

## Supplemental Text

### Promoter Screen

Five different combinations of AARS and tRNA promoter were constructed and tested for activity, as it has been previously demonstrated that optimization of expression levels is necessary for good activity<sup>1</sup>. These combinations were tested and a pVeg constitutive promoter in front of the synthetase and a pSer promoter in front of the tRNA were found to be the most effective (Supp. Fig. 1A).

### Reporter optimization

Initial experiments with the mNeongreen reporter expressed by the IPTG-inducible pHyperspank showed high levels of background in the absence of nsAA & synthetase. Follow-up experiments indicated that the background was due to a secondary start codon at Methionine10 of mNeongreen, driven by a ribosomal binding site ~1/3 of the strength of the canonical pHyperspank contained in residues 4-8 (Supp. Fig. 1B-C). Ribosomal binding site presence and strength was calculated from the Salis lab ribosomal binding site calculator<sup>2</sup>. These findings could drive reinterpretation of subcellular microscopy experiments that used C-terminal or sandwich fusion mNeongreen tags<sup>3,4</sup>, as the commonly used mNeongreen sequence is capable of initiating translation independently. An M10S mutation suppressed the background and reported 30-50 fold increase in mNeongreen fluorescence upon addition of the nsAA (Supp. Fig. 1C). All subsequent usage of mNeongreen used the M10S variant.

### Synthetase Promiscuity

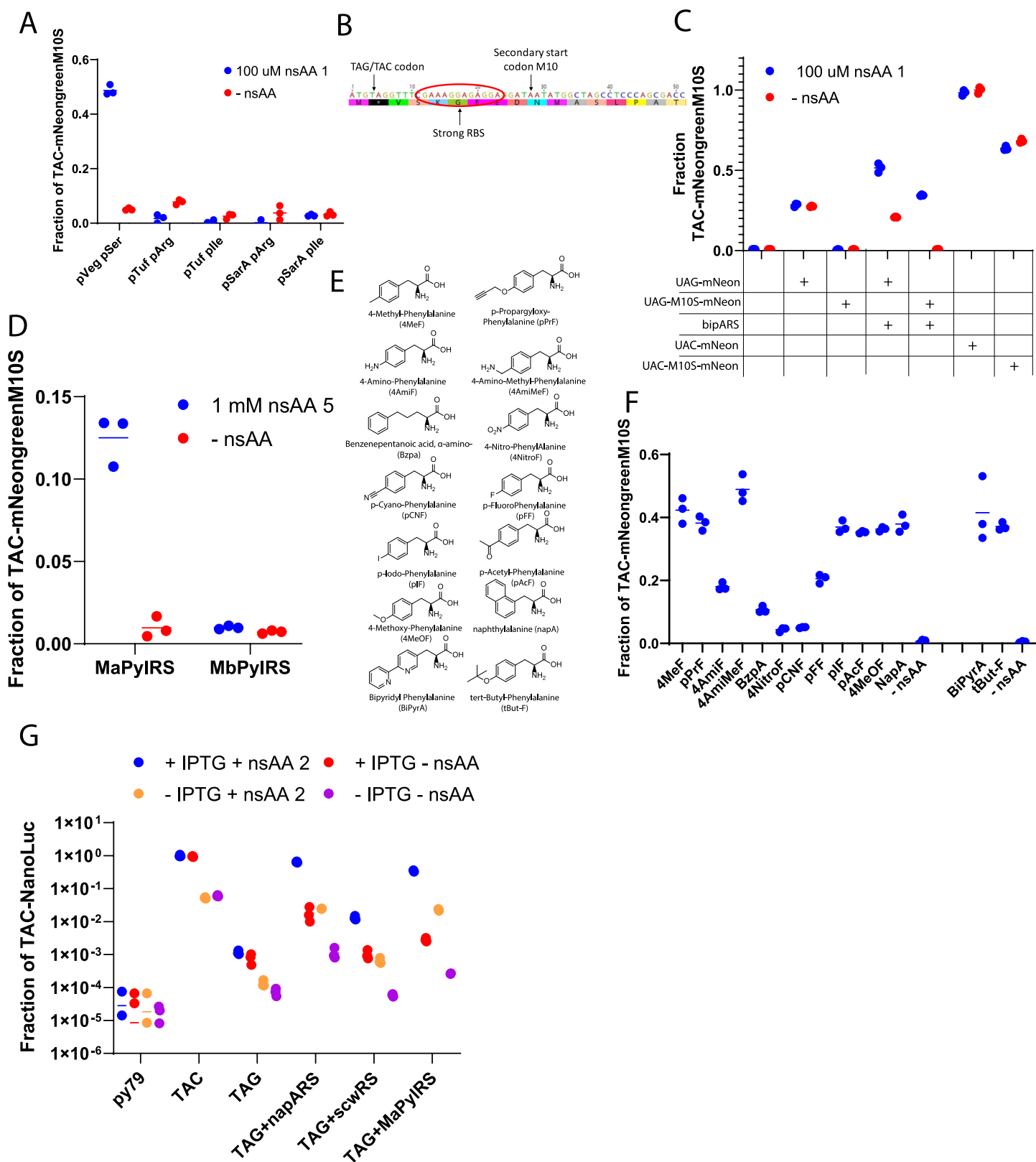
Further exploration of general nsAA incorporation in *B. subtilis* with the extremely sensitive nanoluciferase reporter<sup>5</sup> showed subtleties of incorporation efficiency and promiscuous background incorporation. The MjTyrRSs are known to promiscuously incorporate native amino acids in the absence of the target nsAAs<sup>6</sup>, which is reflected here by some background incorporation in the absence of nsAA. When tested with the TAG-nanoluciferase reporter, the presence of napARS increased TAG-luciferase expression 22-fold in the absence of nsAA. The addition of nsAA increased expression another 25-fold, for a total of 556-fold increase over the TAG-nanoluciferase reporter alone. Both the ScwRS and MaPylRS showed minimal background incorporation, with addition of the synthetase causing 1.3 and 3.6-fold over the reporter in the absence of nsAA, respectively. Addition of nsAA increased expression 11 and 295-fold over the reporter in the presence of corresponding nsAAs, respectively (Supp. Figure 1G).

### nsAA concentration

In general, nsAAs are used at 1 mM concentration. However, some AARSs are capable of functioning effectively at lower concentrations, especially the MjTyrRS-based synthetases. In this study, we found 1 mM to be necessary for all non-tyrosine-based nsAAs and nsAA 3, likely due to its structural distance from the canonical phenyl-group common to other tyrosine-based nsAAs. However, the other tyrosine nsAAs were used at 100 uM as they retained full activity at that level. Some nsAAs cannot be used at 1 mM, especially nsAA 1, which precipitates above 500 uM in LB, and BiPyrA, which causes toxicity at 1 mM. This toxicity may be due to the metal-chelating nature of this nsAA, though we have done no experiments to confirm this.

## Supplemental Figures

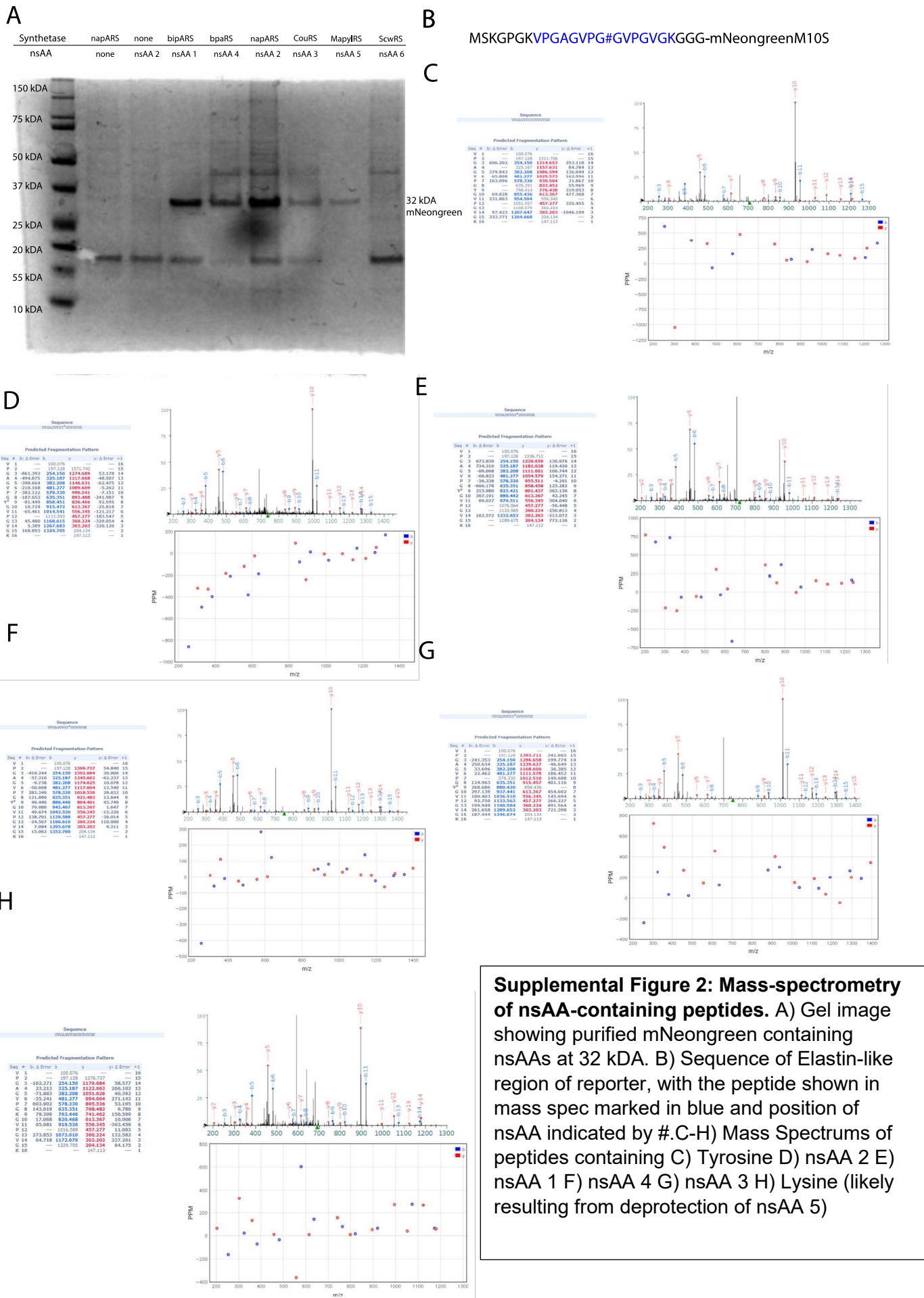
### Supplemental Figure 1



**Supplemental Figure 1: Extended nsAA incorporation in *B. subtilis*.** For all graphs three replicates are shown with a horizontal bar at mean. 100 uM of nsAA was used for all tyrosine-based nsAAs, but 1 mM was used for nsAAs 3, 5 & 6 A). Assay of 5 AARS/tRNA promoter combinations, with the identity of the promoter indicated below the bars. Reported by an pHyperspank-inducible mNeogreen containing a UAG codon in an N-terminal linker and normalized to maximum fluorescence from the experiment. B) Schematic of first 17 residues of TAG-mNeogreen reporter, with the M10 capable of secondary translational start indicated. Secondary RBS strength was calculated as approximately 1/3 the strength of the pHyperspank RBS with the

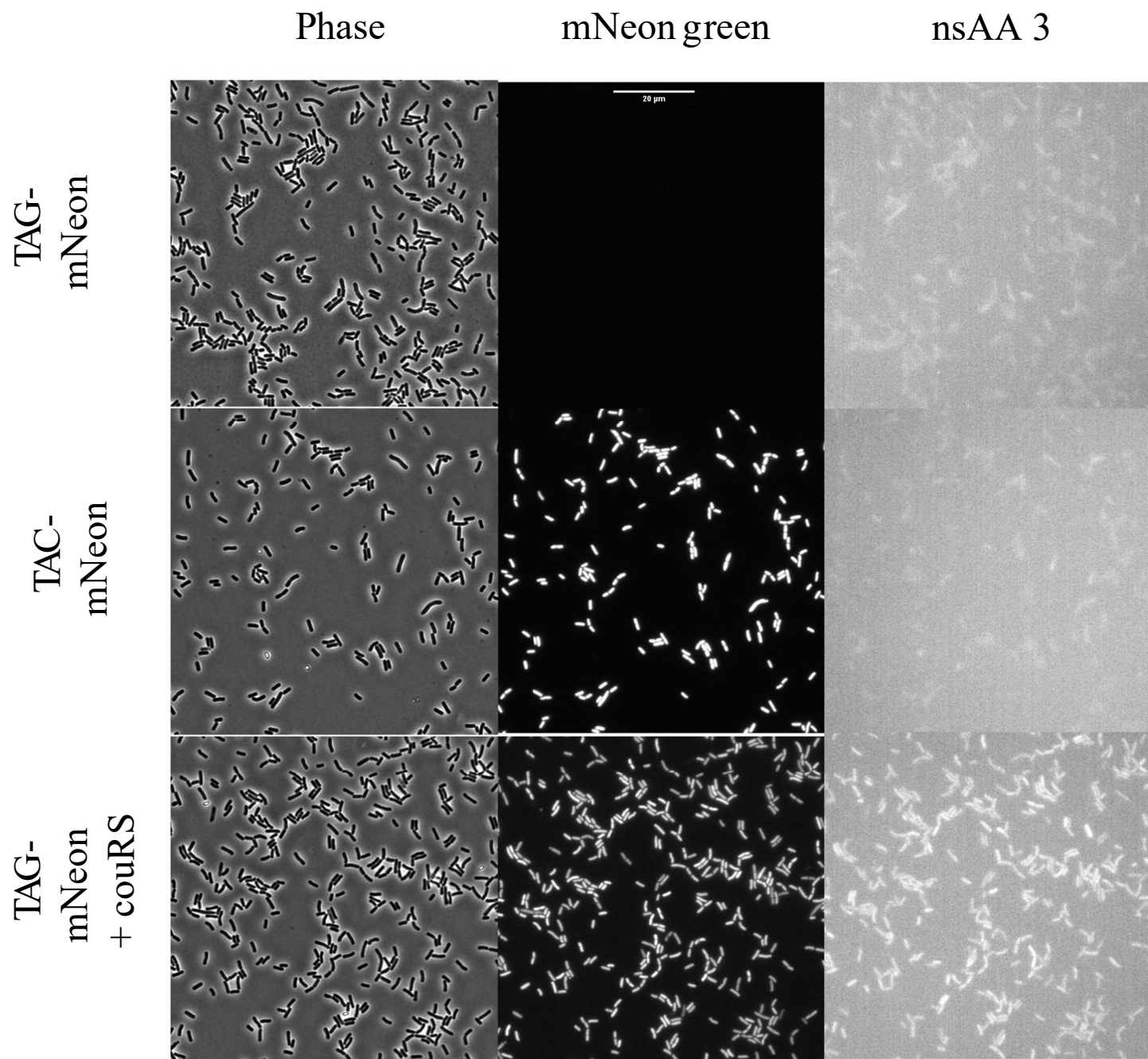
Salis Lab Ribosomal Binding site calculator. C) Assay of the mNeongreen and the M10S mNeongreen reporters with and without associated synthetase. The reported TAG or TAC codon is at position 2, after the start methionine. D) Comparison of *B. subtilis* activity of MaPylRS and MbPylRS activity with the TAG-M10SmNeongreen reporter, normalized to TAC-M10SmNeongreen. MbPylRS is homologous. E) Structures and names of additional nsAAs incorporated in F) using the napARS synthetase (bars on left) or the bipARS synthetase (bars on right). G) Assay of 3 different synthetases incorporating nsAA **1** for napARS, **5** for MaPylRS and **6** for ScwRS using a sensitive IPTG-inducible TAG-nanoluciferase reporter capable of reporting over 5 orders of magnitude. Log plot of pHyperspank-Nanoluciferase levels with a TAG and a TAC codon to demonstrate accurate levels of promiscuity vs. native amino acids.

## Supplemental Figure 2



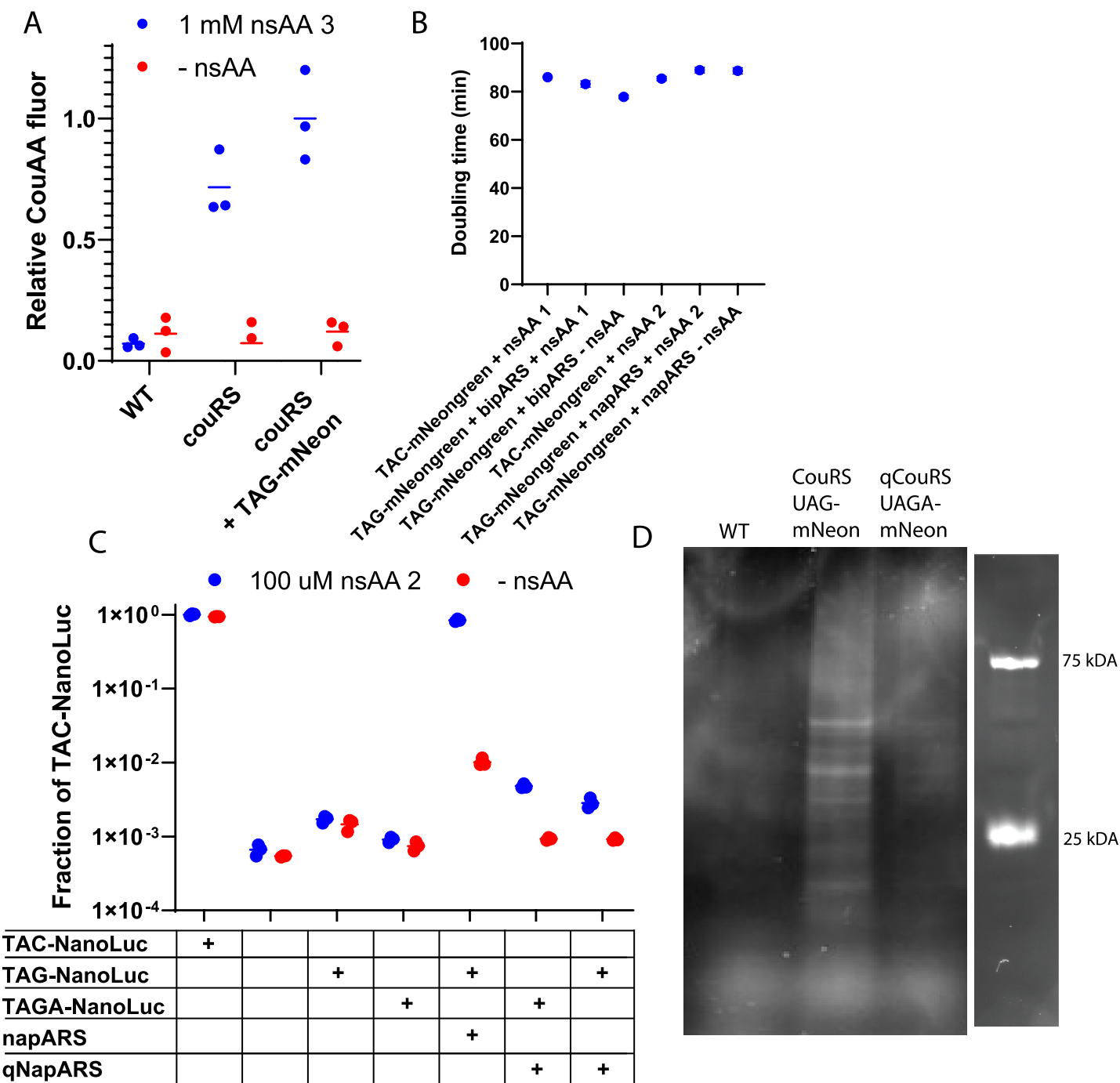
**Supplemental Figure 2: Mass-spectrometry of nsAA-containing peptides.** A) Gel image showing purified mNeogreen containing nsAAs at 32 kDa. B) Sequence of Elastin-like region of reporter, with the peptide shown in mass spec marked in blue and position of nsAA indicated by #. C-H) Mass Spectrums of peptides containing C) Tyrosine D) nsAA 2 E) nsAA 1 F) nsAA 4 G) nsAA 3 H) Lysine (likely resulting from deprotection of nsAA 5)

### Supplemental Figure 3



**Supplemental Figure 3: Fluorescence imaging using CouAA** Images of *B. subtilis* containing mNeongreenM10S reporters with and without a stop codon and with and without CouRS taken in phase, GFP (mNeongreen), and DAPI (nsAA 3) wavelengths. nsAA 3 was supplied to all conditions. CouRS was co-expressed with cognate tRNA. Each column has identical imaging conditions and brightness/contrast settings. A 20 μm scale bar is shown at the top of the figure, and applies to all panels.

Supplemental Figure 4



**Supplemental Figure 4: Genomic incorporation and doubling times.** A) Fluorescence of the CouAA amino acid remaining in bulk cells after washing. Three independent biological replicates shown with mean as a horizontal line, normalized to fraction of TAG signal. CouRS was co-expressed with cognate tRNA. B) Doubling times of *B. subtilis* cells with and without synthetase and 100 uM of nsAA. Single timecourses were fit to exponential growth curves, with 95% confidence intervals from the fits shown as error bars C) High-sensitivity detection of nanoluciferase reporter with UAC, UAG or UAGA codons inserted at position 2 of NanoLuc. Triplicates shown with mean as horizontal bar. Normalized to fraction of expression of UAC construct. D) Whole-cell lysate of cells grown with CouAA run on SDS-page gels and imaged for CouAA fluorescence in the proteome.

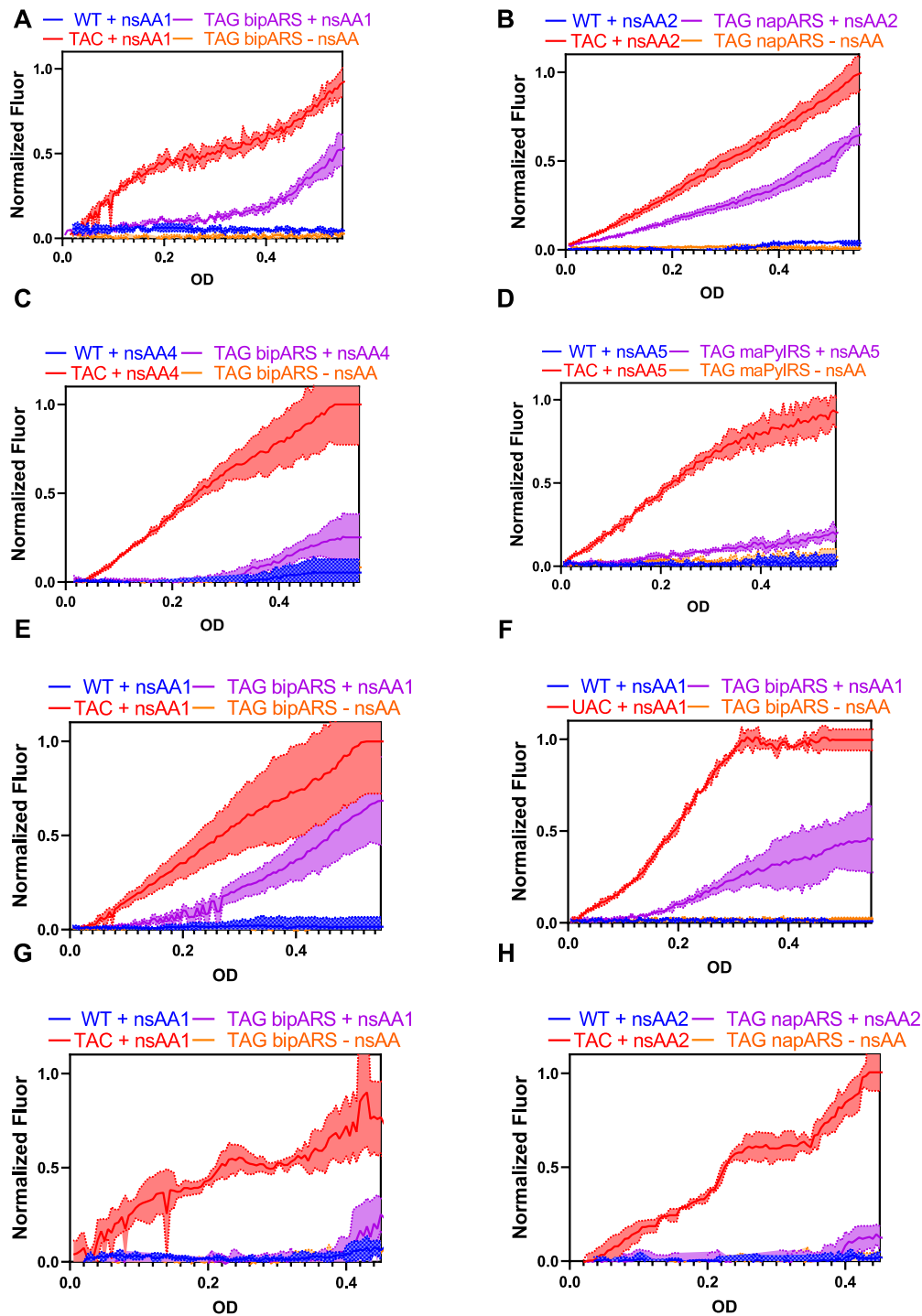
## Supplemental Table 1

| #  | Gene Name | Percentage of nsAA-containing UAG proteins | Gene function                                | Essential? | Ends with TAGA? |
|----|-----------|--------------------------------------------|----------------------------------------------|------------|-----------------|
| 1  | alsS      | 13.1                                       | acetolactate synthase                        | No         | No              |
| 2  | purB      | 10.0                                       | purine biosynthesis                          | No         | Yes             |
| 3  | rplX      | 5.1                                        | ribosomal protein L24                        | Yes        | No              |
| 4  | rpsG      | 4.5                                        | ribosomal protein S7                         | Yes        | No              |
| 5  | yfmC      | 4.0                                        | iron uptake                                  | No         | No              |
| 6  | secA      | 3.6                                        | protein secretion                            | No         | No              |
| 7  | accD      | 3.4                                        | acetyl-CoA carboxylase                       | Yes        | Yes             |
| 8  | ytpR      | 3.2                                        | unknown (possible tRNA synthetase)           | No         | Yes             |
| 9  | atpG      | 3.0                                        | ATP synthase                                 | No         | Yes             |
| 10 | pyrAA     | 3.0                                        | pyrimidine biosynthesis                      | No         | Yes             |
| 11 | ilvD      | 2.9                                        | biosynthesis of branched-chain amino acids   | No         | Yes             |
| 12 | yloV      | 1.7                                        | fatty acid kinase                            | No         | Yes             |
| 13 | dapB      | 1.6                                        | biosynthesis of lysine and peptidoglycan     | Yes        | Yes             |
| 14 | ywlF      | 1.5                                        | ribose-5-phosphate isomerase                 | No         | No              |
| 15 | ywhA      | 1.4                                        | unknown (probable transcriptional regulator) | No         | No              |
| 16 | yaaK      | 1.4                                        | unknown (possible DNA-binding protein)       | No         | No              |
| 17 | murE      | 1.3                                        | peptidoglycan precursor biosynthesis         | Yes        | No              |
| 18 | ylxF      | 1.2                                        | unknown                                      | No         | No              |
| 19 | noc       | 1.2                                        | control of cell division                     | No         | Yes             |
| 20 | ylxM      | 1.1                                        | presecreatory protein translocation          | No         | No              |
| 21 | aroC      | 1.1                                        | biosynthesis of aromatic amino acids         | No         | Yes             |
| 22 | gsaB      | 1.0                                        | biosynthesis of heme, modification of Efp    | No         | Yes             |
| 23 | yeeB      | 1.0                                        | unknown (possible helicase)                  | No         | No              |

**Supplemental Table 1: High-abundance proteins containing nsAAs.** Proteins containing UAG stop codons ranked by abundance in the nsAA-incorporation enrichment by click-pulldown and quantified by mass-spectrometry. Only proteins above 1% abundance are shown and make up 71% of all nsAA-containing proteins found in the enrichment.

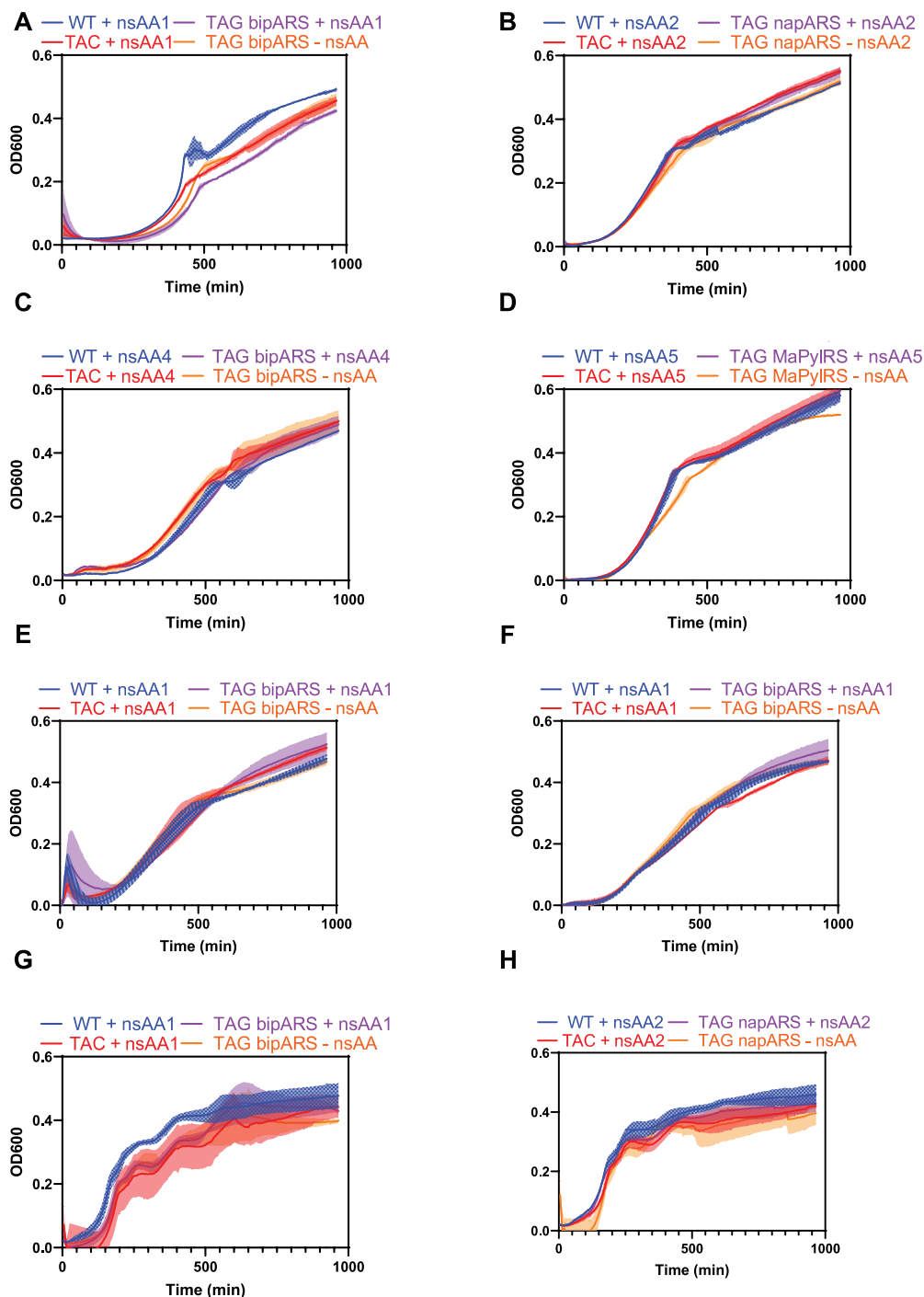


## Supplemental Figure 5



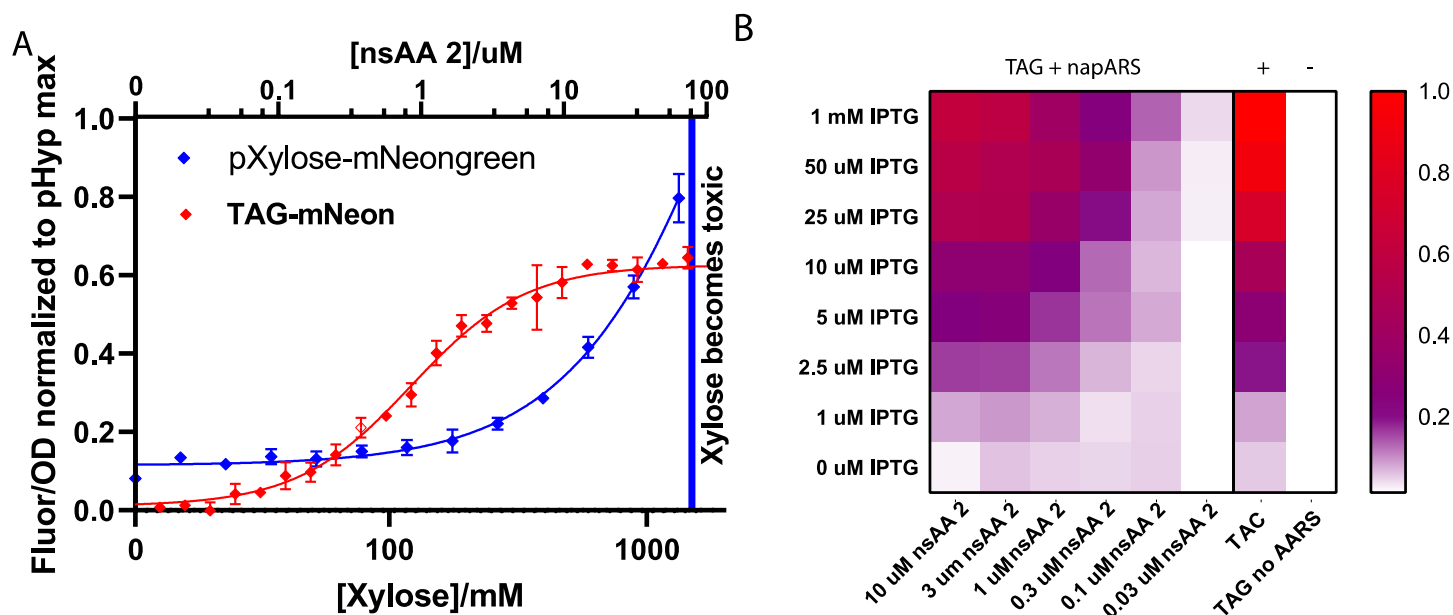
**Supplemental Figure 5: Fluorescence vs. OD time courses for various nsAAs.** Fluorescence and OD curves for different nsAAs and media conditions. A-D) S750 minimal media. E) S750 minimal media plus 1% w/w Pluronic F-68 F) S750 media modified to replace all amino acids with 0.3% buffered ammonium sulfate. G-H) CH rich media. Shaded area represents standard deviation between three biologically independent cultures at each time point.

## Supplemental Figure 6



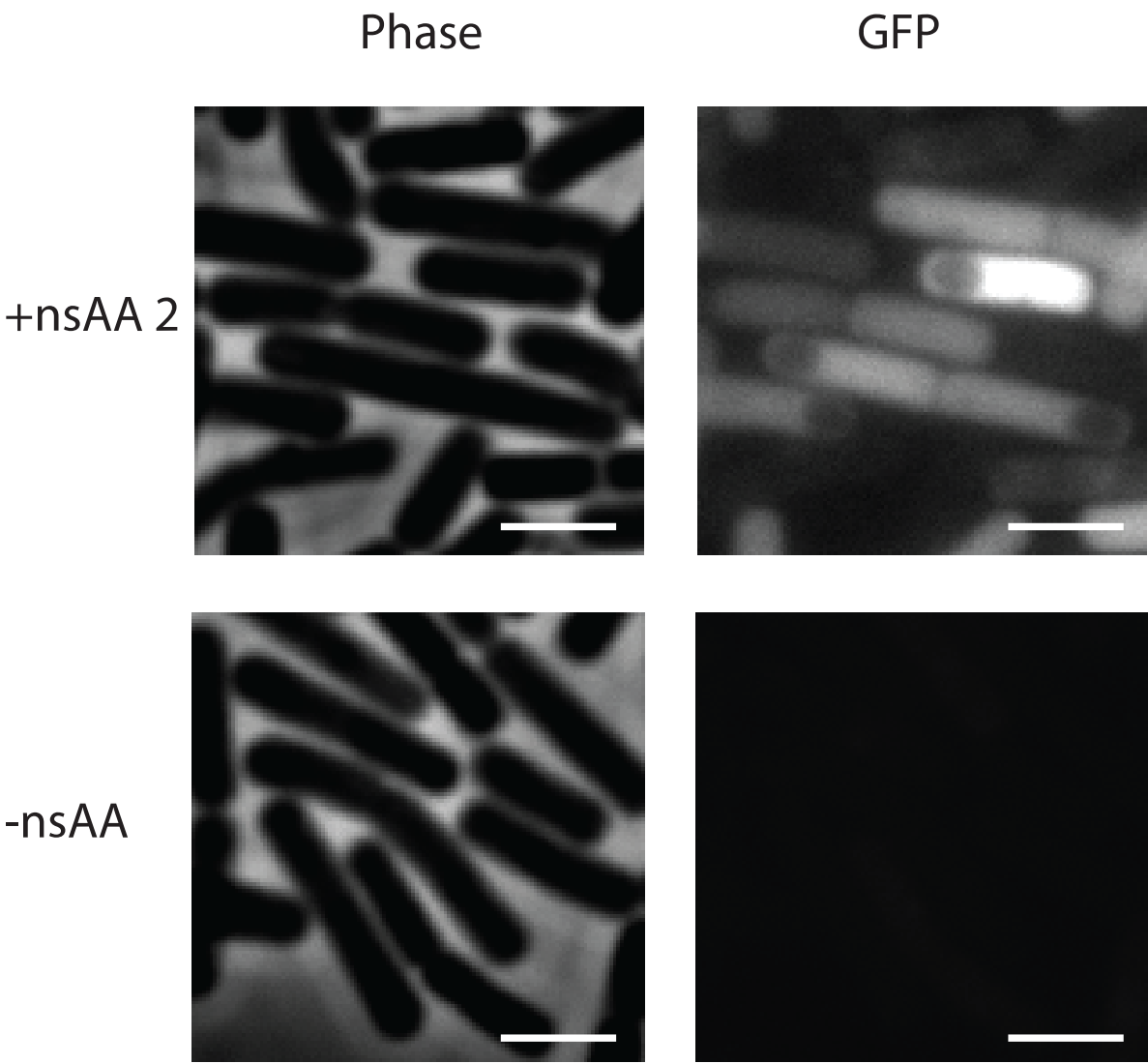
**Supplemental Figure 6. OD time courses for various nsAAs.** OD curves for different nsAAs and media conditions shown in Supplemental figure 5. A-D) S750 minimal media. E) S750 minimal media plus 1% w/w Pluronic F-68 F) S750 media modified to replace all amino acids with 0.3% ammonium sulfate. G-H) CH rich media. Shaded area represents standard deviation between three biologically independent cultures at each time point.

## Supplemental Figure 7



**Supplemental Figure 7:** Extended titration data. A) Titration and sigmoidal fit of pXylose-mNeongreen, with a nsAA 2-induced TAG-mNeongreen from Figure 4A overlaid. 3M xylose significantly reduces growth rate. N=3 biologically independent cultures, and error bars represent standard deviation. B) 2-dimensional titration data, alternate display for the dataset in figure 4B.

Supplemental Figure 8



**Supplemental Figure 8: Incorporation of nsAA 2 in sporulating *B. subtilis* cells.** Cells with GFP(F27TAG) under control of a mother-cell specific promoter, *P<sub>spoIIIE</sub>*, were induced to sporulate by resuspension. At the time of resuspension, the culture was split in two, and nsAA 2 was added to the experimental sample. An example image of sporulating cells at 150 minutes after resuspension shows the GFP signal in the mother cell compartment of engulfed sporulating cells. GFP images shown were taken with identical acquisition times and settings. The control sample shows fluorescent signal at background levels when nsAA 2 is not present. Scale bar shown is 2 microns.

Supplemental Table 2

| Alias  | 5' -> 3' Sequence                              |
|--------|------------------------------------------------|
| oDS066 | TAGGCTGATGCTCCGCTC                             |
| oDS068 | TTCAGGTCGGAAACAGGGAGCACTGGTCAAC                |
| oDS074 | CGAGGGAGCAGAATTGATTTTGCCGCTTAAC                |
| oDS076 | CGAGGGAGCAGAAGAGAGAATATAAAAAGCCAGATTATTAATC    |
| oDS078 | AGGAGGAACTCATATGGATGAATTTGAGATGATAAAACGTAACACG |

|        |                                                               |
|--------|---------------------------------------------------------------|
| oDS086 | TCAGCGGCGTTTTCAAAAAGCTGGTCTGATATG                             |
| oDS088 | TCAGCGGCGTTTTAACCTTTTAGGAGCCAGC                               |
| oDS091 | ACGCAGTCTATATCCGGCGGTAGTTCAGCAG                               |
| oDS092 | AATAAGTTAAGATCCGGCGGTAGTTCAGCAG                               |
| oDS093 | AAAGCTTGC GCCTGCATGCAAAAAAGCCTGCTC                            |
| oDS094 | CTTTTTTGCATGCAGGCGCAAGCTTTAATCC                               |
| oDS096 | GGGCAAGGCTAGACGGGA                                            |
| oDS098 | AACGAGTATGTGATTCTGCTCCCTCGCTCAG                               |
| oDS099 | AGGAAATCCATTACAGGGAGCACTGGTCAAC                               |
| oDS101 | CCAGTGCTCCCTGTAATGGATTTCTTACGC                                |
| oDS102 | CAAATATCTGCATGGTAGTTCCTCCTTAAAG                               |
| oDS106 | CTTTCGAAACCATAAGCTCTTGAACGAACAAC                              |
| oDS107 | CGTTCAAGAGCTTATGGTTTCGAAAGGAGAG                               |
| oDS108 | GAATCCATCCAAATCACTTATAGAGTTCATCCATAC                          |
| oDS109 | ACTCTATAAGTGATTTGGATGGATTAGCCC                                |
| oDS110 | TCTTGACACTCCTTATTTGATTTTTTG                                   |
| oDS135 | TCTTGACACTCCTTATTTGATTTTTTGAAGACTTACTTCGG                     |
| oDS213 | ATCTCAAATTCATCCATATGAGTTCCTCCTTACGCACTACATTTA                 |
| oDS215 | AACGCGGCGTTTTTTAAAGTCTCTTCCGTATTGGCTCCA                       |
| oDS296 | AGAGGAGGATAATTCTGCTAGCCTCCCAGCG                               |
| oDS297 | GGGAGGCTAGCAGAATTATCCTCCTCTCCTTTG                             |
| oDS402 | CATGGTAGTTCCTCCTTAAAGCTTAATTGTTATCC                           |
| oDS406 | TTTGGATGGATTAGCCCCGATTG                                       |
| oDS438 | CAAGGCTAGACGGGACTTACCGAAAGAAACCATCAA                          |
| oDS448 | AGGCGCAAGCTTTAATCCCGGCAAC                                     |
| oDS449 | GTTGACCAGTGCTCCCTGATTCTCCTCCTTGTCTCTTAGCCC                    |
| oDS450 | CAAGGAGGAGAATCAGGGAGCACTGGTCAACTACCG                          |
| oDS451 | GCATCAGCCTAGCATGCAAAAAAGCCTGCTCGTTGAG                         |
| oDS452 | GAGCAGGCTTTTTTGCATGCTAGGCTGATGCTCCGCTCGATAT                   |
| oDS453 | TTTCCGACCTGAACCATGCCGATG                                      |
| oDS454 | GTCTTCAAAAAATCAAATAAGGAGTGCAAGATTCTGCTCCCTCGCTCAGTACCG        |
| oDS455 | TCACATACTCGTTTCAAACGGATCATACAA                                |
| oDS457 | TCTTTCGGTAAGTCCCGTCTAGCCTTGCCCTCACTTATAGAGTTCATCCATACCCATC    |
| oDS608 | GGTGTTCCGGGTGTAGGTAAGGGTGGAGGGACGGTTTCGAAAGGAGAGGAGGATAATTCT  |
| oDS612 | CCTTGTAATCATGATGATGATGATGGTGCTTATAGAGTTCATCCATACCCATCACGTCTG  |
| oDS613 | AAGGACCACGACATCGACTACAAAGACGATGATGATAAATGAGGGCAAGGCTAGACGGGA  |
| oDS614 | CTTGATGTCGCCGTCGTGATCCTTGTAATCATGATGATGATGATGGTGCTTATAG       |
| oDS615 | ATTACAAGGATCACGACGGCGACTACAAGGACCACGACATCGACTACAAAGACGA       |
| oDS618 | AGCTTGACGGAAGTCAATATTGGTAAAGGAGGTTTTTTTATGAGCAAAGGTCCTGGCAAAG |
| oDS619 | CTTTACCAATATTGACTTCCGTCAAGCTTAATTGTTATCCGCTCACAATTACACACATTA  |
| oDS624 | CACAATTCCACACATTATGCCACACCTTGTAGATAAAGTC                      |
| oDS628 | CCCAGCAATCGGGCTGAATCCATCCAAATTACGCCAGAATCCGCTCGCAAA           |
| oDS636 | GTTCCCTCCTTAAGCTTAATTGTTATCCGCTCACAATT                        |
| oTS170 | AGCGATGGCGAAGCAATACGGCTAGGAAAGCCAAGAAGTATTAAATC               |
| oTS172 | GAATGATCTCTGATTTGAAAAGCATGTAGGAAGGTGCTTCAA                    |
| oWM28  | ATGGTTTCGAAAGGAGAGGAGG                                        |
| oAB139 | tatcctcctcctttcgaaacccatGCTAAATCCTCCTAATCTGCCGAATG            |
| oAB197 | AGGCGCAAGCTTTAATCCCG                                          |

|        |                                               |
|--------|-----------------------------------------------|
| oSW251 | gtagttgaccagtgtccctgATTCTCCTCCTTGTTCTCTT      |
| oSW40  | CAGGGAGCACTGGTC                               |
| oSW42  | TTCTGCTCCCTCGC                                |
| oAB76  | gaacggtactgagcgaggagcagaaGTATTGTTTCCGGTTTC    |
| oWM248 | gcccataatcgagcggagcatcagcTTAGCCGCGTTTATTACGGT |
| oSW254 | TAGGCTGATGCTCCGCT                             |
| oAB196 | CCGACCTGAACCATGCCGAT                          |
| oSW250 | AGGCGCAAGCTTTAATC                             |
| oAB13  | ccagtaccgatttctgcatGCTAAATCCTCCTAATCTGCCGAATG |
| oWM24  | ATGGCAGAAATCGGTACTG                           |
| oWM248 | gcccataatcgagcggagcatcagcTTAGCCGCGTTTATTACGGT |
| oVP005 | ggtgatgttaatgggcacaaatAGtctgtcagtggagagggt    |

**Supplemental Table 2: Primers used in this study.** Details on how primers are used is contained in attached strain construction excel document.

## Methods

### References:

1. Scheidler, C. M., Vrabel, M. & Schneider, S. Genetic Code Expansion, Protein Expression, and Protein Functionalization in *Bacillus subtilis*. *ACS Synth. Biol.* **9**, 486–493 (2020).
2. Salis, H. M., Mirsky, E. A. & Voigt, C. A. Automated design of synthetic ribosome binding sites to control protein expression. *Nature Biotechnology* **27**, 946–950 (2009).
3. Bisson-Filho, A. W. *et al.* Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *Science* **355**, 739–743 (2017).
4. Subramanian, S., Gao, X., Dann, C. E. & Kearns, D. B. MotI (DgrA) acts as a molecular clutch on the flagellar stator protein MotA in *Bacillus subtilis*. *PNAS* **114**, 13537–13542 (2017).
5. England, C. G., Ehlerding, E. B. & Cai, W. NanoLuc: A Small Luciferase is Brightening up the Field of Bioluminescence. *Bioconjug Chem* **27**, 1175–1187 (2016).
6. Kunjapur, A. M. *et al.* Engineering posttranslational proofreading to discriminate nonstandard amino acids. *PNAS* **115**, 619–624 (2018).
7. Youngman, P. J., Perkins, J. B. & Losick, R. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. *Proc Natl Acad Sci U S A* **80**, 2305–2309 (1983).
8. Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**, 343–345 (2009).
9. Zhang, M. S. *et al.* Biosynthesis and genetic encoding of phosphothreonine through parallel selection and deep sequencing. *Nature Methods* **14**, 729–736 (2017).
10. Squyres, G. R. *et al.* Dynamics of bacterial cell division: Z ring condensation is essential for cytokinesis. *bioRxiv* 2020.06.30.180737 (2020) doi:10.1101/2020.06.30.180737.
11. Harwood, C. R. & Cutting, S. M. *Molecular biological methods for Bacillus*. (Wiley, 1990).
12. Burton, B. M., Marquis, K. A., Sullivan, N. L., Rapoport, T. A. & Rudner, D. Z. The ATPase SpoIIIE Transports DNA across Fused Septal Membranes during Sporulation in *Bacillus subtilis*. *Cell* **131**, 1301–1312 (2007).
13. Sysoeva, T. A., Zepeda-Rivera, M. A., Huppert, L. A. & Burton, B. M. Dimer recognition and secretion by the ESX secretion system in *Bacillus subtilis*. *PNAS* **111**, 7653–7658 (2014).

14. Huppert, L. A. *et al.* The ESX System in *Bacillus subtilis* Mediates Protein Secretion. *PLOS ONE* **9**, e96267 (2014).