) Ishida, S.; Ngo, P. H. T.; Gundlach, A.; Ellington, A. Engineering Ribosomal Machinery for Noncanonical Amino Acid Incorporation. *Chem. Rev.* **2024**, *124* (12), 7712–7730. <https://doi.org/10.1021/acs.chemrev.3c00912>.
- (7) Ward, F. R.; Watson, Z. L.; Ad, O.; Schepartz, A.; Cate, J. H. D. Defects in the Assembly of Ribosomes Selected for β -Amino Acid Incorporation. *Biochemistry* **2019**, *58* (45), 4494–4504. <https://doi.org/10.1021/acs.biochem.9b00746>.
- (8) Chubiz, L. M.; Rao, C. V. Computational Design of Orthogonal Ribosomes. *Nucleic Acids Res.* **2008**, *36* (12), 4038–4046. <https://doi.org/10.1093/nar/gkn354>.
- (9) Dunkelmann, D. L.; Piedrafita, C.; Dickson, A.; Liu, K. C.; Elliott, T. S.; Fiedler, M.; Bellini, D.; Zhou, A.; Cervettini, D.; Chin, J. W. Adding α,α -Disubstituted and β -Linked Monomers to the Genetic Code of an Organism. *Nature* **2024**, *625* (7995), 603–610. <https://doi.org/10.1038/s41586-023-06897-6>.
- (10) Rackham, O.; Chin, J. W. A Network of Orthogonal ribosome-mRNA Pairs. *Nat. Chem. Biol.* **2005**, *1* (3), 159–166. <https://doi.org/10.1038/nchembio719>.
- (11) Metelev, M.; Lundin, E.; Volkov, I. L.; Gynnå, A. H.; Elf, J.; Johansson, M. Direct Measurements of mRNA Translation Kinetics in Living Cells. *Nat. Commun.* **2022**, *13* (1), 1852. <https://doi.org/10.1038/s41467-022-29515-x>.
- (12) Saito, K.; Green, R.; Buskirk, A. R. Translational Initiation in E. Coli Occurs at the Correct Sites Genome-Wide in the Absence of mRNA-rRNA Base-Pairing. *eLife* **2020**, *9*, e55002. <https://doi.org/10.7554/eLife.55002>.
- (13) Schmied, W. H.; Tnimov, Z.; Uttamapinant, C.; Rae, C. D.; Fried, S. D.; Chin, J. W. Controlling Orthogonal Ribosome Subunit Interactions Enables Evolution of New Function. *Nature* **2018**, *564* (7736), 444–448. <https://doi.org/10.1038/s41586-018-0773-z>.
- (14) Fried, S. D.; Schmied, W. H.; Uttamapinant, C.; Chin, J. W. Ribosome Subunit Stapling for Orthogonal Translation in E. Coli. *Angew. Chem. Int. Ed.* **2015**, *54* (43), 12791–12794. <https://doi.org/10.1002/anie.201506311>.

- (15) Aleksashin, N. A.; Szal, T.; d'Aquino, A. E.; Jewett, M. C.; Vázquez-Laslop, N.; Mankin, A. S. A Fully Orthogonal System for Protein Synthesis in Bacterial Cells. *Nat. Commun.* **2020**, *11* (1), 1858. <https://doi.org/10.1038/s41467-020-15756-1>.
- (16) Delvillani, F.; Papiani, G.; Dehò, G.; Briani, F. S1 Ribosomal Protein and the Interplay between Translation and mRNA Decay. *Nucleic Acids Res.* **2011**, *39* (17), 7702–7715. <https://doi.org/10.1093/nar/gkr417>.
- (17) Tedin, K.; Resch, A.; Bläsi, U. Requirements for Ribosomal Protein S1 for Translation Initiation of mRNAs with and without a 5' Leader Sequence. *Mol. Microbiol.* **1997**, *25* (1), 189–199. <https://doi.org/10.1046/j.1365-2958.1997.4421810.x>.
- (18) Loveland, A. B.; Korostelev, A. A. Structural Dynamics of Protein S1 on the 70S Ribosome Visualized by Ensemble Cryo-EM. *Methods* **2018**, *137*, 55–66. <https://doi.org/10.1016/j.ymeth.2017.12.004>.
- (19) D'Urso, G.; Chat, S.; Gillet, R.; Giudice, E. Structural Insights into the Binding of bS1 to the Ribosome. *Nucleic Acids Res.* **2023**, *51* (7), 3410–3419. <https://doi.org/10.1093/nar/gkad126>.
- (20) Liu, Q.; Fredrick, K. Intersubunit Bridges of the Bacterial Ribosome. *J. Mol. Biol.* **2016**, *428* (10 Pt B), 2146. <https://doi.org/10.1016/j.jmb.2016.02.009>.
- (21) Pulk, A.; Maiväli, Ü.; Remme, J. Identification of Nucleotides in E. Coli 16S rRNA Essential for Ribosome Subunit Association. *RNA* **2006**, *12* (5), 790–796. <https://doi.org/10.1261/rna.2275906>.
- (22) Watson, Z. L.; Ward, F. R.; Méheust, R.; Ad, O.; Schepartz, A.; Banfield, J. F.; Cate, J. H. Structure of the Bacterial Ribosome at 2 Å Resolution. *eLife* **2020**, *9*, e60482. <https://doi.org/10.7554/eLife.60482>.
- (23) Liiv, A.; O'Connor, M. Mutations in the Intersubunit Bridge Regions of 23 S rRNA*. *J. Biol. Chem.* **2006**, *281* (40), 29850–29862. <https://doi.org/10.1074/jbc.M603013200>.
- (24) Ozer, S.; Doshi, K. J.; Xu, W.; Gutell, R. R. rCAD: A Novel Database Schema for the Comparative Analysis of RNA. In *2011 IEEE Seventh International Conference on eScience*; IEEE: Stockholm, Sweden, 2011; pp 15–22. <https://doi.org/10.1109/eScience.2011.11>.
- (25) Nakagawa, S.; Niimura, Y.; Miura, K.; Gojobori, T. Dynamic Evolution of Translation Initiation Mechanisms in Prokaryotes. *Proc. Natl. Acad. Sci.* **2010**, *107* (14), 6382–6387. <https://doi.org/10.1073/pnas.1002036107>.
- (26) Beck, H. J.; Moll, I. Leaderless mRNAs in the Spotlight: Ancient but Not Outdated! *Microbiol. Spectr.* **2018**, *6* (4), 10.1128/microbiolspec.rwr-0016–2017. <https://doi.org/10.1128/microbiolspec.rwr-0016-2017>.
- (27) Wakabayashi, H.; Warnasooriya, C.; Ermolenko, D. N. Extending the Spacing between the Shine–Dalgarno Sequence and P-Site Codon Reduces the Rate of mRNA Translocation. *J. Mol. Biol.* **2020**, *432* (16), 4612–4622. <https://doi.org/10.1016/j.jmb.2020.06.008>.
- (28) Ringquist, S.; Shinedling, S.; Barrick, D.; Green, L.; Binkley, J.; Stormo, G. D.; Gold, L. Translation Initiation in Escherichia Coli: Sequences within the Ribosome-Binding Site. *Mol. Microbiol.* **1992**, *6* (9), 1219–1229. <https://doi.org/10.1111/j.1365-2958.1992.tb01561.x>.

- (29) Kolber, N. S.; Fattal, R.; Bratulic, S.; Carver, G. D.; Badran, A. H. Orthogonal Translation Enables Heterologous Ribosome Engineering in *E. Coli*. *Nat. Commun.* **2021**, 12 (1), 599. <https://doi.org/10.1038/s41467-020-20759-z>.
- (30) Gutell, R. R.; Lee, J. C.; Cannone, J. J. The Accuracy of Ribosomal RNA Comparative Structure Models. *Curr. Opin. Struct. Biol.* **2002**, 12 (3), 301–310. [https://doi.org/10.1016/S0959-440X\(02\)00339-1](https://doi.org/10.1016/S0959-440X(02)00339-1).
- (31) Ram, J. L.; Karim, A. S.; Sendler, E. D.; Kato, I. Strategy for Microbiome Analysis Using 16S rRNA Gene Sequence Analysis on the Illumina Sequencing Platform. *Syst. Biol. Reprod. Med.* **2011**, 57 (3), 162–170. <https://doi.org/10.3109/19396368.2011.555598>.
- (32) Molinaro, M.; Tinoco, I. Use of Ultra Stable UNCG Tetraloop Hairpins to Fold RNA Structures: Thermodynamic and Spectroscopic Applications. *Nucleic Acids Res.* **1995**, 23 (15), 3056–3063. <https://doi.org/10.1093/nar/23.15.3056>.
- (33) Huang, L.; Lilley, D. M. J. The Kink-Turn in the Structural Biology of RNA. *Q. Rev. Biophys.* **2018**, 51, e5. <https://doi.org/10.1017/S0033583518000033>.
- (34) Liu, D.; Shao, Y.; Piccirilli, J. A.; Weizmann, Y. Structures of Artificially Designed Discrete RNA Nanoarchitectures at Near-Atomic Resolution. *Sci. Adv.* **2021**, 7 (39), eabf4459. <https://doi.org/10.1126/sciadv.abf4459>.
- (35) Youngman, E. M.; Green, R. Affinity Purification of in Vivo-Assembled Ribosomes for in Vitro Biochemical Analysis. *Methods* **2005**, 36 (3), 305–312. <https://doi.org/10.1016/j.ymeth.2005.04.007>.
- (36) Shasmal, M.; Dey, S.; Shaikh, T. R.; Bhakta, S.; Sengupta, J. E. Coli Metabolic Protein Aldehyde-Alcohol Dehydrogenase-E Binds to the Ribosome: A Unique Moonlighting Action Revealed. *Sci. Rep.* **2016**, 6 (1), 19936. <https://doi.org/10.1038/srep19936>.
- (37) Kimanius, D.; Dong, L.; Sharov, G.; Nakane, T.; Scheres, S. H. W. New Tools for Automated Cryo-EM Single-Particle Analysis in RELION-4.0. *Biochem. J.* **2021**, 478 (24), 4169–4185. <https://doi.org/10.1042/BCJ20210708>.
- (38) Liang, S.-T.; Xu, Y.-C.; Dennis, P.; Bremer, H. mRNA Composition and Control of Bacterial Gene Expression. *J. Bacteriol.* **2000**, 182 (11), 3037–3044. <https://doi.org/10.1128/jb.182.11.3037-3044.2000>.
- (39) Baez, W. D.; Roy, B.; McNutt, Z. A.; Shatoff, E. A.; Chen, S.; Bundschuh, R.; Fredrick, K. Global Analysis of Protein Synthesis in *Flavobacterium Johnsoniae* Reveals the Use of Kozak-like Sequences in Diverse Bacteria. *Nucleic Acids Res.* **2019**, 47 (20), 10477–10488. <https://doi.org/10.1093/nar/gkz855>.
- (40) Cambray, G.; Guimaraes, J. C.; Arkin, A. P. Evaluation of 244,000 Synthetic Sequences Reveals Design Principles to Optimize Translation in *Escherichia Coli*. *Nat. Biotechnol.* **2018**, 36 (10), 1005–1015. <https://doi.org/10.1038/nbt.4238>.
- (41) Ho, J. M. L.; Miller, C. A.; Parks, S. E.; Mattia, J. R.; Bennett, M. R. A Suppressor tRNA-Mediated Feedforward Loop Eliminates Leaky Gene Expression in Bacteria. *Nucleic Acids Res.* **2021**, 49 (5), e25. <https://doi.org/10.1093/nar/gkaa1179>.
- (42) Studier, F. W. Stable Expression Clones and Auto-Induction for Protein Production in *E. Coli*. In *Structural Genomics*; Chen, Y. W., Ed.; Methods in Molecular Biology; Humana Press: Totowa, NJ, 2014; Vol. 1091, pp 17–32. https://doi.org/10.1007/978-1-62703-691-7_2.
- (43) Travin, D. Y.; Watson, Z. L.; Metelev, M.; Ward, F. R.; Osterman, I. A.; Khven, I. M.; Khabibullina, N. F.; Serebryakova, M.; Mergaert, P.; Polikanov, Y. S.; Cate, J. H. D.;

- Severinov, K. Structure of Ribosome-Bound Azole-Modified Peptide Phazolicin Rationalizes Its Species-Specific Mode of Bacterial Translation Inhibition. *Nat. Commun.* **2019**, *10* (1), 4563. <https://doi.org/10.1038/s41467-019-12589-5>.
- (44) Schorb, M.; Haberbosch, I.; Hagen, W. J. H.; Schwab, Y.; Mastronarde, D. N. Software Tools for Automated Transmission Electron Microscopy. *Nat. Methods* **2019**, *16* (6), 471–477. <https://doi.org/10.1038/s41592-019-0396-9>.
- (45) Punjani, A.; Rubinstein, J. L.; Fleet, D. J.; Brubaker, M. A. cryoSPARC: Algorithms for Rapid Unsupervised Cryo-EM Structure Determination. *Nat. Methods* **2017**, *14* (3), 290–296. <https://doi.org/10.1038/nmeth.4169>.
- (46) Rohou, A.; Grigorieff, N. CTFFIND4: Fast and Accurate Defocus Estimation from Electron Micrographs. *J. Struct. Biol.* **2015**, *192* (2), 216–221. <https://doi.org/10.1016/j.jsb.2015.08.008>.
- (47) Liebschner, D.; Afonine, P. V.; Baker, M. L.; Bunkóczi, G.; Chen, V. B.; Croll, T. I.; Hintze, B.; Hung, L.-W.; Jain, S.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R. D.; Poon, B. K.; Prisant, M. G.; Read, R. J.; Richardson, J. S.; Richardson, D. C.; Sammito, M. D.; Sobolev, O. V.; Stockwell, D. H.; Terwilliger, T. C.; Urzhumtsev, A. G.; Videau, L. L.; Williams, C. J.; Adams, P. D. Macromolecular Structure Determination Using X-Rays, Neutrons and Electrons: Recent Developments in Phenix. *Acta Crystallogr. Sect. Struct. Biol.* **2019**, *75* (10), 861–877. <https://doi.org/10.1107/S2059798319011471>.
- (48) Kimanius, D.; Dong, L.; Sharov, G.; Nakane, T.; Scheres, S. H. W. New Tools for Automated Cryo-EM Single-Particle Analysis in RELION-4.0. *Biochem. J.* **2021**, *478* (24), 4169–4185. <https://doi.org/10.1042/BCJ20210708>.
- (49) Goddard, T. D.; Huang, C. C.; Meng, E. C.; Pettersen, E. F.; Couch, G. S.; Morris, J. H.; Ferrin, T. E. UCSF ChimeraX: Meeting Modern Challenges in Visualization and Analysis. *Protein Sci.* **2018**, *27* (1), 14–25. <https://doi.org/10.1002/pro.3235>.
- (50) Carlson, E. D.; d’Aquino, A. E.; Kim, D. S.; Fulk, E. M.; Hoang, K.; Szal, T.; Mankin, A. S.; Jewett, M. C. Engineered Ribosomes with Tethered Subunits for Expanding Biological Function. *Nat. Commun.* **2019**, *10* (1), 3920. <https://doi.org/10.1038/s41467-019-11427-y>.
- (51) Gersteuer, F.; Morici, M.; Gabrielli, S.; Fujiwara, K.; Safdari, H. A.; Paternoga, H.; Bock, L. V.; Chiba, S.; Wilson, D. N. The SecM Arrest Peptide Traps a Pre-Peptide Bond Formation State of the Ribosome. *Nat. Commun.* **2024**, *15* (1), 2431. <https://doi.org/10.1038/s41467-024-46762-2>.
- (52) Nissley, A. J.; Kamal, T. S.; Cate, J. H. D. Interactions between Terminal Ribosomal RNA Helices Stabilize the E. Coli Large Ribosomal Subunit. *RNA* **2023**, *29* (10), 1500–1508. <https://doi.org/10.1261/rna.079690.123>.
- (53) Geneious | Bioinformatics Software for Sequence Data Analysis. Geneious. <https://www.geneious.com/> (accessed 2025-05-04).
- (54) Boyko, K. V.; Bernstein, R. A.; Kim, M.; Cate, J. H. D. Role of Ribosomal Protein bS1 in Orthogonal mRNA Start Codon Selection. *Biochemistry* **2025**, *64* (3), 710–718. <https://doi.org/10.1021/acs.biochem.4c00688>.
- (55) Shaikh, T. R.; Yassin, A. S.; Lu, Z.; Barnard, D.; Meng, X.; Lu, T.-M.; Wagenknecht, T.; Agrawal, R. K. Initial Bridges between Two Ribosomal Subunits Are Formed within 9.4 Milliseconds, as Studied by Time-Resolved Cryo-EM. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (27), 9822–9827. <https://doi.org/10.1073/pnas.1406744111>.