An efficient cell-free protein synthesis platform for producing proteins with pyrrolysine-based non-canonical amino acids

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Running header: Cell-free synthesis of proteins containing pyrrolysine based ncAAs

ABSTRACT:

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/biot.202200096.

Incorporation of non-canonical amino acids (ncAAs) into proteins opens new opportunities in biotechnology and synthetic biology. Pyrrolysine (Pyl)-based ncAAs are some of the most predominantly used, but expression systems suffer from low yields. Here, we report a highly efficient cell-free protein synthesis (CFPS) platform for site-specific incorporation of Pyl-based ncAAs into proteins using amber suppression. This platform is based on cellular extracts derived from genomically recoded *Escherichia coli* lacking release factor 1 and enhanced through deletion of endonuclease A. To enable ncAA incorporation, orthogonal translation system (OTS) components (i.e., the orthogonal transfer RNA (tRNA) and orthogonal aminoacyl tRNA synthetase) were co-expressed in the source strain prior to lysis and the orthogonal $tRNA_{CUA}^{Pyl}$ that decodes the amber codon was further enriched in the CFPS reaction via co-synthesis with the product. Using this platform, we demonstrate production of up to 442±23 μg/mL modified superfolder green fluorescent protein (sfGFP) containing a single Pyl-based ncAA at high (>95%) suppression efficiency, as well as sfGFP variants harboring multiple, identical ncAAs. Our CFPS platform can be used for the synthesis of modified proteins containing multiple precisely positioned, genetically-encoded Pyl-based ncAAs. We anticipate that it will facilitate more general use of CFPS in synthetic biology.

NOVELTY STATEMENT:

Incorporation of non-canonical amino acids into proteins opens new opportunities in chemical and synthetic biology, as well as biotechnology. Pyrrolysine-based non canonical amino acids are some of the most predominantly used, but expression systems suffer from low yields. Here, we report a robust and efficient cell-free protein synthesis platform for site-specific incorporation of pyrrolysine-based non-canonical amino acids into proteins using amber suppression. This platform is based on cellular extracts derived from genomically recoded *Escherichia coli* lacking release factor 1 (RF1) and deficient in endonuclease A. We anticipate that the platform will facilitate more general use of cell-free protein synthesis in systems and synthetic biology.

INTRODUCTION:

Cell-free protein synthesis (CFPS) has emerged as a powerful and efficient technology platform for applied biotechnology[1-4]. In recent years, for example, CFPS systems have been applied to high-throughput protein production, enzyme screening, diagnostics, clinical scale production of therapeutics, genetic part and circuit characterization, glycoprotein synthesis, incorporation of non-canonical amino acids (ncAAs) into proteins for expanding the chemistry of life, and educational kits[5-47]. The driving force behind the recent expansion of applications include advances in (i) extract optimization, (ii) source strain engineering, and (iii) the ability to activate cost-effective endogenous metabolism to fuel highly efficient CFPS [3,21,26,48].

When compared to complementary *in vivo* protein production approaches, CFPS enjoys several key advantages. First, it provides an unprecedented ability to monitor, modify, and control reaction conditions by enabling easy substrate addition, product removal, and rapid sampling. For example, direct access to the reaction volume eliminates potential transport barriers interfering with the bioavailability of ncAAs. Second, CFPS systems are not affected by toxicity constraints that would be deleterious in living cells. Third, *in vitro* approaches offer rapid prototyping environments (i.e., hours to days) that are faster than *in vivo* standard testing settings that require time consuming cloning work (i.e., days to weeks) [3,6,49].

Given these advantages and recent technical improvements, CFPS is increasingly adapted for use in new application areas. In particular, the approach has recently been leveraged to advance efforts to expand the chemistry of life via ncAA incorporation into proteins[24,46,50]. Expansion of the amino acid repertoire with ncAAs unlocks otherwise inaccessible protein structures, functions, and sidechain chemistries, and has been used for production of antibody drug conjugates [51,52], fluorescent probes for understanding biological systems[53], more effective therapeutic proteins[54], and phosphoproteins[45], among others. The most established method to generate proteins containing ncAAs involves the use of

engineered orthogonal transfer RNA (o-tRNA)/orthogonal aminoacyl-tRNA synthetase (o-aaRS) pairs sourced from phylogenetically distant organisms to repurpose the amber stop codon (TAG) as a coding channel for the incorporation of ncAAs [55]. These orthogonal translation system (OTS) components are evolved to be parallel to and independent from the host but operate in concert with the chassis organism's native translation machinery to catalyze the co-translational incorporation of ncAAs into nascent peptides in a sequence-defined manner. In *Escherichia coli*, amber suppression is limited by the activity of release factor 1 (RF1) which competes with loaded suppressor tRNAs at amber codons and catalyzes translational termination, often leading to the premature truncation of protein products at positions intended for ncAA incorporation using strains in which functional RF1 is intact.

A prominent, naturally occurring OTS is that for pyrrolysine (Pyl), commonly referred to as the 22nd amino acid. This amino acid is inserted into naturally occurring in-frame amber (TAG) stop codons in transcripts for methyltransferases of select bacteria [56,57], and methanogenic archaea [58]. Notably, Pyl amino-acyl tRNA synthetase (PylRS) and Pyl tRNA are orthogonal to the translation machinery in *E. coli* and other organisms [59-61], enabling the incorporation of Pyl-based ncAAs into proteins in these hosts. Several studies *in vivo* have efficiently incorporated Pyl-based ncAAs into proteins and developed evolved Pyl OTS' [62-65]. CFPS systems for Pyl-based ncAAs have also been reported[66].

Here, we expand upon existing work by developing and optimizing an efficient CFPS platform to produce proteins containing one or more Pyl-based ncAAs (**Figure 1a**). Specifically, we tested the incorporation of two ncAAs: (i) N^6-(5-Norbornen-2-yloxycarbonyl)-L-lysine hydrochloride (hereafter referred to as pLysN) and (ii) N^6-(propargyloxycarbonyl)-L-lysine hydrochloride (hereafter referred to as proCarb). Leveraging the open nature of the cell-free system, we first optimized the expression of modified proteins (i.e., those containing ncAAs) by adjusting the compositions of purified exogenous OTS components (e.g., $tRNA_{CUA}^{Pyl}$, PylRS, etc.). Next, we assessed the efficacy of enriching PylRS and $tRNA_{CUA}^{Pyl}$

in the extracts via expression in the source strain. These optimizations were performed in CFPS systems derived from two different strains, one with and one without RF1. Compared to previous work using CFPS from RF1-deficient extracts[66], our effort is unique in its use of strains that lack the nuclease gene *endA*, which stabilizes DNA concentration[24]. To our knowledge, the resulting CFPS platform synthesized the highest yields of modified proteins harboring single and multiple identical Pyl-based ncAAs yet reported, underscoring the importance of using modified extracts from genomically recoded organisms lacking RF1 for accurate and high-yielding ncAA incorporation.

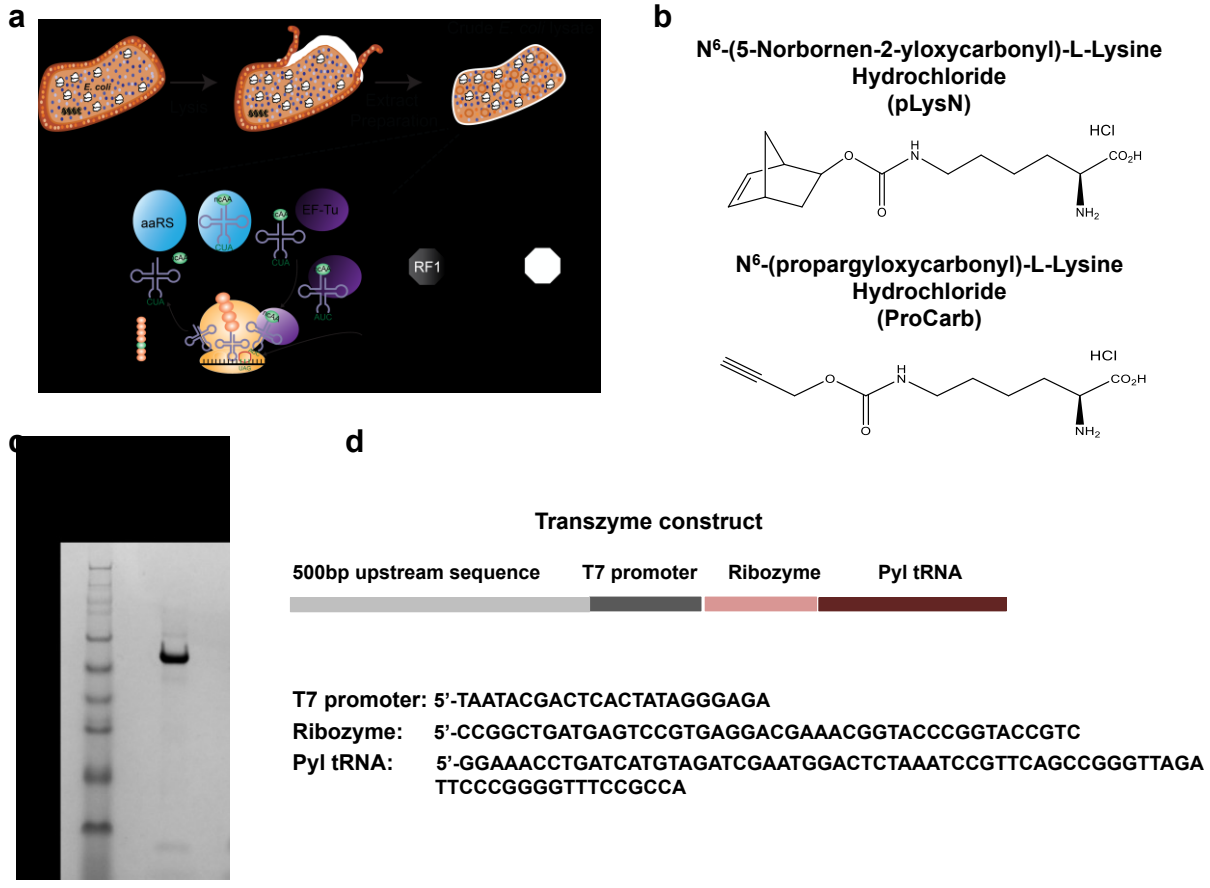

Figure 1. Development of an efficient cell-free platform for incorporation of pyrrolysine (Pyl)-based ncAAs into proteins. (a) Scheme showing the creation of a Pyl-based cell-free platform. Cell extracts containing cellular components required for transcription and translation of proteins are prepared from *E. coli* strains. DNA template encoding sfGFP containing single or multiple amber codon sites, Pyl tRNA, PylRS, Pyl-derived ncAA, T7 RNA polymerase, and other cofactors are added as necessary to activate the cell-free protein synthesis reaction. (b) Structures of the ncAAs used in this study are shown. (c) Purified PylRS was run on a 4-12% PAGE SDS-Polyacrylamide gel to assess for purity. The 51kDa protein is >95% pure. Gel representative of n = 3 independent purifications. (d) The linear DNA template for the transzyme consisting of the T7 promoter, hammerhead Ribozyme and Pyl tRNA is shown. Sequences of the individual components of the transzyme are indicated.

RESULTS:

Expression of the Pyl aaRS and Pyl tRNA during extract preparation is important for protein production in the CFPS reaction

The goal of this work was to demonstrate high-level Pyl-based ncAA incorporation into proteins in a CFPS system. As a model, we tested the incorporation of pLysN and proCarb into superfolder green fluorescent protein (sfGFP) (**Figure 1b**). Creation of an efficient CFPS platform for incorporation of these ncAAs required supplementation of purified PylRS and a purified $tRNA_{CUA}^{Pyl}$ construct into a suitable crude cell extract (**Figure 1c, 1d**). We first assessed the capacity for lysates derived from BL21 (DE3) supplemented with purified PylRS and $tRNA_{CUA}^{Pyl}$ to incorporate pLysN into an in-frame amber stop codon in sfGFP at position 216 (sfGFPT216). Results indicated that this extract did not produce any measurable level of sfGFP as measured by fluorescence (**Figure 2a**). We hypothesized that this lack of full-length sfGFP expression was due to the OTS being outcompeted by active RF1 in the lysates, and that this could be addressed by further enriching the OTS components in the reactions. To test this hypothesis, we expressed Pyl OTS components in the strain off of a plasmid during the exponential phase of cell growth[67]. The resulting extract, named BL21pEvol, was able to catalyze incorporation of pLysN into sfGFP in CFPS reactions as measured by fluorescence (**Figure 2a**).

PylRS is limiting in CFPS reactions derived from BL21 pEvol extracts

We next set out to optimize the concentrations of supplemented PylRS and $tRNA_{CUA}^{Pyl}$ in reactions performed using BL21pEvol extracts. Previous characterizations of PylRS have demonstrated up to a 10-fold reduction in binding affinity for non-Pyl substrates, and the enzyme's low solubility is well-characterized[68]. Based on these observations, we hypothesized that a high concentration of PylRS would be required in CFPS reactions to

overcome these limitations and increase ncAA incorporation. To test this, increasing concentrations of purified PylRS and $tRNA_{CUA}^{Pyl}$ were added to reactions in various combinations, and the resulting yields of pLysN-containing protein (as measured via sfGFP fluorescence) were measured and plotted in MatLab in a two-point lattice (**Figure 2b**). As hypothesized, fluorescence values were observed to increase with increasing concentrations of PylRS but not with increasing $tRNA_{CUA}^{Pyl}$. These data supported our hypothesis that PylRS and not $tRNA_{CUA}^{Pyl}$ was limiting in the reactions even at the highest concentration of PylRS evaluated. We were unable to concentrate the PylRS further due to solubility constraints, and therefore attempted to increase the concentration of PylRS in the extract by having the host strain further overexpress it during cell growth. To achieve this, BL21pEvol was transformed with a second plasmid encoding a single inducible copy of PylRS. As expected, when we attempted pLysN incorporation using the resulting extract (BL21pEvolpET) we observed a slight improvement over the BL21pEvol extract, suggesting that the additional PylRS facilitates incorporation of the ncAA (**Figure 2a**).

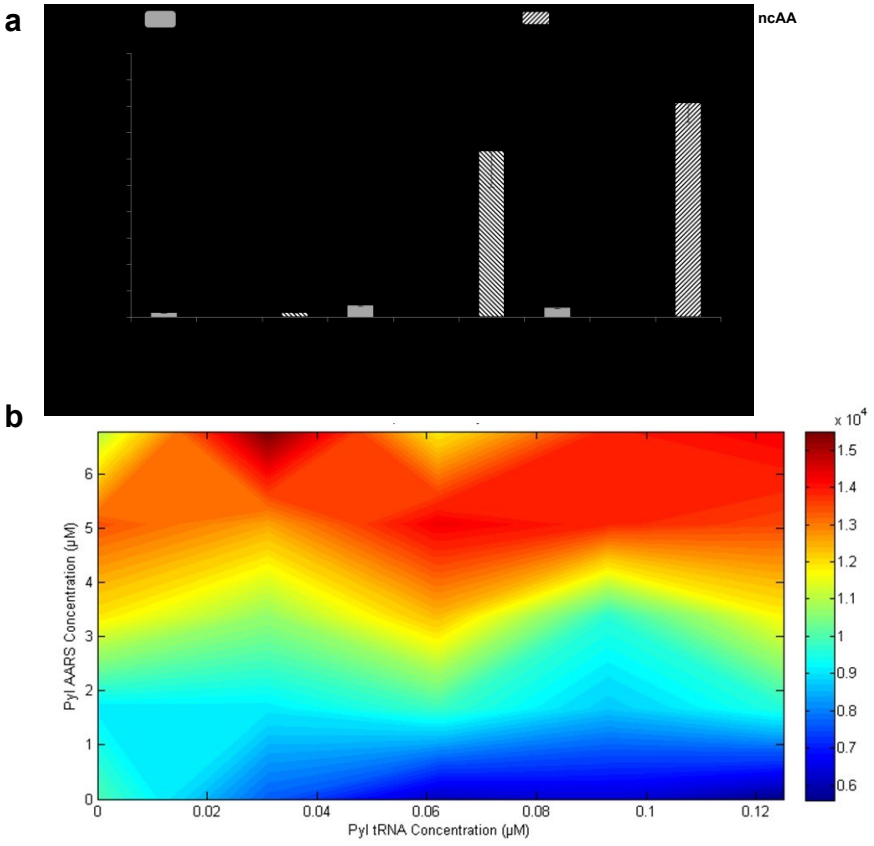

Figure 2. Expression of PylRS in cells before making the extract is important for amber suppression. All CFPS reactions were conducted with plasmid sfGFPT216 (amber position at 216) at 30°C for 20 hours, and incorporated the Pyl-based ncAA pLysN. (a) sfGFP Relative Fluorescence Units (RFUs) are graphed for CFPS reactions performed using lysates derived from the denoted strains in the presence and absence of ncAAs and supplemental OTS components as indicated. Three independent reactions (n = 3) were performed for each data point and one standard deviation is shown. (b) sfGFP yields (RFUs) of samples in BL21pEvol extract are plotted using MatLab software with increasing PylRS concentration on the y-axis and increasing $tRNA_{CUA}^{Pyl}$ concentrations on the x-Axis. Red color indicates the highest fluorescence values with blue indicating the lowest. Two independent reactions (n = 2) were performed for each data point.

We set out to determine whether expressing PylRS during chassis strain growth is equivalent to or more or less effective than adding exogenous purified PylRS to reactions. To assess this, extracts were prepared from cultures with varying amounts of PylRS overexpression and utilized in CFPS both with and without supplementation with purified PylRS to incorporate pLysN into sfGFPT216. Results indicate that initially it is important to express the Pyl OTS components in the chassis strain, as adding exogenous PylRS alone is not sufficient to facilitate pLysN incorporation (**Figure 2a, Figure 3a**). However, once the extract has a certain concentration of PylRS and $tRNA_{CUA}^{Pyl}$ (as in the BL21pEvol extract), further addition of exogenous PylRS is approximately equivalent to expressing a second PylRS-encoding plasmid during chassis strain growth (as in the BL21pEvolpET extract). The system was further improved by supplementing BL21pEvolpET extract with additional purified exogenous PylRS to yield an optimized system (**Figure 3a**).

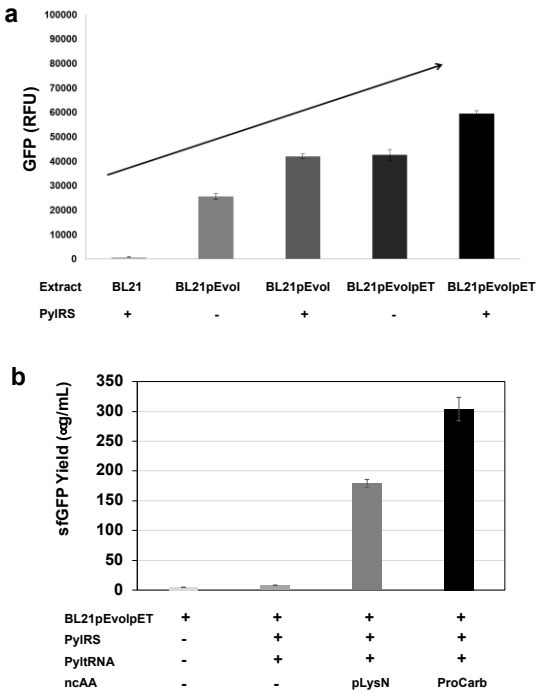

Figure 3. Additional PylRS expression in-strain further increases production of proteins with Pyl-ncAAs. (a) sfGFP produced from samples in the presence (+) and absence (-) of supplemental purified PylRS are compared. Strains used to prepare extracts are indicated and all samples contain pLysN. (b) sfGFP yields (in μg/mL) are compared for samples in the presence (+) and absence (-) of ncAAs pLysN and ProCarb in BL21pEvolpET Extract. Mass amounts of sfGFP produced in each sample are calculated based on a standard curve. PylRS and $tRNA_{CUA}^{Pyl}$ presence (+) and absence (-) in each sample are indicated. These data show the sfGFP produced from samples in which a single amber codon is suppressed (position T216). Three independent reactions (n = 3) were performed for each data point and one standard deviation is shown.

CFPS enables distinct incorporation of Pyl-based ncAAs at high levels

We next applied BL21pEVOLpET-derived lysates supplemented with $tRNA_{CUA}^{Pyl}$ transzyme for the incorporation of the ncAA proCarb and compared its incorporation to that of pLysN. For each of these ncAAs reactions were assembled directed to synthesize sfGFPT216 both in the presence and absence of the ncAA of interest (**Figure 3b**). Synthesis of 304 ± 17 μg/mL and 180 ± 9 μg/mL of modified sfGFP was observed when proCarb and pLysN were added to reactions, respectively. Next, we assembled reactions using all three BL21-derived extracts that were supplemented with radioactive ^{14}C-Leucine and used

autoradiography to visualize radiolabeled sfGFP produced with and without the addition of proCarb and pLysN. This analysis revealed only truncated sfGFP in the BL21 extract both with and without the ncAAs (**Supplementary Figure 1a**). This is expected as in the absence of Pyl OTS components this extract is unable to incorporate the ncAAs into the amber position at amino acid 216 and therefore translation stalls at this position yielding no full-length product. The other two extracts (BL21pEVOL and BL21pEVOLpET) do generate full-length sfGFP upon addition of the ncAAs; however, most of the product is still truncated which is to be expected in an extract that still has active RF1 competing with ncAA incorporation at amber codons (**Supplementary Figure 1a**).

Genomically recoded strain lacking RF1 and endonuclease A circumvents problem of protein truncation

We next pursued a strategy to circumvent product truncation due to premature RF1-mediated translational termination. We reasoned that this could be achieved by preparing lysates from a chassis organism lacking the gene encoding RF1, as has been reported before[66]. One such organism is *E. coli* strain C321.Δ*A*, which has been genomically recoded to replace all 321 UAG amber codons with UAA codons enabling the complete knockout of the RF1-encoding gene *prfA* from the strain [24,46,69,70]. Previous works have demonstrated that lysates derived from this strain and its derivatives show a significantly increased ability to incorporate ncAAs in CFPS[24,46]. We therefore selected a derivative of C321.Δ*A* deficient in endonuclease A (C321.Δ*A* Δ*endA*, henceforth known as rEcoli) and compared extracts derived from this strain to the BL21-derived extracts. Of note, extracts from C321.Δ*A* Δ*endA* (i.e., rEcoli)produce more protein than those from C321.Δ*A* [24].

We produced crude S30 extract from rEcoli cells carrying a plasmid encoding Pyl OTS components (strain rEcolipEvol). Experiments to determine if this extract was limited by PylRS or $tRNA_{CUA}^{Pyl}$ revealed that addition of extra PylRS or Pyl $tRNA_{CUA}^{Pyl}$ did not

significantly improve the incorporation of ncAAs (**Supplementary Figure 2**). As with the BL21-derived extracts, we tested the amount of sfGFPT216X produced in the rEcoli extracts both with and without the addition of pLysN or proCarb. We observed synthesis of 349 ± 79 µg/mL and 442 ± 22.8 µg/mL of sfGFPT216 with pLysN and proCarb, respectively (**Figure 4a**). As expected, these yields were higher than what was produced by the best performing BL21 extract (**Figure 3**). Autoradiography experiments to visualize protein showed a reduction in the amount of truncated protein and a corresponding increase in the amount of full-length sfGFP (**Supplementary Figure 1b**).

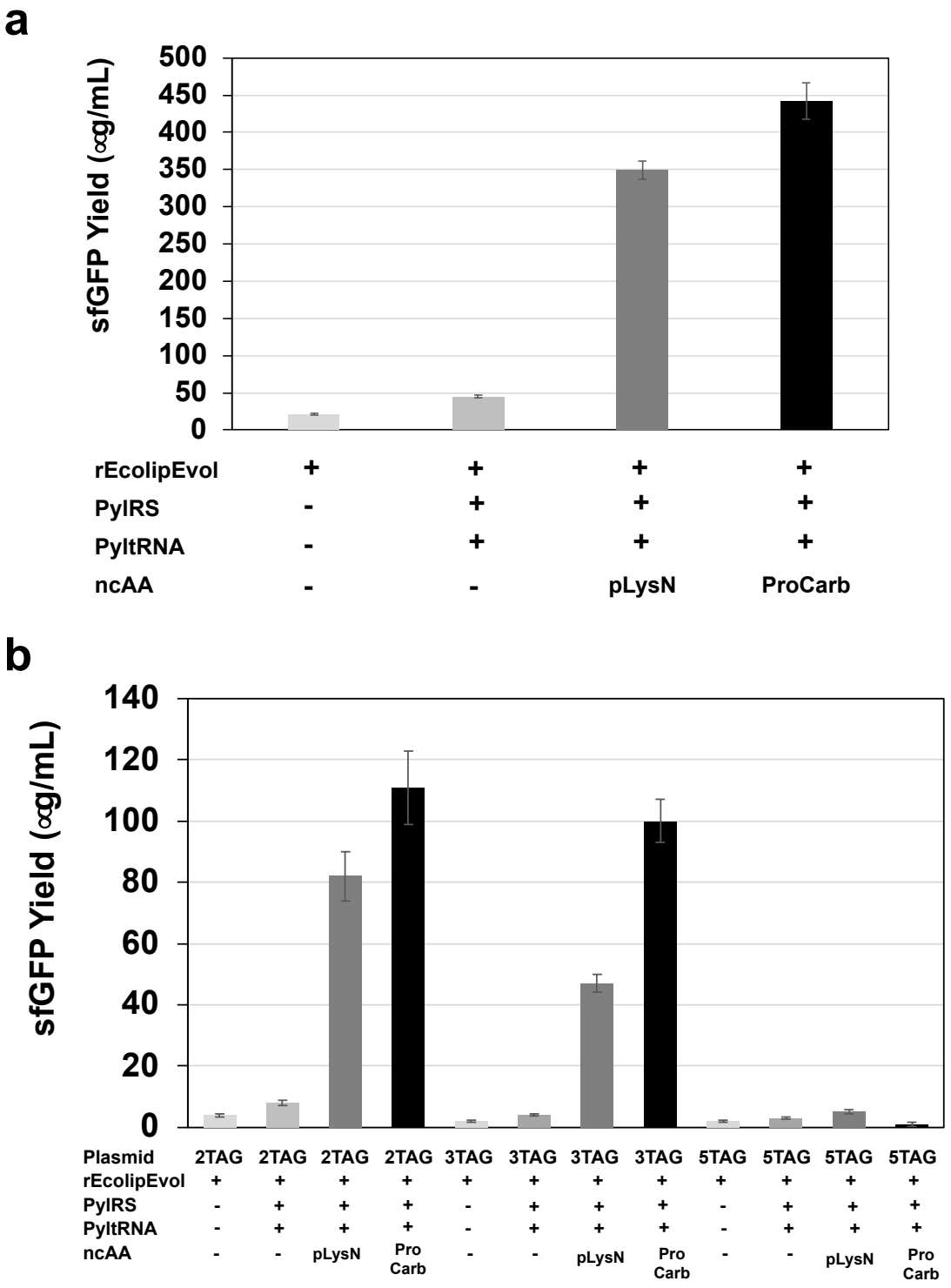

Figure 4: rEcoli extract allows for high yielding biosynthesis of proteins containing Pyl-based ncAAs. (a) sfGFP produced from cell-free reactions in which a single amber codon is suppressed (position T216). The presence/absence of the listed OTS components is indicated for each condition. (b) sfGFP produced from cell-free reactions in which 2 (2TAG), 3 (3TAG), and 5 (5TAG) amber codons are suppressed. The presence/absence of the listed OTS components is indicated for each condition. All reactions were performed using lysates derived from strain rEcolipEvol. Three independent reactions (n = 3) were performed for each data point and one standard deviation is shown.

Cell-free rEcolipEvol extracts can incorporate multiple ncAAs

We next assessed if cell-free extracts from C321.Δ*A* Δ*endA* had the ability to incorporate multiple, identical ncAAs into sfGFP. To test this, we used our rEcolipEvol lysate to synthesize sfGFP constructs featuring 2, 3, or 5 TAG codons in the presence of either pLysN or proCarb (**Figure 4b**). The system was able to synthesize 82 and 111 μg/mL of sfGFP2TAG and 46 and 99 μg/mL of sfGFP3TAG featuring pLysN or proCarb, respectively. sfGFP5TAG synthesis was not detected above background using either ncAA. These results were confirmed and quantified using top-down mass spectrometry (i.e., MS analysis of whole, intact proteins), which showed >95% ncAA incorporation at amber codons in the sfGFP variants containing 1, 2, and 3 TAGs (**Figure 5, Supplementary Table 1**).

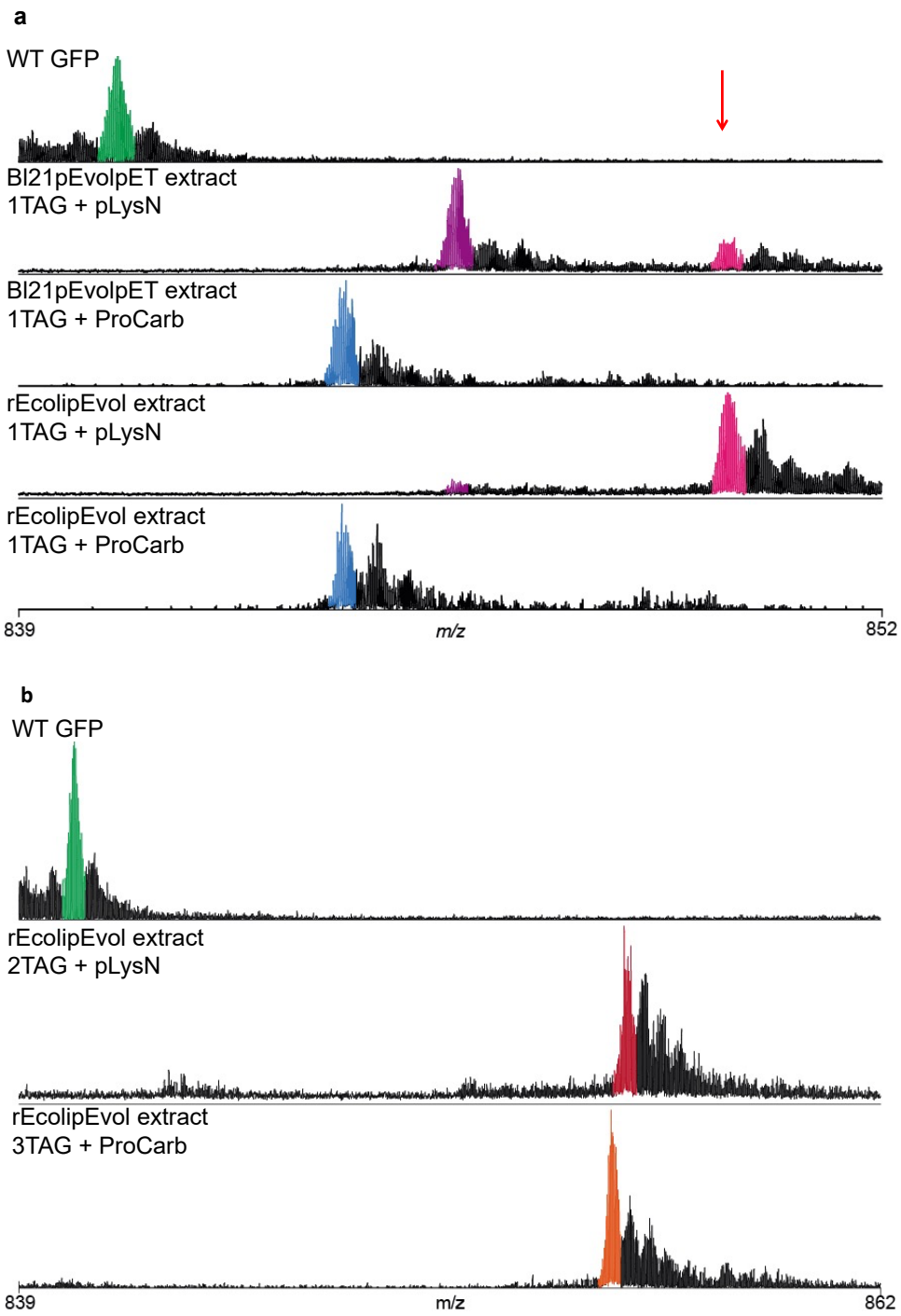

Figure 5: Top-down mass spectrometry demonstrates efficient incorporation of ncAAs into sfGFP. pLysN and ProCarb were incorporated into sfGFP using the indicated extracts sfGFP variants containing (a) 1TAG (sfGFPT216) (b) 2TAG (sfGFP S2T216) or 3TAG (sfGFPS2N212T216) were synthesized by the various extracts as indicated. In each example, colored peaks correspond to the expected mass. The second peak under the red arrow in panel (a) corresponds to the mass of the modified protein retaining the initiator methionine residue. All other samples have this methionine cleaved off. Exact masses and mass shifts are indicated in **Supplementary Table 1.** Data representative of three independent reactions.

DISCUSSION:

Here we present the creation of a cell-free platform for site-specific genetically-encoded incorporation of single or multiple Pyl-based ncAAs into proteins based on RF1 deleted extracts. Expression of the orthogonal PylRS and $tRNA_{CUA}^{Pyl}$ in the cells prior to preparation of the extract was instrumental in creating a robust and efficient S30 extract that was then optimized by supplementation with exogenous purified components. Furthermore, use of extracts derived from the genomically recoded RF1-deficient *E. coli* strain C321.Δ*A*.Δ*endA* resulted in the ability to generate significantly larger quantities of full-length protein, as well as the ability to incorporate more than one ncAA into proteins, as compared to BL21-based extracts. Top-down mass spectrometry confirmed the high degree of ncAA incorporation and purity of the full-length samples.

Our final optimized CFPS system can produce up to ~440 µg/ml or ~349 µg/ml of sfGFP featuring a single incorporation of proCarb or pLysN, respectively, as determined by active fluorescence (i.e., active protein). The described CFPS system may provide several advantages over *in vivo* approaches, namely, improved bioavailability of the Pyl-derived ncAAs and increased yields. Reports in the literature for incorporation of ncAAs using the Pyl OTS *in vivo* suggest yields between ~1 - 40 µg/mL [63,71]. As a result, we anticipate that the cell-free gene expression platform will be useful for synthetic and chemical biology.

MATERIALS AND METHODS:

PylRS purification. An overnight culture of BL21(DE3) transformed with pET21aMmPyl was inoculated into 1L of LB (1:100 dilution) and grown at 250 rpm and 37°C until OD 0.1 (600 nm). At this point the cells were moved to 42°C for heat shock treatment and grown at 250 rpm until OD 0.5. Protein production was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (Sigma, St. Louis, MO) and cells were moved to a 33°C incubator and grown at 250 rpm for 2.5 hours. Cells were harvested at 6,000 × g for 15 min at 4°C, washed

with 1X PBS buffer, and stored at -80°C. The frozen cell pellet was thawed in lysis buffer (100mM HEPES pH 7.2, 500 mM NaCl, 5mM BME) and lysed using a homogenizer at 20,000 ~ 25,000 psi. After clarification by centrifuging at 12,000 × g at 4°C for 15 min, imidazole was added to the supernatant at a final concentration of 10mM and it was loaded onto 2ml of Ni-NTA agarose slurry (Qiagen) that had been washed twice with 1X PBS. The beads were rotated for 1 hour at room temp and spun down at 5,000 × g at 4°C for 4 min. The beads were washed twice with 10 mL wash buffer (100mM Hepes pH 7.2, 500 mM NaCl, 50mM Imidazole) by slow rotation at room temp for 25 minutes followed by pelleting the beads as before. The His-tagged PylRS was eluted in 1mL of elution buffer (100mM Hepes pH 7.2, 500 mM NaCl, 750mM imidazole) with rotation for 20 minutes. The beads were spun down and the eluate was collected and dialyzed against an excess of dialysis buffer (100mM Hepes pH 7.2, 10mM $MgCl_2$, 10mM KCl) overnight at 4°C to remove imidazole. Buffer was exchanged once in the middle of this dialysis. Protein purity was confirmed by 4~12% PAGE SDS-PAGE (Life Technologies, Grand Island, NY). Concentrations were determined by Quick-Start Bradford protein assay kit (Bio-Rad, Hercules, CA) and the protein was stored at -80°C.

Cell extract preparation. The engineering of *E. coli* strain C321.Δ*A*.Δ*endA* was described previously[24]. All *E. coli* cells were grown in 2xYTPG media (Tryptone 16g/L, Yeast extract 10g/L, NaCl 5g/L, K_2HPO_4 7g/L KH_2PO_4 3g/L, glucose 18g/L) at 34°C. To enable in-cell expression of Pyl OTS components, strains were transformed with plasmid pEVOL-Pyl[67]. To drive higher overexpression levels of PylRS, some strains were additionally transformed with plasmid pET21aMmPyl. Cells harboring pEVOL-Pyl were induced at OD (600nm) 0.4 with 0.1% arabinose. Cells harboring pET21aMmPyl were additionally induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. After induction, cell were grown further until OD (600nm) 3.0. Cells were pelleted by centrifuging for 15 min at 6000 × g at 4°C, washed twice with cold S30 buffer (10 mM tris-acetate pH 8.2, 14 mM magnesium acetate, 60 mM

potassium acetate, 1 mM dithiothreitol)[72], and stored at -80°C. Thawed cells were suspended in 1 mL of S30 buffer per gram cells and lysed using a sonicator (Q-Sonica Model CL-18) using 50% amplitude and three pulses (45 sec on and 59 sec off) on ice. 3µl of DTT (1M) was added per mL of sample and the lysate was clarified by spinning at 12000 X *g* for 10 min at 4°C. Clarified supernatant was transferred to a fresh tube and incubated for 80 min at 120 rpm at 37°C to optimize the extract activity, after which it was again centrifuged for 15 min at 15,000 × g at 4°C. The final clarified supernatant was flash-frozen using liquid nitrogen and stored at -80°C until use. Total protein concentration of the extracts was approximately 55 mg/mL, as measured by Quick-Start Bradford protein assay kits (Bio-Rad, Hercules, CA).

CFPS. CFPS reactions were performed as described previously [73] using a modified PANOx-SP system [21]. Briefly, 15 µL of CFPS reaction in a 1.5 mL microcentrifuge tube was prepared by mixing the following components: 1.2 mM ATP; 0.85 mM each of GTP, UTP, and CTP; 34.0 µg/mL folinic acid; 170.0 µg/mL of *E. coli* tRNA mixture; 100 µg/mL T7 RNA polymerase; 2 mM each of 20 standard amino acids; 0.33 mM nicotinamide adenine dinucleotide (NAD); 0.27 mM coenzyme-A (CoA); 1.5 mM spermidine; 1 mM putrescine; 4 mM sodium oxalate; 130 mM potassium glutamate; 10 mM ammonium glutamate; 12 mM magnesium glutamate; 33 mM phosphoenolpyruvate (PEP); 200ng of plasmid DNA, 6.8 µM PylRS (where indicated); 0.045 µM $tRNA_{CUA}^{Pyl}$ (where indicated); 2 mM pLysN or proCarb (where indicated) and 27% v/v of cell extract. Assembled reactions were incubated for 20 h at 30°C.

Quantification of the synthesized sfGFP. Total protein yields were quantified by determining radioactive ^{14}C-Leu incorporation using trichloroacetic acid (TCA) precipitation onto paper strips [74]. Radioactivity of TCA-precipitated samples was measured using liquid

scintillation counting, (MicroBeta2, PerkinElmer, Waltham, MA). Active sfGFP protein yields were quantified by measuring fluorescence of the product and converting it to concentration (μg/mL) according to a standard curve [73]. For quantification via fluorescence, 2 μL of CFPS reaction was added in the middle of the flat bottom of 96-well half area black plates along with 48μl nuclease –free water (Costar 3694; Corning Incorporated, Corning, NY). sfGFP was excited at 485 nm while measuring emission at 528 nm with a 510 nm cut-off filter. The fluorescence of sfGFP was converted to concentration (μg/mL) according to a standard curve described previously [73].

Autoradiography analysis. Radioactive ^{14}C-Leucine was added in CFPS reactions. 5 μL of each reaction was heated at 90°C with 10mM DTT and LDS sample loading buffer from Novex (Life Technologies) and loaded onto a 4~12% NuPAGE SDS-PAGE gel. The gel was stained using simply blue safe stain (Invitrogen), destained in water, and soaked in Gel Drying Solution (Bio-Rad, Hercules, CA) for 30 min, fixed with cellophane films, dried without applying heat overnight in GelAir Dryer (Bio-Rad, Hercules, CA), and exposed for 48 hours on a Storage Phosphor Screen (GE Healthcare Biosciences, Pittsburgh, PA). The autoradiogram was scanned using a Storm Imager (GE Healthcare Biosciences, Pittsburgh, PA) and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

Preparation of sfGFP proteins in vitro for mass spectrometry. Multiple CFPS reactions were set up (up to 20 reactions per sample) as described above. After CFPS samples were combined and the product was purified using 0.2 mL gravity-flow Strep-Tactin Sepharose mini-columns (IBA GmbH, Gottingen, Germany). Eluted protein samples were concentrated using Microcon centrifugal filter columns YM-10 (Millipore, Billerica, MA). Concentrations were determined by Quick-Start Bradford protein assay kit (Bio-Rad, Hercules, CA). The samples were analyzed by top-down mass spectrometry as detailed in the following section.

Mass spectrometry. The purified protein was analyzed by nano-capillary LC-MS using a 100 mm x 75 µm ID PLRP-S column in-line with an Orbitrap Elite (ThermoFisher, Waltham, MA). All MS methods included the following events: (i) FT scan, m/z 400–2,000, 120,000 resolving power and (ii) data-dependent MS/MS on the top 2 peaks in each spectrum from scan event 1 using higher-energy collisional dissociation (HCD) with normalized collision energy of 25, isolation width 50 m/z, and detection of ions with resolving power of 60,000. All data were analyzed using QualBrowser, part of the Xcalibur software packaged with the ThermoFisher Orbitrap Elite.

Preparation of ncAAs. N^6-(5-Norbornen-2-yloxycarbonyl)-L-Lysine Hydrochloride (pLysN) and N^6-(propargyloxycarbonyl)-L-Lysine Hydrochloride (proCarb) were synthesized by the Center for Molecular Innovation & Drug Discovery. The methods are detailed in the **Supplementary Information** section. Both ncAAs were soluble in nuclease-free water. Stock solutions of 500mM were made for use in experiments.

Plasmids and Cloning. Plasmid pEvolPylRSWT was a kind gift from Dr. E. Lemke [75]. Plasmids sfGFPWT and sfGFPT216 were a kind gift from Dr. B. Bundy [76]. Plasmid sfGFP-5TAG (sfGFP D36xK101xE132xD190x E213x) was described previously[73]. Plasmid sfGFP-2TAG (sfGFPS2xT216x) was created by introducing an amber codon at the S2 site in sfGFPT216x and sfGFP-3TAG (sfGFPS2xT216x) was created by introducing an amber codon at the S2 site in sfGFPN212xT216x that has been previously described [73]. The amber codon was introduced at S2 by performing PCR using primers S2-f forward primer and S2-r reverse primer (**Supplementary Table 2**) with Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) at 98°C for 30 sec, with 30 cycles of 98°C for 10 sec, 49°C for 30 sec, and 72°C for 3 min, and a final extension of 72°C for 5 min followed by gel

extraction of the band and ligation. Plasmid pET21aMmPyl was created as follows. The wildtype PylRS DNA sequence was amplified using primers AR108 and AR109 (**Supplementary Table 2**) from pEvolPylRSWT and the PCR product was digested with Not1 and Nde1. This digested product was ligated with vector pET21a (EMD Millipore) that had also been digested with Not1 and Nde1 to get the PylRS gene upstream of a fused C-terminal His tag. Sequences for the plasmids described here can be found in the Supplementary Information (**Supplementary Sequences**).

Construction of linear DNA templates for expressing Pyl tRNA. A plasmid pY71-GB1f was created to contain the transzyme sequence[70] composed of the DNA sequence of the T7 promoter followed by the hammerhead Ribozyme and the Pyl tRNA. Briefly, gBlock GB1 (**Supplementary Table 2**) was obtained (Integrated DNA technologies, Coralville, IA) and digested with BglII and Sal1 and ligated into cloned into pY71 plasmid using BglII and SalI restriction sites. The final sequence for plasmid pY71-GB1f can be found in the Supplementary Information (**Supplementary Sequences**). The linear DNA template was created by amplifying the transzyme sequence as well as 500 base pairs of upstream sequence in the plasmid using primers T7500up forward primer (**Supplementary Table 2**) and AR045 reverse primer. The PCR was performed using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) at 98°C for 30 sec, with 30 cycles of 98°C for 40 sec, 58°C for 40 sec, and 72°C for 1 min, and a final extension of 72°C for 5 min. The PCR was purified using the E.Z.N.A. Cycle Pure Kit (Omega biotech) and quantified using a nanodrop 2000c (Thermo Scientific).

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A.R. and M.C.J. conceived the study. A.R. carried out experiments and analyzed the data. I.N. and B.J.D. carried out mass spectrometry. A.R., B.J.D., and M.C.J. wrote the paper. N.K. and M.C.J. supervised the study. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS:

M.C.J. has a financial interest in Pearl Bio. M.C.J.'s interests are reviewed and managed by Northwestern University in accordance with their conflict-of-interest policies. All other authors declare no competing interests.

ACKNOWLEDGMENTS:

This work was supported by the National Science Foundation (NSF) (MCB-1716766), the Army Research Office (W911NF-20-1-0195, W911NF-18-1-0200), Army Contracting Command (W52P1J-21-9-3023), and the David and Lucile Packard Foundation (2011-37152). This research was also carried out in collaboration with the National Resource for Translational and Developmental Proteomics under Grant P41 GM108569 from the National Institute of General Medical Sciences, National Institutes of Health and supported by the Sherman Fairchild Foundation. The ncAA synthesis was completed by Christopher Holmquist at ChemCore in the Center for Molecular Innovation and Drug Discovery which is supported in part by the Chicago Biomedical Consortium with support from The Searle Funds at The Chicago Community Trust. We would like to thank Dr. Farren Isaacs for the kind gift of the genomically recoded *E. coli* strain. We would like to acknowledge Dr. Javin Oza for cloning of the sfGFP2TAG plasmid and Dr. Seok Hoon Hong for cloning the sfGFP5TAG plasmid. We would like to thank Alaksh Choudhury for help with Matlab coding and Mark Anderson for assistance with Chemdraw. We would also like to thank Dr. E. Lemke for plasmid pEvolPylRSWT.

DATA AVAILABILITY STATEMENT:

Data available upon request from authors.

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