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1. Construction of Plasmids

Phusion high-fidelity DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, and restriction enzymes were purchased from New England Biolabs. Oligonucleotide primers were ordered from Integrated DNA Technologies, Inc. All the plasmid structures were confirmed with DNA sequencing by Eton Bioscience, Inc. Ni-NTA superflow resins were purchased from Qiagen. BocK and AlocK were purchased from Chem-Impex International, Inc, ProK was from Wuhan Peptech Pharmaceutical co., LTD.

1.1 Construction of pETDuet-mbPylRS-pylTCCU-GFPuv149AGG, pETDuet-mbPylRS-pylTUCCU-GFPuv149AGGA and pETDuet-mbPylRS-pylTCCU-sfGFP134AGG.

pETDuet-mbPylRS-pylTCCU-GFPuv149AGG was derived from pETDuet–AcKRS –pylTCUA-GFP149TAG [1] and contains a pylTCCU gene under control of the lpp promoter and the rrnC terminator, the codon optimized *Methanosarcina barkeri (M.barkery)* PylRS gene and GFPuv gene with an AGG mutation at position 149, both are under control of the T7 promoter and terminator. The standard QuikChange site-directed mutagenesis was used to generate PstI and AgeI restriction sites of pETDuet-AcKRS-pylTCUA-GFP149TAG (pDUET-pstI-QC-F, 5'- CTGTAACTGCAGGTCGACAAGCTTGCGGCC-3' and pDUET-AgeI-QC-R, 5'-TGACATACCGGTGGTATATCTCCTTCTTAAAGTTAAAC-3'), then ligated with optimized *M.barkery* PylRS (mbpylRS-AgeI-F, 5'-CTGTAAACCGGTATG GATAAGAAGCCACTGGATGTTC-3' and mbpylRS-PstI-R, 5'-GTCGACCTGCAGTTA CAGGTTAGTGGAGATGCCATTG-3') with corresponding restrictive sites to generate pETDuet -mbPylRS- pylTCUA-GFP149TAG first, the resulting plasmid then underwent the second QuikChange site-directed mutagenesis to mutate the TAG codon at position 149 of GFPuv to the AGG codon (GFPuv-AGG-QC-F, 5'-AGGGTATACATCACGGCAGA CAAACAA-3' and GFPuv-AGG-QC-R, 5'-GTGTGA GTTATAGTTGTACTCGAGTT-3'). And PylTCUA was replaced by PylTCCU from SphI sites of pETtrio-pylTCCU -PylRS-sfGFP2AGG [2]. Similarly, pylTCCU gene was replaced by PylTUCCU gene from SphI sites of pETtrio-pylTUCCU-PylRS-sfGFP134AGGA and GFPuv149AGG was replaced by GFPuv149AGGA gene from NdeI and KpnI sites of pETDuet-AckRS–PylTUCCU -GFPuv149AGGA to generate pETDuet-mbPylRS–pylTUCCU-GFPuv149AGGA.

1.2. Construction of pEVOL-mbPylRS-pylTCUA, pEVOL-mbPylRS-pylTCCU and pEVOL-AntisenseArgW.

These plasmids pEVOL-mbPylRS-pylTTAG, pEVOL-mbPylRS-pylTAGG and pEVOL-AntisenseArgW were derived from pEVOL-pylTTAG-MCS. mbPylRS gene (mbpylRS-SpeI-F, 5'-GGAATTACTAGTATGGATAAGAAGCCACTGGATGTTC-3' and mbpylRS-SalI-R, 5'-ATGATGGTCGACTTACAGGTTAGTGGAGATGCCATTG-3') was cloned to SpeI and SalI sites of pEVOL-pylTTAG-MCS to construct pEVOL-mbPylRS-pylTTAG under the control of pBAD promoter and terminator. pEVOL-mbPylRS-pylTAGG was constructed by pylTCUA to pylTCCU quickchange with pEVOL-pylTCCU-QC-F, 5'-CCGTTCAGCCGGGTTAGATTCCCGGGGTTTCCGCCAAA TTCGAAAAGCCTGCTCAACG-3' and pEVOL-pylTCCU-QC-R, 5'-ATTAGGAGTCCAT

TCGATCTACATGATCAGGTTTCCAATGCGGGGCGCATCTTACTGC-3'. pEVOL-AntisenseArgW was constructed by quickchange ArgW gene antisence fragment to pEVOL to be controlled by pBAD promoter and terminator with primers AntiArgW-QC-F, 5'-TAGGAGGGGCTCGTTATATCCATTTAACTAAGAGGACAATTCGAAAAGCCTGC TCAA-3' and AntiArgW-QC-R, 5'-AGGGCTAATTGCAGGTTCGATTCCTGCAGGGG ACACCAAATGCGGGGCGCATC TTACT-3'.

1.3. Construction of pETrio-mmPylRS-pylTUCCU-sfGFP134AGGA-5aa-upstream -non-optimal and pETrio-mmPylRS-pylTUCCU-GFPuv149AGGA

pETrio-mmPylRS-pylTUCCU-sfGFP134AGGA-5aa-upstream-non-optimal was constructed by quickchange pETrio-mmPylRS-pylTUCCU-sfGFPAGGA [2] with primer pairs sfGFP-134AGGA-5AA-UP-QC-F, 5'-CGCATTGAACTGAAAGGTATAGACTTCAAGGA GAGGAGGCAACATTCTGGGT-3', sfGFP-134AGGA-5AA-UP-QC-R, 5'-GTTCACCAG GGTATCGCCTTCAAATTTAAC-3'. pETrio-mmPylRS-pylTUCCU-GFPuv149AGGA was generated by replacing sfGFP134AGGA with GFPuv149AGGA at AflII and KpnI sites.

2. DNA and Protein Sequences

2.1 DNA Sequences

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***methanosarcina barkeri* PylRS:**

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2.2 Protein sequence

sfGFP134AGGA:mvskgeelftgvvpilveldgdvnghkfsvrgegegdatngkltlkficttgklpvpwptlvttltygvq cfsrypdhmkrhdffksampegyvqertisfkddgtyktraevkfegdtlvnrielkgidfkexgnilghkleynfnshnvyitad kqkngikanfkirhnvedgsvqladhyqqntpigdgpvllpdnhylstqsvlskdpnekrdhmvllefvtaagithgmdelykg shhhhhh

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(x is the incorporated noncanonical amino acid)

3. Protein Expression and Purification

3.1 AGG sense Codon Suppression

Plasmid pETDuet-mbPylRS-pylTCCU-GFPuv149AGG was transformed to *E. coli* BL21 (DE3) cells. A single colony was then picked and allowed to grow in LB medium (5 mL) with ampicillin (100 mg/mL) at 37°C overnight. A 0.5ml aliquot from the overnight culture was used to inoculate 2YT (50 mL) or GMML (50 mL) (1xM9 Salts, glycerol (1%), Leucine (300uM), $MaSO_4$ (2mM), $CaCl_2$ (0.1 mM), NaCl (0.2%)) medium with ampicillin (100 mg/mL). After growth to OD600 of 0.5, IPTG (final 0.5mM) was added to induce GFPuv expression with or without BocK (10mM). The induced cells were allowed to grow at 37°C overnight and then the cells were collected by centrifugation (6,000 rpm for 15 min). The cells were resuspended lysis buffer (NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (10 mM), pH 8.0, 25 mL) and sonicated in an ice water bath three times (4 min each, with a 5 min interval between each run). The lysate were clarified by centrifugation (10,000 rpm for 30min), and the supernatant was decanted into a tube containing Ni-NTA superflow resin (2mL). After incubation at 4°C for 1 h, the mixture was loaded into an empty Qiagen Ni-NTA superflow cartridge. The resin was washed with 5 times volume each of lysis buffer and wash buffer (NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (20 mM), pH 8.0), GFPuv was then eluted with elution buffer (NaH_2PO_4 (50 mM), NaCl (300 mM), imidazole (250 mM), pH 8.0). To further purify the expressed GFPuv, the protein was equilibrated against buffer A (Bis-Tris (20 mM), pH 6.1) and then loaded onto a monoS column from GE Health Science. The protein was eluted out by running a gradient from buffer A to 100% of buffer B (Bis-Tris (20 mM), NaCl (1 mM), pH 6.1). The eluted protein was then concentrated and analyzed by SDS-PAGE. To analyze the purified protein by electrospray ionization mass spectrometry (ESI-MS), the buffer of the purified protein was changed to ammonium bicarbonate (10 mM) using an Amicon Ultra-15 Centrifugal Filter Devices (10,000 MWCO cut, Millopore). Protein concentrations were determined by BCA method. Without further indication, protein purification and characterization in the following experiments were the same.

Plasmids pETDuet-mbPylRS-pylTCCU-GFPuv149AGG and pEVOL-AntisenseArgW carrying antisense tRNAArgccu gene ArgW were cotransformed to E. coli BL21 (DE3) cells. A single colony was then used to express GFPuv with GMML media with IPTG (0.5mM), Arabinose (0.2%) and BocK (10mM).

Plasmids pETDuet-mbPylRS-pylTCCU-GFPuv149AGG and pEVOL-mbPylRS-pylTCCU were cotransformed to E. coli BL21-AI cells [3]. A single colony was then used to express GFPuv with GMML media, noncananicol amino acids BocK, AlocK or ProK was added with IPTG (0.5mM) and Arabinose (0.2%).

Plasmid pDUET-mbPylRS-pylTCCU-sfGFP134AGG and pEVOL-mbPylRS-pylTCCU were cotransformed to E. coli BL21-AI cells. A single colony was then used to express sfGFP with GMML media with IPTG (0.5mM), Arabinose (0.2%) and BocK (10mM).

3.2 AGGA quadruplet Codon Suppression

Plasmid pETrio-mmPylRS-pylTUCCU-sfGFP134AGGA-5aa-upstream-non-optimal was used to transform E. coli BL21(DE3) cells. A single colony was selected and allowed to grow in LB medium (5 mL) with ampicillin (100 mg/mL) at 37°C overnight. This overnight culture was then inoculated into LB medium (50 mL) or GMML media (50 mL) with ampicillin (100 mg/mL) and allowed to grow to OD600 0.5. Two temperatures, 16°C and 37°C, were used to induce sfGFP expression overnight supplied with IPTG (0.5 mM) and BocK (5 mM). Each sample has control only supplied with IPTG (0.5 mM).

Plasmid pETrio-mmPylRS-pylTUCCU-GFPuv149AGGA was used to transform E.coli BL21 (DE3) cells. A single colony was selected and allowed to grow in LB medium (5 mL) with ampicillin (100 mg/mL) at 37°C overnight. This overnight culture was then inoculated into GMML, LB, 2YT or self-synthetic medium[4] (50 mL) (refer to nature protocol no Arginine supply, Glycerol (10% (wt/vol)) 25 ml ,Glucose (40% (wt/vol)) 0.6 ml, Arabinose (20% (wt/vol)) 1.25 ml, 50x M salts 10 ml, $MgSO_4$ (1 M) 1 ml, Aspartate (5%, pH 7.5) 25 ml, Leucine (4 mg/ ml, pH 7.5) 10 ml, 17-amino-acid mix (25x) 20 ml, Trace metal stock solution 100 ul, Water To 500 ml.) with ampicillin (100 mg/mL) and allowed to grow to OD600 0.5. GFP expression overnight supplied with IPTG (0.5 mM) and BocK (5 mM). Induction with autosynthetic media containing different Arg concentrations of 0,100uM, 500uM, 1mM, and 5mM was also performed.

4. Protein labelling

GFPuv (1mg/ml in 1×PBS buffer, pH 7.4) was incubated with $CuSO_4$ (100 μM), NiCl (1 mM), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (0.5 mM), and FlAz (2.5 mM) sequentially, followed by Sodium ascorbate (5 mM) at room temperature for 3 h., then TCA (10%) was added to precipitate the proteins on ice for 30 min, followed by 2V aceton wash, and SDS-PAGE analysis[5].

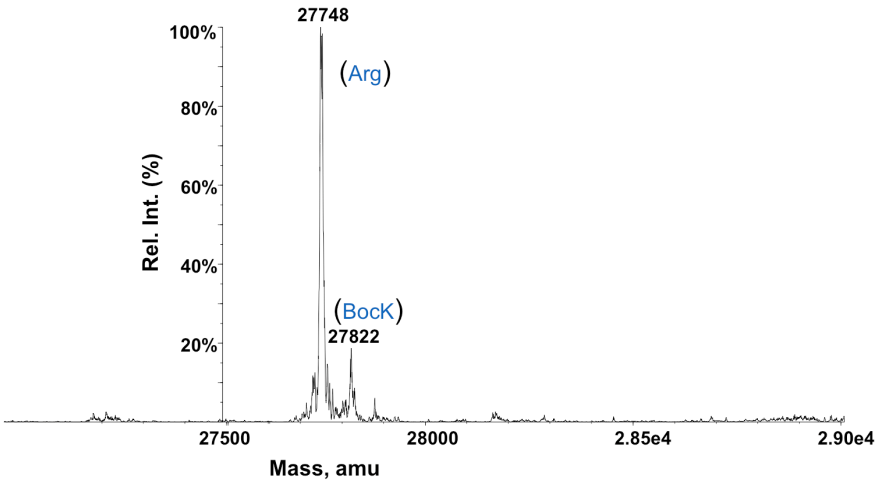
5. Non-selective incorporation of ProK in the *E. coli* proteome

Cells were grown in the presence or in the absence of ProK (10 mM) overnight in minimal media. Cell lysates were then labeled with AzC (0.1 mM) in the presence of $CuSO_4$ (1 mM), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (0.1 M) and TCEP (1 mM) for 1 h before they were analyzed by SDS-PAGE and fluorescent imaging[6].

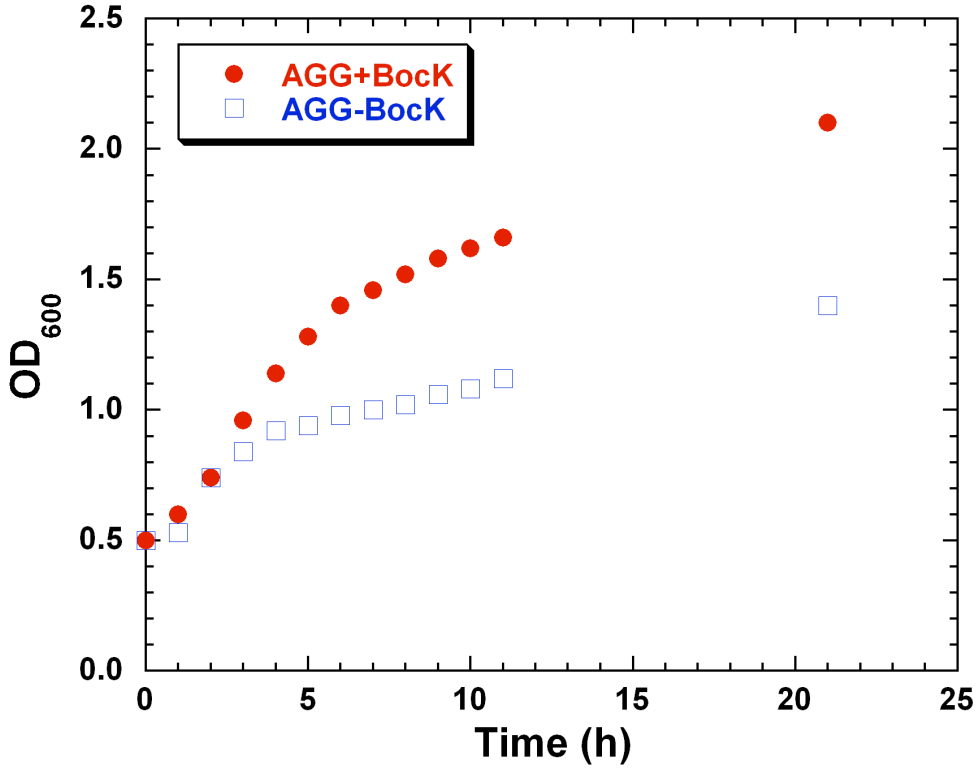
6. ESI-MS analysis

Nanoelectrospray ionization in positive mode was performed using an Applied Biosystems QSTAR Pulsar (Concord, ON, Canada) equipped with a nanoelectrospray ion source. Solution was flowed at 700 nL/min through a 50 µm ID fused-silica capillary that was tapered at the tip. Electrospray needle voltage was held at 2100 V.

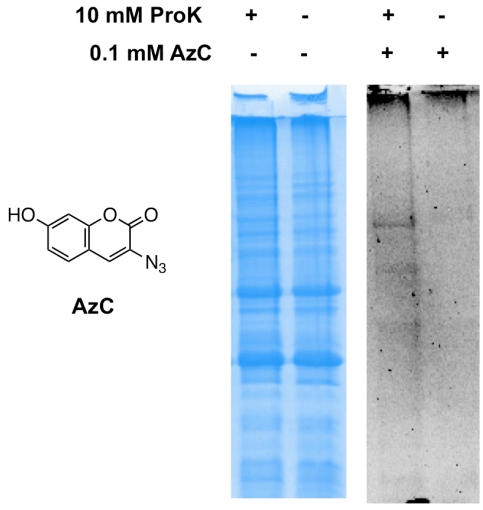
7. Supplementary Figures



Supplementary Figure 1. ESI-MS spectra of GFP_{UV} variants expressed in the presence of BocK (5 mM). *E. coli* BL21(DE3) cells with pET-AGG-GFPUV149AGG were grown in 2×YT medium and induced with the addition of IPTG (0.5 mM) and BocK (5 mM).



Supplementary Figure 2: Growth patterns of BL21-AI cells transformed with pEVOL-AGG and pET-AGG-GFPUV149AGG in media supplied with or without BocK (10 mM). Cells were grown in GMML to OD_{600} as 0.5, arabinose, BocK, and IPTG were added sequentially in 60 min apart to induce the expression of GFP_{UV}.



Supplementary Figure 3: Non-selective incorporation of ProK in the *E. coli* proteome. Cells were grown in the presence or in the absence of ProK (10 mM) overnight in minimal media. Cell lysates were then labeled with AzC (0.1 mM) in the presence of $CuSO_4$ (1 mM), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (0.1 M) and TCEP (1 mM) for 1 h before they were analyzed by SDS-PAGE and fluorescent imaging. The left side shows the Coomassie blue staining of the gel and the right shows the fluorescent imaging of the same

gel.

8. References

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