# Orthogonal Translation Enables Heterologous Ribosome Engineering in *E. coli*

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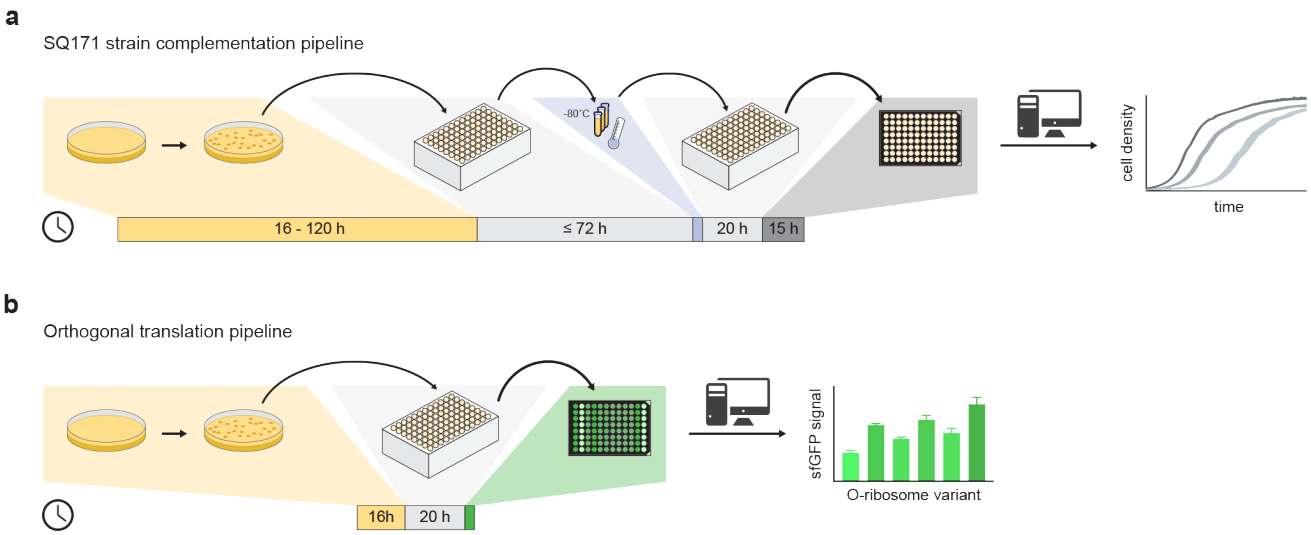
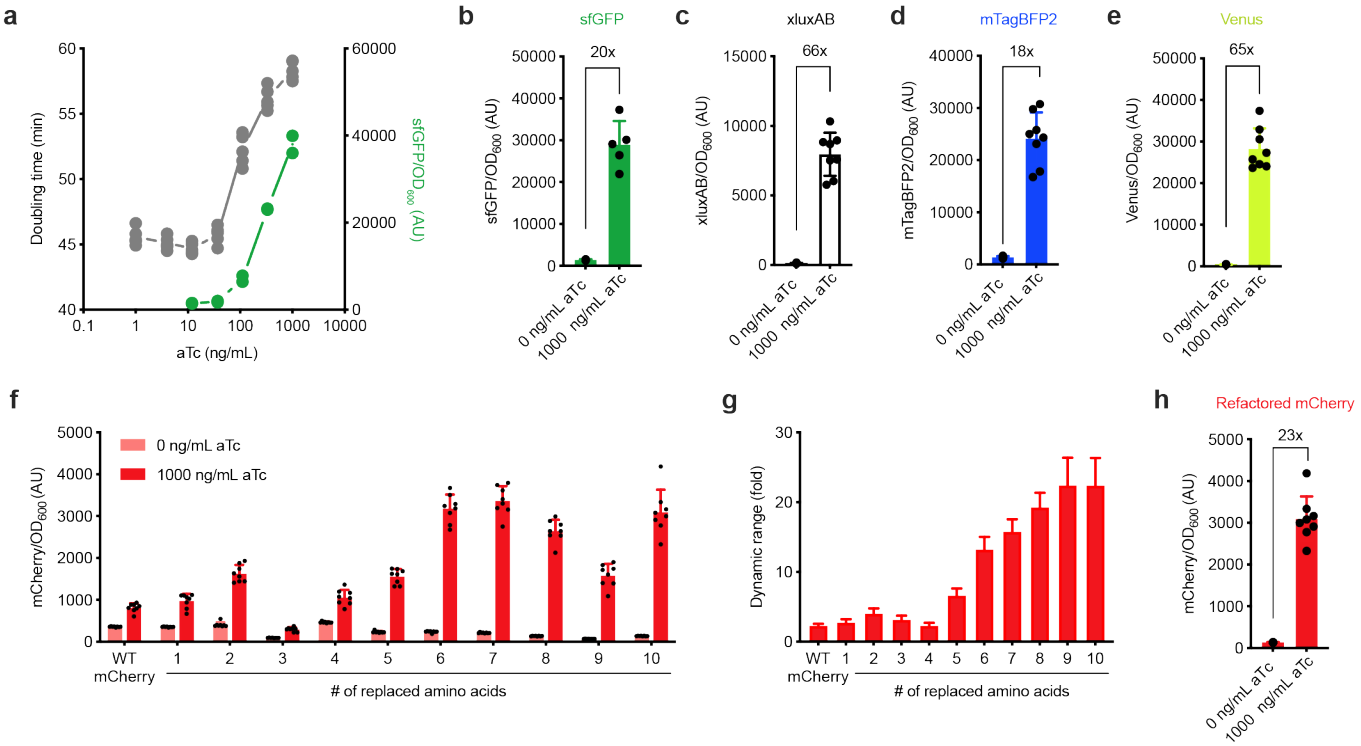
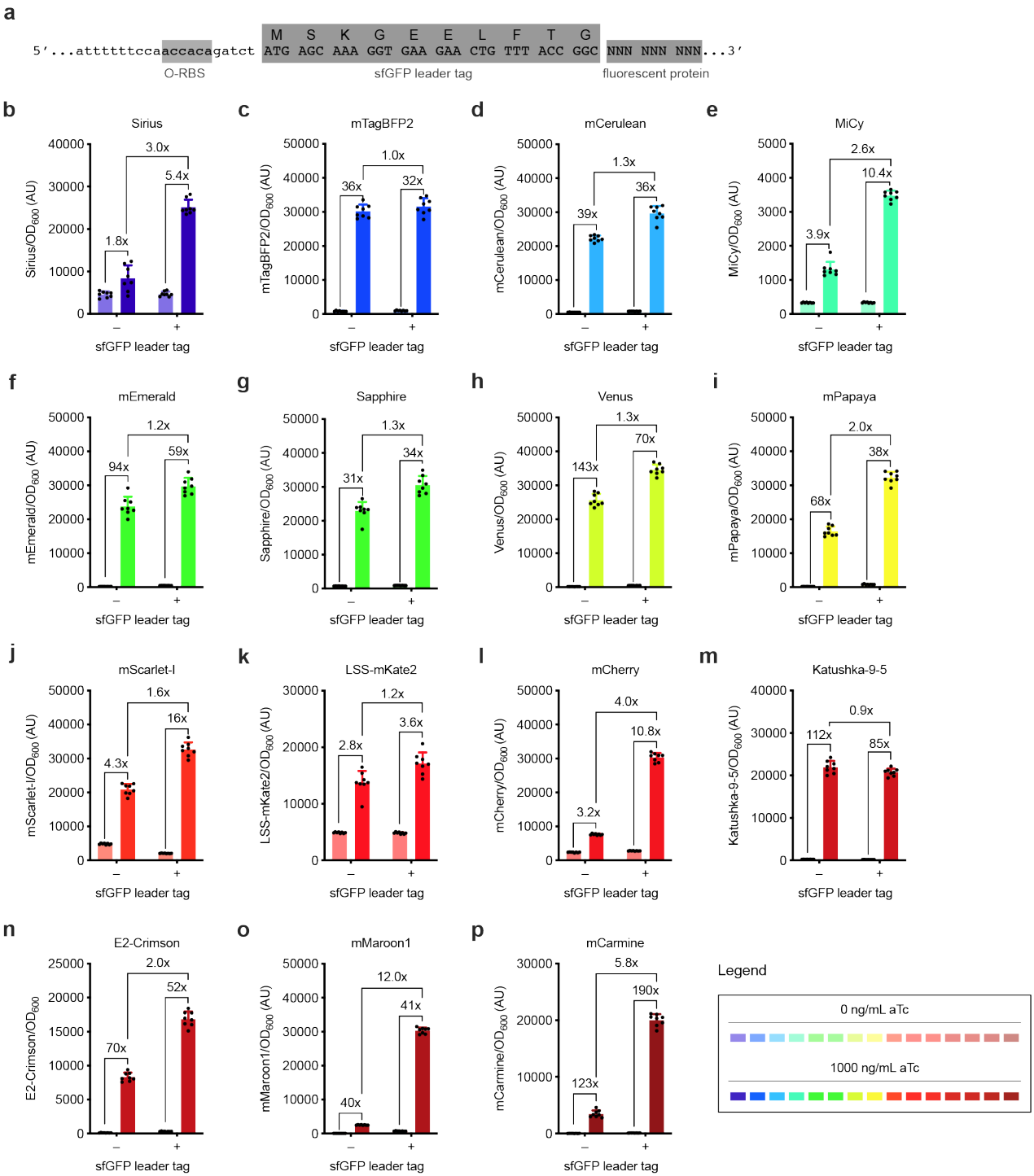


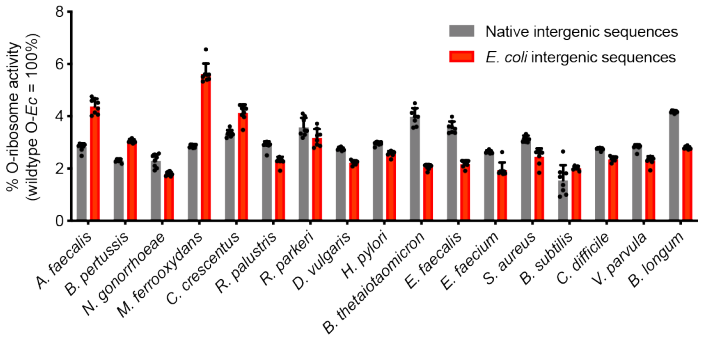

**Supplementary Figure 1 | Comparison of SQ strain complementation and orthogonal translation pipelines.** a) To evaluate heterologous rRNAs (ribosomal RNAs) via SQ strain complementation, rRNA plasmids are transformed into the SQ171 strain after which it may take up to 120 hours to observe colonies. Colonies are grown up in liquid medium +/- kanamycin to evaluate for pSacB persistence, which may take up to 3 days, after which cultures are glycerol stocked. Finally, stocked strains are revived, grown overnight in liquid medium and back-diluted to initiate a growth curve. b) To evaluate heterologous rRNAs via orthogonal translation, O-rRNA plasmids are transformed alongside the reporter plasmid and plates are incubated overnight. Colonies are then picked into media and grown overnight, after which sfGFP (superfolder GFP) fluorescence is quantified. Detailed experimental conditions for both assays are described in the Methods section. h = hours.



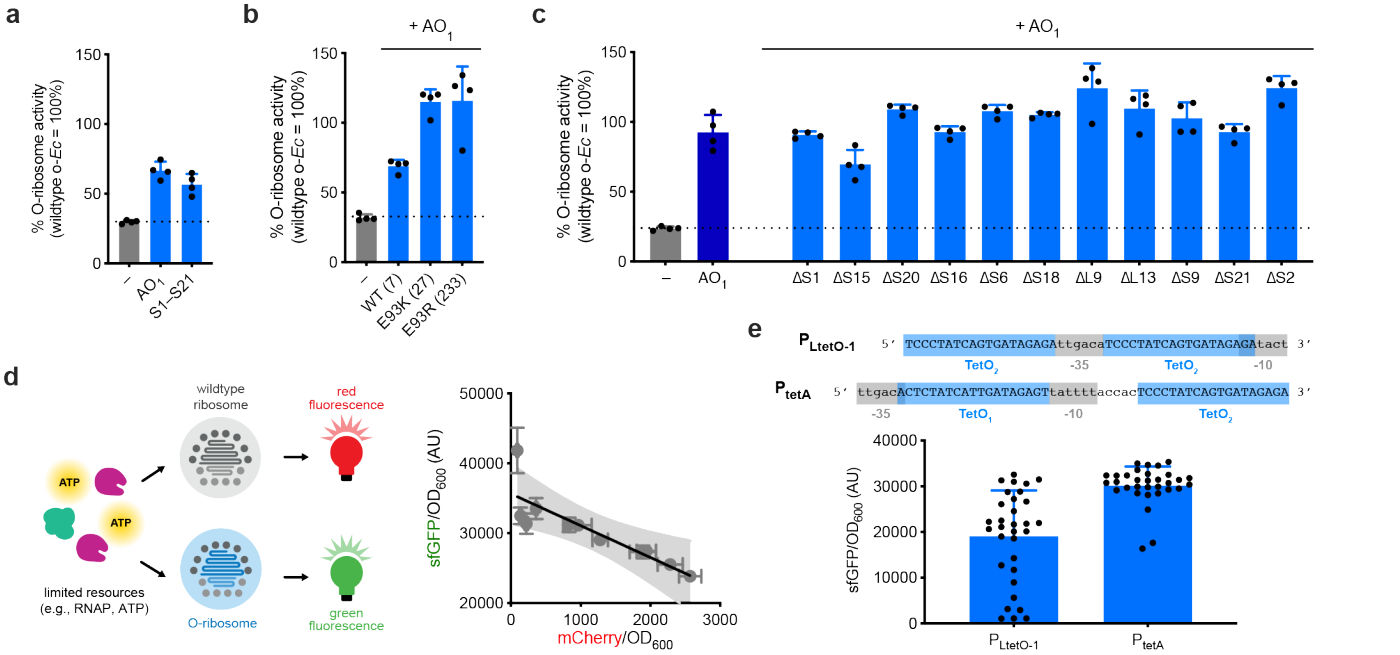

**Supplementary Figure 2 | Benchmarking and extending the orthogonal translation reporter system.** a) Induction of *E. coli* O-rRNA does not have a substantial effect on host growth rate. OD (optical density), n=5 and sfGFP (superfolder GFP), n=2. b) The O-sfGFP[1] reporter used throughout this study shows robust signal-to-noise upon O-rRNA induction (n=5). c-e) Additional orthogonal reporters show dynamic ranges comparable to or exceeding that of sfGFP: covalently-linked *Photorhabdus luminescens* luxAB (xluxAB)[2] (c), mTagBFP2[3] (d), and (e) Venus[4] (n=8). f-g) Conversely, an orthogonal reporter incorporating mCherry[5] showed low signal-to-noise. Replacement of successive codons at the mCherry N-terminus with their sfGFP counterparts yielded a gradual improvement in signal (f) and dynamic range (g) (n=8). h) A refactored mCherry orthogonal reporter in which the first 10 codons are replaced with the cognate sfGFP signal has improved dynamic range (n=8). Data reflect the mean and standard deviation of 2-8 biological replicates. AU = arbitrary units; aTc = anhydrotetracycline. Source data are available in the Source Data File.



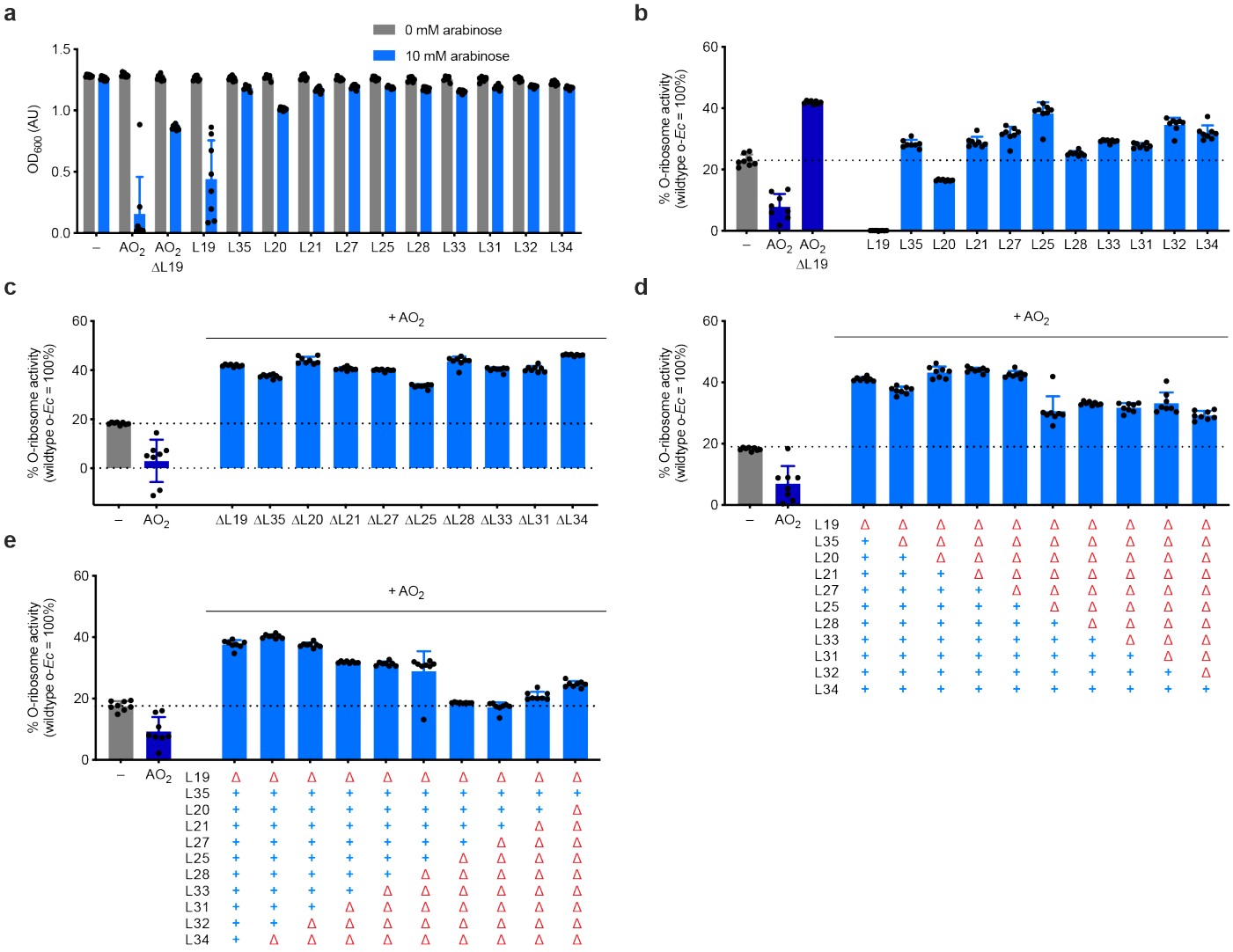

**Supplementary Figure 3 | A superfolder GFP-derived leader sequence improves the function of orthogonal reporters**. a) Schematic illustrating the O-RBS (orthogonal ribosome binding site), 10 amino acid sfGFP (superfolder GFP)-derived tag, and N-terminus of a fluorescent protein. When appended to the N-terminus of 15 fluorescent proteins, the tag limited reporter-dependent effects on orthogonal translation activity: b) Sirius[6], c) mTagBFP2[3], d) mCerulean[7], e) MiCy[8], f) mEmerald[9], g) Sapphire[9], h) Venus[4], i) mPapaya[10], j) mScarlet-I[11], k) LSS-mKate2[12], l) mCherry[5], m) Katusha-9-5[13], n) E2-Crimson[14], o) mMaroon1[15], p) mCarmine[16]. Generally, addition of the leader tag led to an improvement in absolute signal (average improvement 2.7-fold) and/or dynamic range (average improvement 1.5-fold). Data reflect the mean and standard deviation of 8 biological replicates. OD = optical density; AU = arbitrary units; aTc = anhydrotetracycline. Source data are available in the Source Data File.



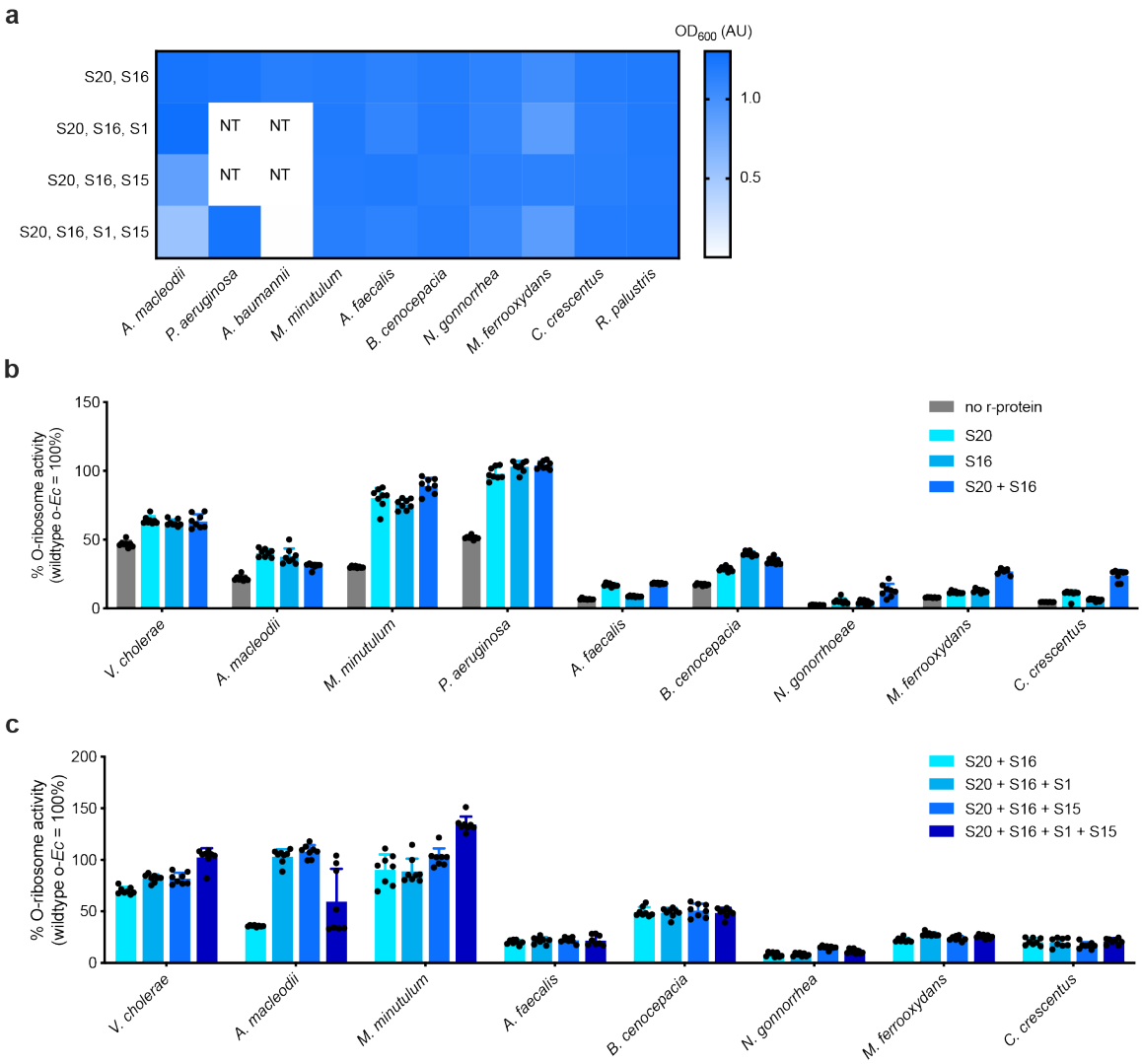

**Supplementary Figure 4 | Divergent O-rRNA activities are not improved following intergenic sequence replacement.** Comparison of O-sfGFP (orthogonal superfolder GFP) translation activity before and after intergenic sequence replacement for O-rRNAs derived from increasingly divergent microorganisms (69.8-82.3% 16S rRNA sequence identity to *E. coli*), showing limited improvement following intergenic sequence replacement. Data reflect the mean and standard deviation of 8 biological replicates. Comprehensive O-translation data reported in Supplementary Table 1. O-ribosome = orthogonal ribosome; wildtype O-*Ec* = wildtype orthogonal *E. coli* rRNA. Source data are available in the Source Data File.



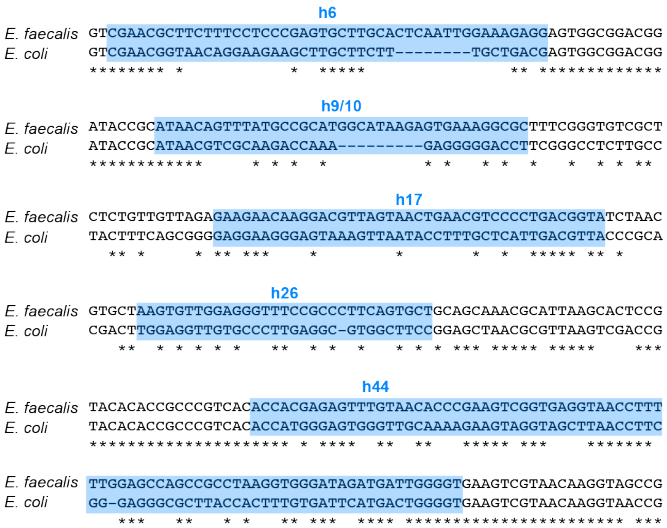

**Supplementary Figure 5 | R-protein supplementation improves *A. baumannii* O-rRNA function.** a) *A. baumannii* heterologous O-rRNA activity is improved following $AO_1$ induction, yielding comparable activity levels as supplementation with all cognate SSU (small subunit) r-proteins (S1-S21; n=4). b) *A. baumannii* heterologous O-rRNA activity improvement further depends upon $AO_1$ copy number, suggesting insufficient r-protein production at low copy numbers. Labels indicate RepA genotypes and numbers in parentheses indicate the approximate corresponding copy numbers[17] (n=4). c) Single r-protein deletion from $AO_1$ does not adversely affect *A. baumannii* heterologous O-rRNA activity, indicating that more than a single r-protein on this plasmid can complement O-rRNA function (n=4). d) O-sfGFP (orthogonal superfolder GFP) production using an *E. coli* O-rRNA is inversely proportional to mCherry production using *E. coli* native ribosomes, suggesting that r-protein overexpression may have pleiotropic effects on O-ribosome activity (99% CI, $R^2$ = 0.73, n=8). e) *E. coli* O-rRNA regulation using two related aTc-inducible promoters, $P_{LtetO-1}$[18] and $P_{tetA}$, highlights the improved signal and reduced variability using $P_{tetA}$, the native promoter found in the Tn10 transposon. $P_{LtetO-1}$-dependent variability is a consequence of promoter recombination between identical TetR operators (not shown) during cell passaging (n=32). Data reflect the mean and standard deviation of 4-32 biological replicates. OD = optical density; AU = arbitrary units; O-ribosome = orthogonal ribosome; wildtype O-*Ec* = wildtype orthogonal *E. coli* rRNA; ATP = adenosine triphosphate; RNAP = RNA polymerase. Source data are available in the Source Data File.



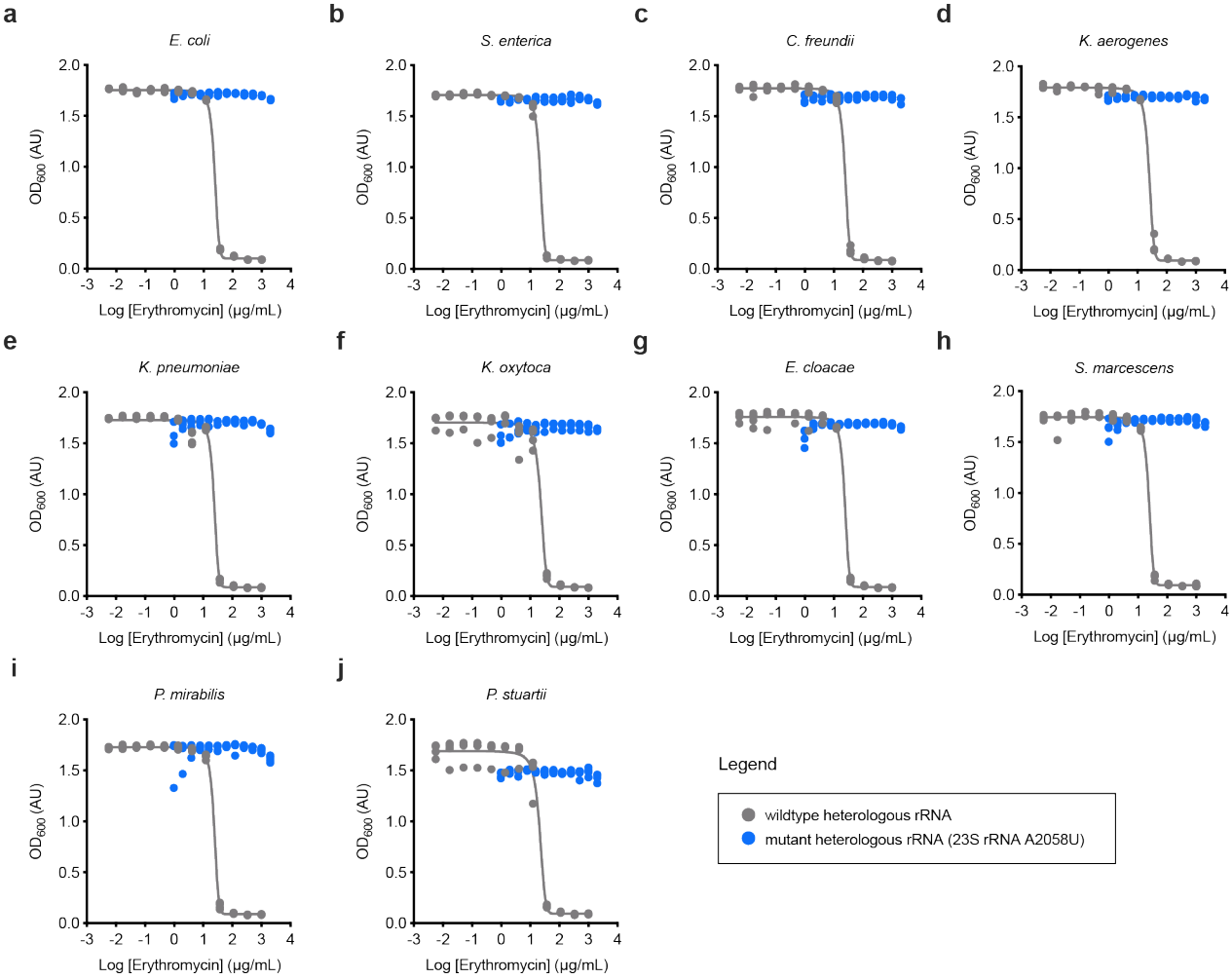

**Supplementary Figure 6 | Dissection of LSU r-proteins that improve *A. macleodii* O-rRNA activity.** a) Single r-proteins (from $AO_2$) expressed alongside cognate *A. macleodii* O-rRNA reveal that L19 is responsible for the observed toxicity from $AO_2$, where removal of L19 ($AO_2$ ΔL19) mitigates the observed growth reduction (n=7 for L25 +10 mM arabinose; otherwise n=8). b) No single r-protein from $AO_2$ enhances *A. macleodii* O-rRNA activity. c-e) Single deletions from $AO_2$ do not reveal any variants that differ in effect on O-rRNA activity, nor do truncation variants from the 5' end (d) or 3' end (e) of the artificial operon. These data collectively suggest that the observed improvement relies on the concerted action of numerous r-proteins from $AO_2$. Data reflect the mean and standard deviation of 8 biological replicates. OD = optical density; AU = arbitrary units; O-ribosome = orthogonal ribosome; wildtype O-*Ec* = wildtype orthogonal *E. coli* rRNA. Source data are available in the Source Data File.



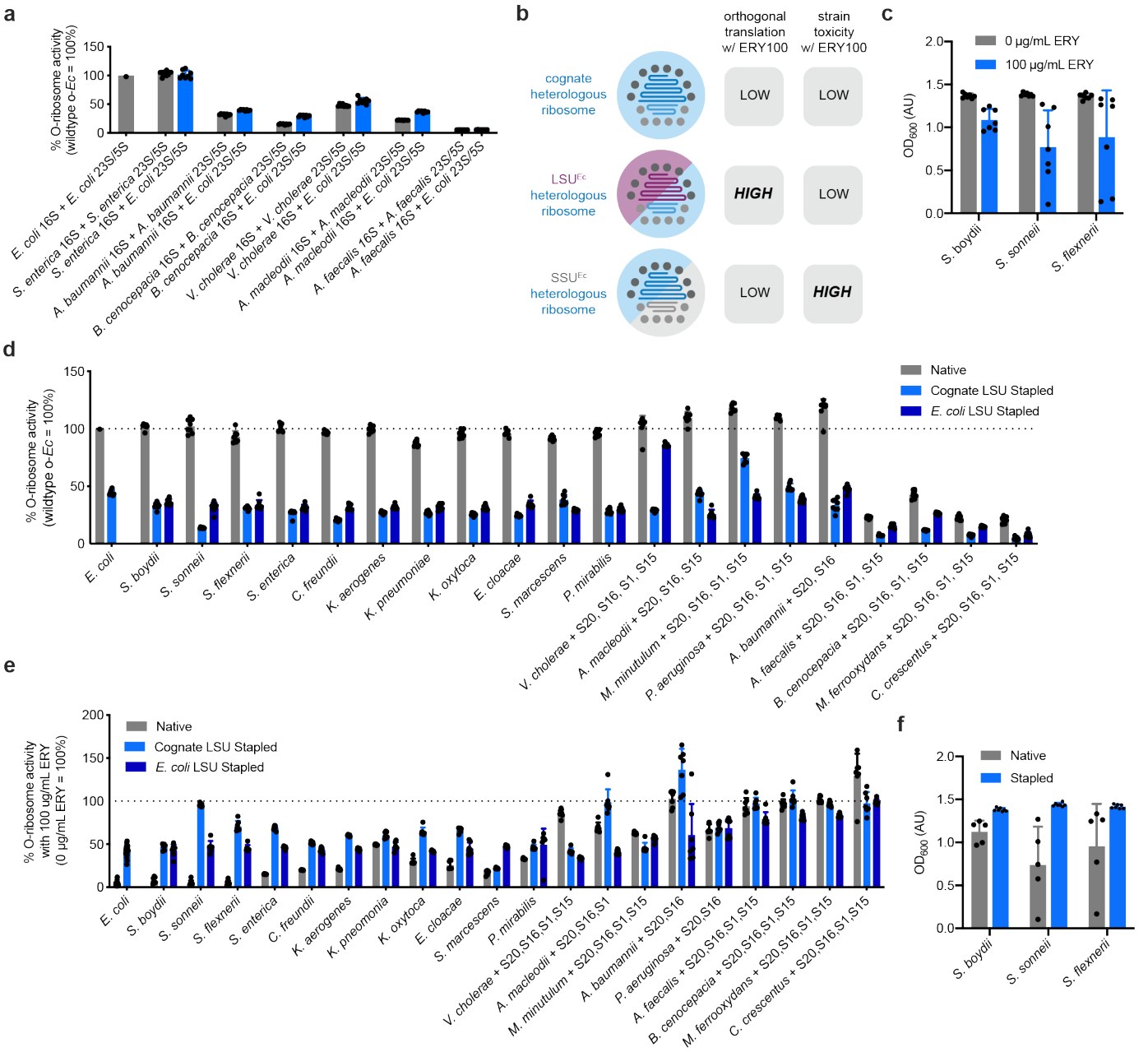

**Supplementary Figure 7 | Investigation of the contributions of the identified r-proteins S20, S16, S1, and S15.** a) Expression of cognate r-proteins S20, S16, S1, and S15 combinations alongside numerous heterologous O-rRNAs. *A. macleodii* and *A. baumannii* cognate S20, S16, S1, and S15 limit the growth of the *E. coli* host when co-expressed, as indicated by culture density after overnight growth, whereas most other r-proteins evaluated are well tolerated. NT = not tested. b) Both cognate r-proteins S20 and S16 are necessary for maximal sfGFP expression using O-rRNAs from more divergent microorganisms: *N. gonorrheae* (81.8% 16S rRNA sequence identity to *E. coli*), *M. ferrooxydans* (80.1%), and *C. crescentus* (79.3%). However, S20 and S16 are functionally redundant when expressed alongside more related O-rRNAs to *E. coli*. c) The combination of S20, S16, S1, and S15 is necessary for maximal activity using *V. cholerae* (90.3% 16S identity to *E. coli)* and *M. minutulum* (85.3%) O-rRNAs. For more phylogenetically distant O-rRNAs, no additional improvement is observed upon supplementation with S1 or S15 beyond the effect of S20 and S16. Data reflect the mean and standard deviation of 8 biological replicates. Comprehensive O-translation data reported in Supplementary Table 1. OD = optical density; AU = arbitrary units; O-ribosome = orthogonal ribosome; wildtype O-*Ec* = wildtype orthogonal *E. coli* rRNA. Source data are available in the Source Data File.




**Supplementary Figure 8 | *Enterococcus faecalis* 16S rRNA helices with low sequence identity to *E. coli*.** *E. faecalis* and *E. coli* rRNAs were aligned using Clustal Omega with default parameters[19], and regions with low sequence identity were manually identified. Elements that were later transplanted into the *E. coli* 16S O-rRNA are identified in blue. h = 16S rRNA helix.

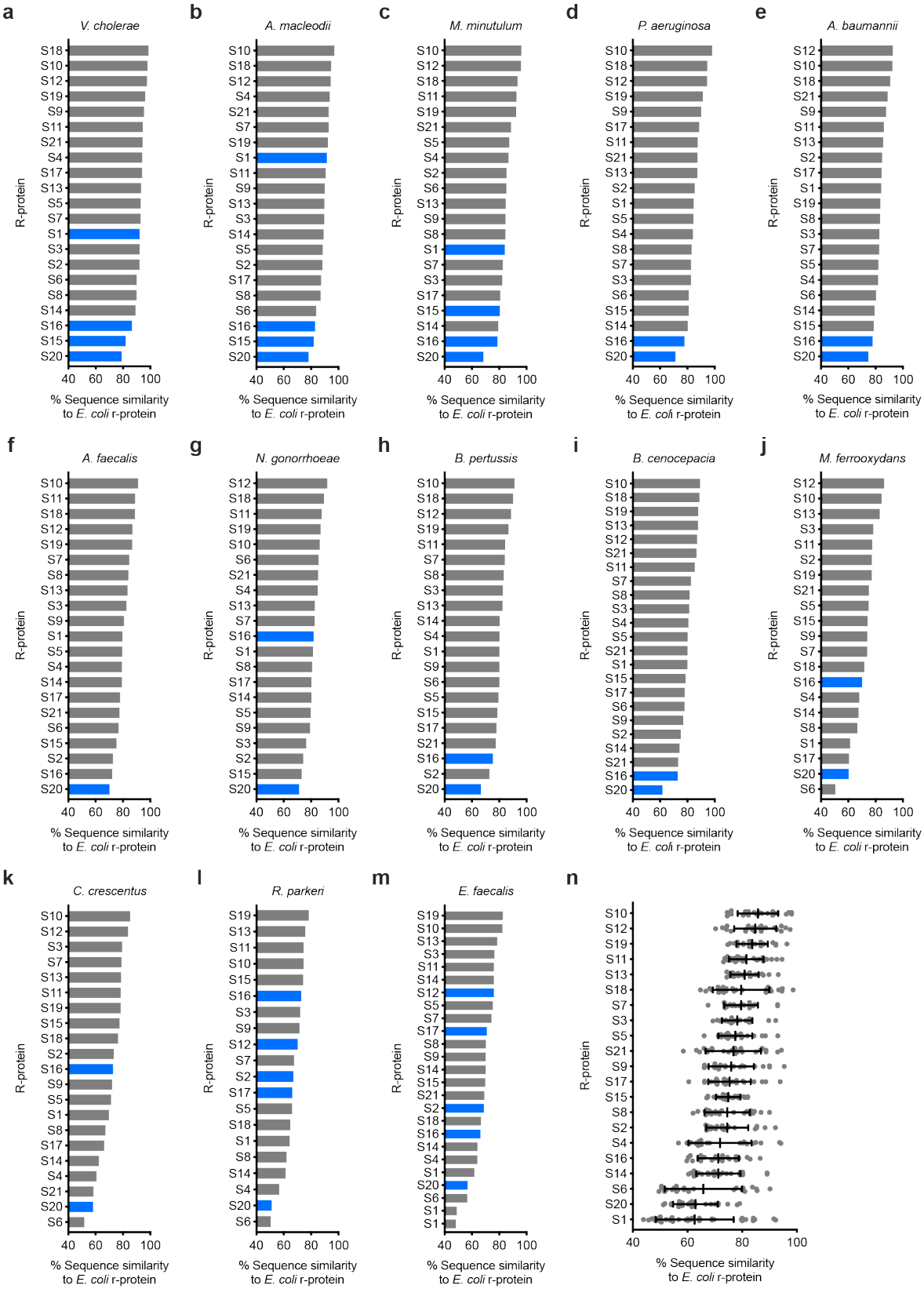



**Supplementary Figure 9 | Erythromycin sensitivity of wildtype and A2058U 23S rRNAs in SQ171 cells.** a-j) Erythromycin titration for SQ171 strains exclusively supported by the heterologous rRNA of interest. All strains show similar erthyromycin sensitivities using wildtype rRNAs (gray, n=4 biological replicates; best fit curves generated using GraphPad Prism version 8) or using 23S rRNA A2058U mutants (blue, n=4 biological replicates). All $IC_{50}$ values are reported in Supplementary Table 2. OD = optical density; AU = arbitrary units. Source data are available in the Source Data File.




**Supplementary Figure 10 | Benchmarking the ERY-dependent orthogonal translation reporter system**. a) Substitution of the heterologous 23S/5S rRNAs with the *E. coli* counterparts yielded functional orthogonal translation, suggesting that exchange may occur in cases where the cognate LSU is not produced (n=8). b) The ERY (erythromycin)-dependent reporter allows discriminates between three possible subunit assembly scenarios. When an orthogonal SSU (small subunit) assembles with a cognate LSU (large subunit), the ribosome is unable to translate the orthogonal sfGFP (superfolder GFP) reporter due to ERY-sensitivity. Alternatively, heterologous SSUs may assemble with *E. coli* LSUs, resulting in robust sfGFP translation in the presence of ERY. Finally, *E. coli* SSUs may assemble with heterologous LSUs, resulting in strain toxicity due to an inability to translate essential *E. coli* genes in the presence of ERY and low sfGFP signal as a result. c) Heterologous ribosomes closely related to *E. coli* (>99.2% 16S sequence ID) re-sensitize S4246 cells to ERY treatment due to usage of sensitive LSUs for translating host genes (n=7). d) Orthogonal translation activities for native ribosomes and ribosomes stapled to cognate LSUs vs. *E. coli* LSUs (n=7 for *V. cholerae*, *E. coli* LSU stapled and *A. macleodii, E. coli* LSU stapled; otherwise n=8). e) ERY-dependent reporter data for native ribosomes and ribosomes stapled to cognate LSUs vs. *E. coli* LSUs at 100 µg mL$^{-1}$ ERY. Data for each ribosome is normalized to its sfGFP fluorescence at 0 µg mL$^{-1}$ ERY (n=35 for native *E. coli*, n=21 for stapled *E. coli*; otherwise n=7). f) $OD_{600}$ for heterologous ribosomes with high 16S sequence identity to *E. coli* (≥99.2%) at 100 µg mL$^{-1}$ ERY increases after subunit

stapling, indicating a decrease in intersubunit exchange (n=5 for native constructs, n=7 for stapled constructs). Data reflect means and standard deviations of the indicated biological replicates. Comprehensive data reported in Supplementary Table 3. OD = optical density; AU = arbitrary units; wildtype O-*Ec* = wildtype orthogonal *E. coli* rRNA. Source data are available in the Source Data File.




**Supplementary Figure 11 | Analysis of heterologous r-protein sequence similarity to *E. coli* homologs.** a-m) r-protein sequence similarities to *E. coli* for species evaluated in this study. R-proteins identified as enhancing O-rRNA activity are highlighted in blue. n) Average sequence similarity to *E. coli* for species evaluated in this study which were not immediately functional in *E. coli* prior to r-protein complementation (error bars reflect standard deviations). As protein sequences were identified via BLAST

to *E. coli* sequences (see Methods), we note that in some cases multiple homologs were identified or a full complement of r-proteins was not identified (such that n=22 for S7; n=24 for S8, S15, S17, S9, S5, S11, S19; n=25 for S18; n=29 for S16; n=37 for S1; otherwise n=23.). Source data are available in the Source Data File.

| Organism Name | Bacterial Class | %16S ID | Doubling time (min) | | Orthogonal translational activity (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | nIS | *Ec*IS | nIS | *Ec*IS | *Ec*IS + S20/S16 | *Ec*IS + S20/S16 + S1/S15 | *Ec*IS + S20/S16 + S2/S12/S17 |
| *Escherichia coli* | Gammaproteobacteria | 100 | 48.1 ± 1.5 (7) | NA | 100 | NA | NA | NA | NA |
| *Shigella boydii* | Gammaproteobacteria | 99.16 | 54.9 ± 2.2 (8) | 43 ± 0.9 (6) | 100.2 ± 3.7 (8) | 102.2 ± 5.6 (8) | NT | NT | NT |
| *Shigella sonnei* | Gammaproteobacteria | 99.61 | 46.3 ± 1.8 (8) | 41.6 ± 1.2 (7) | 80.6 ± 5.2 (8) | 102.3 ± 7.9 (8) | NT | NT | NT |
| *Shigella flexneri* | Gammaproteobacteria | 99.61 | 42.5 ± 2 (4) | 40.9 ± 0.5 (6) | 89.3 ± 4.7 (8) | 93.1 ± 6.8 (8) | NT | NT | NT |
| *Salmonella enterica* | Gammaproteobacteria | 97.02 | 43 ± 0.8 (8) | 42.6 ± 1.3 (8) | 102.0 ± 8.0 (8) | 100.4 ± 5.8 (8) | NT | NT | NT |
| *Citrobacter freundii* | Gammaproteobacteria | 96.23 | 47.6 ± 2 (8) | 40.9 ± 1.2 (8) | 61.7 ± 3.7 (8) | 94.3 ± 3.6 (8) | NT | NT | NT |
| *Klebsiella aerogenes* | Gammaproteobacteria | 95.84 | 40.1 ± 0.5 (5) | 41.9 ± 2.1 (8) | 94.3 ± 4.3 (8) | 99.8 ± 4.3 (8) | NT | NT | NT |
| *Klebsiella pneumoniae* | Gammaproteobacteria | 96.17 | 41.3 ± 1.1 (8) | 46 ± 3.3 (8) | 98.3 ± 4.7 (8) | 86.2 ± 4.5 (8) | NT | NT | NT |
| *Klebsiella oxytoca* | Gammaproteobacteria | 96.23 | 44.3 ± 3.2 (8) | 43.4 ± 1.4 (8) | 97.3 ± 5.1 (8) | 95.8 ± 4.1 (8) | NT | NT | NT |
| *Enterobacter cloacae* | Gammaproteobacteria | 97.54 | 44.5 ± 0.2 (3) | 42.2 ± 1.1 (8) | 69.8 ± 2.4 (8) | 96.2 ± 2.9 (8) | NT | NT | NT |
| *Serratia marcescens* | Gammaproteobacteria | 96.04 | 44.6 ± 1.1 (7) | 42.9 ± 2.8 (6) | 2.9 ± 0.2 (8) | 91.8 ± 2.3 (8) | NT | NT | NT |
| *Proteus mirabilis* | Gammaproteobacteria | 92.92 | 46.3 ± 2.3 (5) | 44.5 ± 1 (6) | 54.4 ± 3.8 (8) | 96.1 ± 4.7 (8) | NT | NT | NT |
| *Providencia stuartii* | Gammaproteobacteria | 93.12 | 52.5 ± 5.6 (7) | 45.7 ± 1.1 (6) | 14.5 ± 0.6 (8) | 76.9 ± 9.4 (8) | NT | NT | NT |
| *Vibrio cholerae* | Gammaproteobacteria | 90.26 | 56.3 ± 0.8 (6) | 60.4 ± 1.4 (8) | 3.3 ± 0.2 (8) | 44.5 ± 2.2 (8) | 60.7 ± 5.6 (8) | 84.3 ± 5.7 (8) | NT |
| *Alteromonas macleodii* | Gammaproteobacteria | 85.91 | 54.8 ± 0.9 (6) | 60.2 ± 1 (4) | 32.0 ± 3.1 (8) | 27.8 ± 2.2 (8) | 34.4 ± 2.8 (8) | 61.1 ± 29.0* (8) | NT |
| *Marinospirillum minutulum* | Gammaproteobacteria | 85.27 | NT | NT | NT | 29.5 ± 1.6 (8) | 82.5 ± 5.3 (8) | 118.7 ± 6.4 (8) | NT |
| *Pseudomonas aeruginosa* | Gammaproteobacteria | 85.15 | 55.1 ± 1.8 (6) | 49.9 ± 1.3 (5) | 3.3 ± 0.1 (8) | 57.4 ± 2.6 (8) | 113.4 ± 8.0 (8) | 109.9 ± 6.9 (8) | NT |
| *Acinetobacter baumannii* | Gammaproteobacteria | 84.31 | 58.5 (2) | 62.1 ± 0.8 (5) | 3.4 ± 0.2 (8) | 30.0 ± 4.6 (8) | 117.4 ± 9.4 (8) | NG* (8) | NT |
| *Alcaligenes faecalis* | Betaproteobacteria | 82.32 | 55 (2) | 63.9 ± 10.7 (4) | 2.8 ± 0.2 (8) | 4.4 ± 0.4 (8) | 23.1 ± 2.7 (8) | 23.0 ± 1.5 (8) | NT |
| *Bordetella pertussis* | Betaproteobacteria | 81.62 | 59 (1) | 56.2 ± 4.5 (3) | 2.3 ± 0.1 (8) | 3.1 ± 0.2 (8) | 6.0 ± 0.4 (8) | 6.2 ± 0.5 (8) | NT |
| *Burkholderia cenocepacia* | Betaproteobacteria | 81.45 | 54.5 ± 5.1 (4) | 86.5 ± 2.0 (6) | 1.9 ± 0.1 (8) | 14.7 ± 0.6 (8) | 36.7 ± 4.7 (8) | 43.0 ± 4.1 (8) | 28.2 ± 1.8 (8) |
| *Neisseria gonorrhoeae* | Betaproteobacteria | 81.81 | 87.7 (1) | 87.8 ± 2.7 (5) | 2.3 ± 0.3 (8) | 1.8 ± 0.1 (8) | 8.5 ± 2.8 (8) | 12 ± 1.8 (8) | NT |
| *Mariprofundus ferrooxydans* | Zetaproteobacteria | 80.72 | 84.5 ± 4.2 (4) | 63.5 ± 4.0 (3) | 2.9 ± 0.1 (8) | 5.6 ± 0.4 (8) | 17.0 ± 0.8 (8) | 22.7 ± 2.4 (8) | 11.5 ± 0.5 (8) |
| *Caulobacter crescentus* | Alphaproteobacteria | 79.31 | NT | NT | 3.3 ± 0.3 (8) | 4.1 ± 0.4 (8) | 20.6 ± 3.7 (8) | 21.0 ± 3.1 (8) | NT |
| *Rhodopseudomonas palustris* | Alphaproteobacteria | 77.55 | NT | NT | 2.9 ± 0.2 (8) | 2.3 ± 0.2 (8) | 3.3 ± 0.4 (8) | 3.2 ± 0.5 (8) | NT |
| *Rickettsia parkeri* | Alphaproteobacteria | 76.83 | NT | NT | 3.6 ± 0.4 (8) | 3.2 ± 0.4 (8) | 4.2 ± 0.6 (8) | 4.3 ± 0.3 (8) | 10.2 ± 2.2 (8) |
| *Desulfovibrio vulgaris* | Deltaproteobacteria | 77.73 | NT | NT | 2.8 ± 0.1 (8) | 2.2 ± 0.1 (8) | NT | NT | NT |
| *Helicobacter pylori* | Epsilonproteobacteria | 75.13 | NT | NT | 3.0 ± 0.1 (8) | 2.6 ± 0.1 (8) | NT | NT | NT |
| *Bacteroides thetaiotaomicron* | Bacteroidia | 69.83 | NT | NT | 4.0 ± 0.4 (8) | 2.1 ± 0.1 (8) | NT | NT | NT |
| *Enterococcus faecalis* | Bacilli | 76.06 | NT | NT | 3.6 ± 0.3 (8) | 2.2 ± 0.2 (8) | 2.1 ± 0.2 (8) | NT | 9.5 ± 0.6 (8) |
| *Enterococcus faecium* | Bacilli | 75.98 | NT | NT | 2.6 ± 0.1 (8) | 1.9 ± 0.3 (8) | NT | NT | NT |
| *Staphylococcus aureus* | Bacilli | 76.01 | NT | NT | 3.1 ± 0.2 (8) | 2.5 ± 0.3 (8) | NT | NT | NT |
| *Bacillus subtilis* | Bacilli | 76.06 | NT | NT | 1.6 ± 0.6 (8) | 2.0 ± 0.1 (8) | NT | NT | NT |
| *Clostridium difficile* | Clostridia | 75.45 | NT | NT | 2.8 ± 0.1 (8) | 2.4 ± 0.1 (8) | NT | NT | NT |
| *Veillonella parvula* | Clostridia | 74.59 | NT | NT | 2.8 ± 0.2 (8) | 2.3 ± 0.2 (8) | NT | NT | NT |
| *Bifidobacterium longum* | Actinobacteria | 74.64 | NT | NT | 4.2 ± 0.2 (8) | 2.8 ± 0.1 (8) | NT | NT | NT |

Data reflect means and standard deviations of biological replicates indicated in parentheses (few replicates were sometimes obtained by SQ complementation; SDs are not reported for n<3).
Note that SQ data in the main text is generally reported as fitness (doublings/h), which is obtained from doubling time as described in the methods.
"NT" = not tested; NA = not applicable; NG = no growth. * indicates toxicity was observed using this combination of rRNA/r-proteins

**Supplementary Table 1 | Summary of heterologous and orthogonal ribosome translation data**. Doubling times in SQ171 cells (minutes) and orthogonal translation activity (normalized to orthogonal *E. coli*) for all heterologous ribosomes tested. nIS = native intergenic sequences; *Ec*IS = *E. coli* intergenic sequences. NA = not applicable; NT = not tested.

| Organism Name | ERY $IC_{50}$ (µg/mL) | |
|---|---|---|
| | Wildtype 23S rRNA | A2058U 23S rRNA |
| *Escherichia coli* | 20.8 ± 1.02 | >2000 |
| *Salmonella enterica* | 18.82 ± 1.02 | >2000 |
| *Citrobacter freundii* | 20.83 ± 1.02 | >2000 |
| *Klebsiella aerogenes* | 21.4 ± 1.02 | >2000 |
| *Klebsiella pneumoniae* | 20.17 ± 1.05 | >2000 |
| *Klebsiella oxytoca* | 19.66 ± 1.06 | >2000 |
| *Enterobacter cloacae* | 19.75 ± 1.06 | >2000 |
| *Serratia marcescens* | 19.98 ± 1.03 | >2000 |
| *Proteus mirabilis* | 20.57 ± 1.02 | >2000 |
| *Providencia stuartii* | 18.35 ± 1.06 | >2000 |

**Supplementary Table 2 | Tabulated erythromycin $IC_{50}$ values for wildtype and 23S rRNA A2058U rRNAs**. Data reflect means and standard deviations of 4 biological replicates. Data are plotted in Supplementary Figure 9. rRNA = ribosomal RNA; ERY = erythromycin; $IC_{50}$ = half maximal inhibitory concentration.

| Organism Name | Bacterial Class | %16S rRNA identity | Orthogonal Translational Activity (%) | | | % O-ribosome Activity at 100 µg/mL ERY (0 µg/mL ERY = 100%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | Native | Cognate LSU Stapled | *E. coli* LSU Stapled | Native | Cognate LSU Stapled | *E. coli* LSU Stapled |
| *Escherichia coli* | Gammaproteobacteria | 100 | 100 | 44.4 ± 2.7 | NA | 3.5 ± 2.7 | 41.3 ± 7.3 | NA |
| *Shigella boydii* | Gammaproteobacteria | 99.16 | 100.2 ± 3.7 | 33.0 ± 2.7 | 36.3 ± 2.8 | 8.1 ± 3.6 | 47.0 ± 1.8 | 42.9 ± 7.0 |
| *Shigella sonnei* | Gammaproteobacteria | 99.61 | 80.6 ± 5.2 | 13.8 ± 0.7 | 31.6 ± 4.5 | 4.8 ± 3.1 | 95.9 ± 1.9 | 47.1 ± 6.7 |
| *Shigella flexneri* | Gammaproteobacteria | 99.61 | 89.3 ± 4.7 | 31.0 ± 1.7 | 33.8 ± 4.3 | 4.7 ± 3.0 | 71.0 ± 6.0 | 45.2 ± 3.9 |
| *Salmonella enterica* | Gammaproteobacteria | 97.02 | 102.0 ± 8.0 | 6.6 ± 2.9 | 31.5 ± 2.7 | 15.3 ± 0.4 | 69.2 ± 2.6 | 46.3 ±1.8 |
| *Citrobacter freundii* | Gammaproteobacteria | 96.23 | 61.7 ± 3.7 | 20.8 ± 1.7 | 31.8 ± 2.7 | 20.1 ± 0.4 | 51.4 ± 1.6 | 43.5 ± 2.7 |
| *Klebsiella aerogenes* | Gammaproteobacteria | 95.84 | 94.3 ± 4.3 | 27.2 ± 1.8 | 31.7 ± 2.1 | 21.2 ±1.3 | 60.2 ± 1.1 | 44.1 ± 1.5 |
| *Klebsiella pneumoniae* | Gammaproteobacteria | 96.17 | 98.3 ± 4.7 | 26.8 ± 1.6 | 32.1 ± 2.8 | 49.4 ± 0.6 | 60.7 ± 3.8 | 47.4 ± 4.9 |
| *Klebsiella oxytoca* | Gammaproteobacteria | 96.23 | 97.3 ± 5.1 | 25.5 ± 1.6 | 31.1 ± 2.4 | 29.8 ± 3.5 | 64.6 ± 5.0 | 41.8 ± 1.1 |
| *Enterobacter cloacae* | Gammaproteobacteria | 97.54 | 69.8 ± 2.4 | 24.7 ± 1.6 | 34.4 ± 3.2 | 25.8 ± 5.0 | 66.5 ± 2.7 | 45.3 ± 6.7 |
| *Serratia marcescens* | Gammaproteobacteria | 96.04 | 2.9 ± 0.2 | 38.4 ± 2.7 | 29.2 ± 1.1 | 17.3 ± 2.6 | 22.0 ± 0.7 | 47.5 ± 1.6 |
| *Proteus mirabilis* | Gammaproteobacteria | 92.92 | 54.4 ± 3.8 | 28.4 ± 2.7 | 30.0 ± 2.0 | 33.0 ± 0.8 | 46.4 ± 3.3 | 56.5 ± 3.3 |
| *Vibrio cholerae + S20, S16, S1, S15* | Gammaproteobacteria | 90.26 | 84.3 ± 5.7 | 29.1 ± 1.5 | 77.8 ± 4.6 | 85.6 ± 4.4 | 41.9 ± 3.3 | 34.0 ± 1.3 |
| *Alteromonas macleodii + S20, S16, S15* | Gammaproteobacteria | 85.91 | 109.5 ± 5.3 | 36.1 ± 2.7 | 17.3 ± 3.8 | 69.5 ± 5.7 | 99.2 ± 14.6 | 41.3 ± 1.7 |
| *Marinospirillum minutulum + S20, S16, S1, S15* | Gammaproteobacteria | 85.27 | 118.7 ± 6.4 | 67.9 ± 4.9 | 39.4 ± 3.7 | 62.9 ± 5.7 | 46.0 ± 5.8 | 55.7 ± 3.9 |
| *Pseudomonas aeruginosa + S20, S16, S1, S15* | Gammaproteobacteria | 85.15 | 109.9 ± 6.9 | 47.2 ± 4.9 | 39.3 ± 4.3 | 103.1 ± 9.5 | 137.0 ± 23.8 | 60.9 ± 35.8 |
| *Acinetobacter baumannii + S20, S16* | Gammaproteobacteria | 84.31 | 117.4 ± 9.4 | 33.4 ± 4.9 | 47.4 ± 3.1 | 66.7 ± 6.5 | 69.4 ± 5.4 | 69.2 ± 10.8 |
| *Alcaligenes faecalis + S20, S16, S1, S15* | Betaproteobacteria | 82.32 | 23.0 ± 1.5 | 7.3 ± 0.7 | 16.0 ± 1.9 | 94.5 ± 9.3 | 97.4 ± 6.5 | 79.5 ± 7.8 |
| *Burkholderia cenocepacia + S20, S16, S1, S15* | Betaproteobacteria | 81.45 | 43.0 ± 4.1 | 11.8 ± 0.5 | 26.4 ± 1.0 | 96.5 ± 5.6 | 103.2 ± 9.3 | 81.6 ± 3.9 |
| *Mariprofundus ferrooxydans + S20, S16, S1, S15* | Zetaproteobacteria | 80.72 | 22.7 ± 2.4 | 7.5 ± 1.0 | 12.1 ± 1.0 | 102.0 ± 9.5 | 96.6 ± 2.8 | 82.9 ± 2.3 |
| *Caulobacter crescentus + S20, S16, S1, S15* | Alphaproteobacteria | 79.31 | 21.0 ± 3.1 | 3.8 ± 2.6 | 5.8 ± 2.0 | 129.6 ± 25.5 | 98.0 ± 12.7 | 99.0 ± 3.2 |

**Supplementary Table 3 | Summary of stapled heterologous ribosome experiments.** Data reflect means and standard deviations of 7-28 biological replicates. ERY = erythromycin; rRNA = ribosomal RNA; LSU = large subunit.

| Name | Function | Sequence |
|---|---|---|
| AB3441 | Universal rRNA amplification primer, FWD | ACCGGCCGCUGTGCCAGCAGCCGCGGTAATAC |
| AB3442 | Universal rRNA amplification primer, REV | AGGGGTTCCGCGCACAUGTGACGGGCGGTGTGTACAAG |
| AB5708 | Recombineering primer, rrlA-H A2058U | C*T*C*A*ATGTTCAGTGTCAAGCTATAGTAAAGGTTCACGGGGTCTTACCGTCTTGCCGCGGGTACACTGCATCTTCACAGCGAGTTCAATTT |
| AB5710 | Recombineering check primer, FWD | GAAATTCCTTGTCGGGTAAGTTCC |
| AB5711 | Recombineering check primer, REV | GAACATCAAACATTAAAGGGTGGTATTTC |

blue = USER junction; red = annealing region; *=phosphothirate bonds; yellow = mutated base

**Supplementary Table 4 | Primers used in this study.** Instances where primers haven been used are described in the Methods section. rRNA = ribosomal RNA; FWD = forward; REV = reverse.

| Reporter Protein | $\lambda_{Ex}$ (nm) | $\lambda_{Em}$ (nm) | sfGFP Leader Tag | |
|---|---|---|---|---|
| | | | – | + |
| Sirius | 355 | 424 | pNK141a1 | pNK141a2 |
| mTagBFP2 | 399 | 454 | pNK133b | pNK141b2 |
| mCerulean | 433 | 475 | pNK141c1 | pNK141c2 |
| MiCy | 472 | 495 | pNK141m1 | pNK141m2 |
| mEmerald | 487 | 509 | pNK141e1 | pNK141e2 |
| Sapphire | 399 | 511 | pNK141d1 | pNK141d2 |
| Venus | 515 | 530 | pNK133d | pNK141f2 |
| mPapaya | 530 | 550 | pNK141g1 | pNK141g2 |
| mScarlet-I | 569 | 593 | pNK141h1 | pNK141h2 |
| LSS-mKate2 | 460 | 605 | pNK141l1 | pNK141l2 |
| mCherry | 587 | 610 | pNK133a | pNK141i2 |
| Katushka-9-5 | 588 | 635 | pNK141j1 | pNK141j2 |
| E2-Crimson | 611 | 646 | pNK141n1 | pNK141n2 |
| mMaroon1 | 609 | 657 | pNK141o1 | pNK141o2 |
| mCarmine | 603 | 675 | pNK141k1 | pNK141k2 |
| ermCL-sfGFP | 485 | 510 | pAB140j8 | N/A |
| sfGFP | 485 | 510 | Plpp5.B.GFP | N/A |

**Supplementary Table 5 | Reporter plasmids used in this study.** Highlighted plasmids (blue) have been deposited in Addgene. Addgene IDs are listed in Supplementary Table 8. Plasmid Plpp5.B.GFP was kindly provided by the Jewett Lab. sfGFP = superfolder GFP; $\lambda_{Ex}$ = excitation wavelength; $\lambda_{Em}$ = emission wavelength.

| Organism Name | NCBI TaxID | NCBI Species TaxID | NCBI Strain Identifier | Accession (GTDB Genome Representative) |
|---|---|---|---|---|
| *Acinetobacter baumannii* | 575584 | 470 | ATCC 19606 | RS_GCF_002811175.1 |
| *Alcaligenes faecalis* | 511 | 511 | ZD02 | RS_GCF_000967305.2 |
| *Alteromonas macleodii* | 529120 | 28108 | ATCC 27126 | RS_GCF_000172635.2 |
| *Burkholderia cenocepacia* | 331272 | 95486 | HI2424 | RS_GCF_000203955.1 |
| *Bifidobacterium longum* | 206672 | 216816 | NCC2705 | RS_GCF_000007525.1 |
| *Bordetella pertussis* | 257313 | 520 | Tohama I | RS_GCF_000195715.1 |
| *Bacillus subtilis* | 224308 | 1423 | 168 | RS_GCF_000009045.1 |
| *Bacteroides thetaiotaomicron* | 818 | 818 | 7330 | RS_GCF_001314975.1 |
| *Caulobacter crescentus* | 565050 | 155892 | NA1000 | RS_GCF_000022005.1 |
| *Clostridium difficile* | 272563 | 1496 | 630 | RS_GCF_000009205.1 |
| *Citrobacter freundii* | 546 | 546 | CAV1321 | RS_GCF_001022155.1 |
| *Desulfovibrio vulgaris* | 882 | 881 | Hildenborough | RS_GCF_000195755.1 |
| *Enterobacter cloacae* | 716541 | 550 | ATCC 13047 | RS_GCF_000025565.1 |
| *Escherichia coli* | 511145 | 562 | K-12 | RS_GCF_000005845.2 |
| *Escherichia coli/Shigella Spp.* | 198214 | 623 | 301 | RS_GCF_000006925.2 |
| *Enterococcus faecalis* | 226185 | 1351 | V583 | RS_GCF_000007785.1 |
| *Enterococcus faecium* | 333849 | 1352 | DO | RS_GCF_000174395.2 |
| *Helicobacter pylori* | 210 | 210 | KH33 | RS_GCF_002832255.1 |
| *Klebsiella aerogenes* | 1028307 | 548 | KCTC 2190 | RS_GCF_000215745.1 |
| *Klebsiella oxytoca* | 571 | 571 | CAV1015 | RS_GCF_001870185.1 |
| *Klebsiella pneumoniae* | 1125630 | 573 | HS11286 | RS_GCF_000240185.1 |
| *Mariprofundus ferrooxydans* | 314345 | 314344 | PV-1 | RS_GCF_000153765.1 |
| *Neisseria gonorrhoeae* | 242231 | 485 | FA 1090 | RS_GCF_000006845.1 |
| *Pseudomonas aeruginosa* | 208964 | 287 | PAO1 | RS_GCF_000006765.1 |
| *Proteus mirabilis* | 529507 | 584 | HI4320 | RS_GCF_000069965.1 |
| *Providencia stuartii* | 1157951 | 588 | MRSN 2154 | RS_GCF_000259175.1 |
| *Rhodopseudomonas palustris* | 316058 | 1076 | HaA2 | RS_GCF_000013365.1 |
| *Rickettsia parkeri* | 1105108 | 35792 | Portsmouth | RS_GCF_000284195.1 |
| *Staphylococcus aureus* | 93061 | 1280 | NCTC 8325 | RS_GCF_000013425.1 |
| *Salmonella enterica* | 90371 | 28901 | LT2 | RS_GCF_002289225.1 |
| *Serratia marcescens* | 911022 | 615 | ATCC 13880 | RS_GCF_000735445.1 |
| *Vibrio cholerae* | 243277 | 666 | N16961 | RS_GCF_000006745.1 |
| *Veillonella parvula* | 479436 | 29466 | DSM 2008 | RS_GCF_000024945.1 |

**Supplementary Table 6 | Species names and GTDB representative genomes used to construct phylogenetic tree.**

| | rRNA Expression Plasmids | | | | | | R-Protein Expression Plasmids | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Individual R-proteins | | | | R-protein Operons | | | | | | | | |
| **Intergenic Regions** | Native | *E. coli* | Native | *E. coli* | *E. coli* | *E. coli* | | | | | | | | | | | | | |
| **Anti-Ribosome Binding Site** | Wildtype | Wildtype | *Anti-B* | *Anti-B* | *Anti-B* | *Anti-B* | | | | | | | | | | | | | |
| **Stapled vs. WT** | WT | WT | WT | WT | Stapled | Stapled | | | | | | | | | | | | | |
| **Co-Produced LSU** | Cognate | Cognate | Cognate | *Cognate* | Cognate | *E. coli* | **S20, S16** | **S20, S16, S15** | **S20, S16, S1, S15** | **S2, S12, S20, S16, S17** | **alpha (α)** | **beta (β)** | **str** | **spc** | **S10** | **AO1** | **AO2** |
| *Shigella sonnei* | pRF001a | pRF001b | pRF001c | pRF001d | pRF112 | pRF112a | - | - | - | - | - | - | - | - | - | - | - |
| *Shigella flexneri* | pRF002a | pRF002b | pRF002c | pRF002d | pRF113 | pRF113a | - | - | - | - | - | - | - | - | - | - | - |
| *Escherichia coli* | pSB19b | pSB19b | pAB172a | pAB172a | pRF100 | pRF100 | - | - | - | - | - | - | - | - | - | - | - |
| *Salmonella enterica* | pRF003a | pRF003b | pRF003c | pNK007p | pRF114 | pRF114a | - | - | - | - | - | - | - | - | - | - | - |
| *Citrobacter freundii* | pRF004a | pRF004b | pRF004c | pRF004d | pRF115 | pRF115a | - | - | - | - | - | - | - | - | - | - | - |
| *Klebsiella aerogenes* | pRF005a | pRF005b | pRF005c | pRF005d | pRF116 | pRF116a | pNK143 | - | - | - | - | - | - | - | - | - | - |
| *Klebsiella pneumoniae* | pRF006a | pRF006b | pRF006c | pRF006d | pRF117 | pRF117a | pNK144 | - | - | - | - | - | - | - | - | - | - |
| *Klebsiella oxytoca* | pRF007a | pRF007b | pRF007c | pRF007d | pRF118 | pRF118a | - | - | - | - | - | - | - | - | - | - | - |
| *Enterobacter cloacae* | pRF008a | pRF008b | pRF008c | pRF008d | pRF119 | pRF119a | - | - | - | - | - | - | - | - | - | - | - |
| *Serratia marcescens* | pRF009a | pRF009b | pRF009c | pNK041p | pAB207a | pAB207a2 | - | - | - | - | - | - | - | - | - | - | - |
| *Proteus mirabilis* | pRF010a | pRF010b | pRF010c | pRF010d | pAB207b | pAB207b2 | - | - | - | - | - | - | - | - | - | - | - |
| *Providencia stuartii* | pRF011a | pRF011b | pRF011c | pNK059p | - | - | pNK083g | - | - | - | - | - | - | - | - | - | - |
| *Vibrio cholerae* | pRF012a | pRF012b | pRF012c | pNK013p | pAB207c | pAB207c2 | pNK083f | - | pAB184h | - | pNK096c | pNK096e | pNK096d | pNK096b | pNK096a | pNK096f | pNK096g |
| *Alteromonas macleodii* | pRF013a | pRF013b | pRF013c | pNK045p | pAB207d | pAB207d2 | pNK083e | pNK108h | pNK102c | - | pNK099c | pNK099e | pNK099d | pNK099b | pNK099a | pNK099f | pNK099g |
| *Marinospirillum minutulum* | pNK112a | pNK112b | pNK112c | pAC027 | pAB207e | pAB207e2 | pAB184e | - | pAB184e2 | - | - | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | pRF014a | pRF014b | pRF014c | pNK003p | pAB207f | pAB207f2 | pNK083d | - | pNK136a | - | - | - | - | - | - | - | - |
| *Acinetobacter baumannii* | pRF015a | pRF015b | pRF015c | pAC018 | pRF101 | pRF101a | pNK083b | - | pNK136b | - | pAB164b3 | pAB164b5 | pAB164b4 | pAB164b2 | pAB164b | pAB164b6 | pAB164b7 |
| *Alcaligenes faecalis* | pRF016a | pRF016b | pRF016c | pAC044 | pAB207g | pAB207g2 | pAB184c | - | pAB184c3 | - | pAB164c3 | pAB164c5 | pAB164c4 | pAB164c2 | pAB164c | pAB164c6 | pAB164c7 |
| *Bordetella pertussis* | pRF017a | pRF017b | pRF017c | pAC037 | - | - | pAB184b | - | pAB184b3 | - | - | - | - | - | - | - | - |
| *Burkholderia cenocepacia* | pRF018a | pRF018b | pRF018c | pNK053p | pRF102 | pRF102a | pNK083c | - | pAB184g | - | pNK098c | pNK098e | pNK098d | pNK098b | pNK098a | pNK098f | pNK098g |
| *Neisseria gonorrhoeae* | pRF019a | pRF019b | pRF019c | pAC036 | - | - | pAB184a | - | pAB184a3 | - | - | - | - | - | - | - | - |
| *Mariprofundus ferrooxydans* | pRF020a | pRF020b | pRF021c | pAC030 | pAB207h | pAB207h2 | pAB184f | - | pAB184f2 | - | - | - | - | - | - | - | - |
| *Caulobacter crescentus* | - | - | pRF022c | pAC042 | pAB207i | pAB207i2 | pNK131a | - | pNK131d | - | - | - | - | - | - | - | - |
| *Rhodopseudomonas palustris* | - | - | pRF023c | pAC038 | - | - | pNK132a | - | pNK132d | - | - | - | - | - | - | - | - |
| *Rickettsia parkeri* | - | - | pRF024c | pAC040 | - | - | pAB184d | - | pAB184d3 | pNK138b | pAB185c | pAB185e | pAB185d | pAB185b | pAB185a | pAB185f | pAB185g |
| *Desulfovibrio vulgaris* | - | - | pRF025c | pAC039 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Helicobacter pylori* | - | - | pRF027c | pAC041 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Bacteroides thetaiotaomicron* | - | - | pRF028c | pRF028d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Enterococcus faecalis* | - | - | pRF029c | pAC019 | - | - | pNK155 | - | - | pNK078a | - | - | - | - | - | - | - |
| *Enterococcus faecium* | - | - | pRF030c | pRF030d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Staphylococcus aureus* | - | - | pRF031c | pRF031d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Bacillus subtilis* | - | - | pRF032c | pRF032d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Clostridium difficile* | - | - | pRF033c | pRF033d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Veillonella parvula* | - | - | pRF034c | pRF034d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Bifidobacterium longum* | - | - | pRF035c | pRF035d | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Mycobacterium tuberculosis* | - | - | pNK036c | pRF036d | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Supplementary Table 7 | rRNA and r-protein expression plasmids used in this study.** rRNA/r-protein combinations yielding the highest degree of activity have been deposited in Addgene and are highlighted in blue. Addgene IDs are listed in Supplementary Table 8. rRNA = ribosomal RNA; r-protein = ribosomal protein; LSU = large subunit.

| rRNA Plasmids | |
|---|---|
| **Name** | **Addgene ID** |
| pNK110d | 156321 |
| pRF001d | 156322 |
| pRF002d | 156323 |
| pAB172a | 156324 |
| pNK007p | 156325 |
| pRF004d | 156326 |
| pRF005d | 156327 |
| pRF006d | 156328 |
| pRF007d | 156329 |
| pRF008d | 156330 |
| pNK041p | 156331 |
| pRF010d | 156332 |
| pNK059p | 156333 |
| pNK013p | 156334 |
| pNK045p | 156335 |
| pAC027 | 156336 |
| pNK003p | 156337 |
| pAC018 | 156338 |
| pAC044 | 156339 |
| pAC037 | 156340 |
| pNK053p | 156341 |
| pAC036 | 156342 |
| pAC030 | 156343 |
| pAC042 | 156344 |
| pAC038 | 156345 |
| pAC040 | 156346 |
| pAC039 | 156347 |
| pAC041 | 156348 |
| pRF028d | 156349 |
| pAC019 | 156350 |
| pRF030d | 156351 |
| pRF031d | 156352 |
| pRF032d | 156353 |
| pRF033d | 156354 |
| pRF034d | 156355 |
| pRF035d | 156356 |
| pRF036d | 156357 |

| R-protein Plasmids | |
|---|---|
| **Name** | **Addgene ID** |
| pAB184a | 156358 |
| pAB184b3 | 156359 |
| pAB184c3 | 156360 |
| pAB184d3 | 156361 |
| pAB184e2 | 156362 |
| pAB184f2 | 156363 |
| pAB184g | 156364 |
| pAB184h | 156365 |
| pNK083b | 156366 |
| pNK083d | 156367 |
| pNK108h | 156368 |
| pNK131d | 156369 |
| pNK132d | 156370 |
| pNK055b | 156371 |

| Reporter Plasmids | |
|---|---|
| **Name** | **Addgene ID** |
| pAB140j8 | 156373 |
| pNK141a2 | 156374 |
| pNK141b2 | 156375 |
| pNK141c2 | 156376 |
| pNK141d2 | 156377 |
| pNK141e2 | 156378 |
| pNK141f2 | 156379 |
| pNK141g2 | 156380 |
| pNK141h2 | 156381 |
| pNK141i2 | 156382 |
| pNK141j2 | 156383 |
| pNK141k2 | 156384 |
| pNK141l2 | 156385 |
| pNK141m2 | 156386 |
| pNK141n2 | 156387 |
| pNK141o2 | 156388 |

| Strains | |
|---|---|
| **Name** | **Addgene ID** |
| S4246 | 156372 |

**Supplementary Table 8 | Addgene IDs of deposited plasmids.**

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